Rx-kinase and protein kinase C in superoxide production from neutrophils.

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Abstract.

The phorbol ester TPA activates PKC in vitro at low (nM) concentrations, whilst a related compound, Rx, only activates PKC at high (μM) concentrations. Another kinase, Rx-kinase, can be activated by low (nM) concentrations of Rx, but is unaffected by TPA. The aim of the project was to purify and characterise this novel Rx-kinase. However, screening of known sources revealed that Rx-kinase activity was sporadic and it was concluded that the enzyme was inducible. Human neutrophils were chosen for further studies, because of their ready availability and easy isolation. Using a ³²P PKC assay and anti-sera raised against specific PKC isoforms, neutrophils were shown to contain β₁-PKC, but not α-, β₂-, δ-, ε-, ζ-, η-, or θ-PKC.

A micro-titre based cytochrome c-reduction assay was developed to measure the phorbol ester stimulated superoxide production from neutrophils. The micro-titre format allowed screening of large numbers of samples, over a prolonged time period. Cells responded to low (nM) concentrations of TPA, but showed two, distinct, sensitivities to Rx. Cells responding to low (nM) concentrations of Rx, insufficient to stimulate PKC, were found to contain Rx-kinase and related protamine sulfate kinase activity. However, cells that only responded to high (μM) concentrations of Rx did not contain such activity. T.L.C. analysis of the purchased Rx revealed contamination with Ro, a known activator of PKC, and accounted for the response of cells to high concentrations of Rx. Indeed, analysis of FPLC fractions from neutrophils revealed that Ro stimulated β₁-PKC. However it was
concluded that the response of cells to low concentrations of Rx was mediated by the Rx/protamine kinases, and not PKC.

The superoxide assay was used to screen for compounds capable of inducing a high sensitivity state in neutrophils (i.e. responding to low Rx concentrations), but substance P, lipopolysaccharide, muramyldipeptide, platelet aggregating factor, plumbagin, GM-CSF and overnight incubation all failed to do so.

The results suggest that experiments successfully inducing high sensitivity to Rx, as measured by phagocytic cell superoxide production, thereby induce a family of related protamine sulfate/Rx kinases in these cells. Elucidation of such mechanisms of induction would be highly rewarding in unravelling the biochemical basis of the respiratory burst.
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List of Abbreviations.

A23187 = a calcium ionophore.
A.a. = arachidonic acid.
ACTN = acetone.
ATP = adenosine triphosphate.
$^{32}$-P-γ-ATP = radiolabelled adenosine triphosphate.
BSA = bovine serum albumin.
Ca$^{2+}$ = calcium ion.
C-fraction = cytosolic subcellular fraction.
CGD = chronic granulomatous disease.
AMP = adenosine monophosphate.
DAG = diacylglycerol (syn. Diglyceride (DG)).
DGK = diacylglycerol kinase.
DMEM = Dulbecco’s modified Eagles’ medium.
DMSO = dimethylsulfoxide.
DOPP = 12-deoxyphorbol-13-phenylacetate.
DOPPA = 12-deoxyphorbol-13-phenylacetate-20-acetate.
DTT = dithiothreitol.
EDTA = Ethylene diamine tetraacetic acid.
EGTA = Ethylene glycol bis (β-aminoethy ether)-N,N,N’,N’-tetraacetic acid.
EtOH = ethanol.
FAD = flavin adenine dinucleotide.
FCS = foetal calf serum.
FMLP = N-formyl-methionyl-leucyl-phenylalanine.
FPLC = Fast protein liquid chromatography.
FSBA = fluorylsulfonylbenzoyladenosine.
GAP = GTPase activating protein.
gp91phox = a membrane bound component of the NADPH oxidase complex.
GTP = guanosine triphosphate.
HEPES = 4(2-hydroxyethyl)-1-piperazine ethane sulphonic acid.
HMNC = human mononuclear cells.
LPS = lipopolysaccharide.
MDP = muramyl dipeptide.
MeOH = methanol.
NADP$^+$ = nicotinamide adenine dinucleotide phosphate (oxidised form).
NADPH = nicotinamide adenine dinucleotide phosphate (reduced form).
O$_2^-$ = superoxide anion radical.
OZ = opsonised zymosan.
p21$^{ox}$ = GTP-binding component of NADPH oxidase.
p47$^{phox}$ = cytosolic factor of NADPH oxidase.
p67$^{phox}$ = cytosolic factor of NADPH oxidase.
p22$^{phox}$ = membrane bound subunit of NADPH oxidase.
PA = phosphatidic acid.
PAF = platelet aggregating factor.
PDBu = phorbol dibutyrate.
Pi = Triton-X100 insoluble membrane subcellular fraction.

PIP$_3$ = phosphatidyl inositol triphosphate

PI3-K = phosphatidyl inositol 3-kinase.

PKA = protein kinase A.

PKC = protein kinase C.

cPKC = classical PKC.

nPCK = novel PKC.

aPKC = atypical PKC.

'n-PK'C = unidentified PKC isotype found in neutrophils by Majumdar.

PKM = proteolytic fragment of PKC.

PLA$_2$ = phospholipase A$_2$.

PLC = phospholipase C.

PLD = phospholipase D.

PLM = plumbagin.

PMSF = phenylmethylsulfonylfluoride.

PMN = polymorphonuclear cells.

PMA = see TPA.

Ps = Triton-X100 soluble membrane subcellular fraction.

PS = phosphatidyserine.

RACKS = receptor for active C kinases.

Rap1a = GTP-binding protein with putative role in regulation of NADPH oxidase.

rhoGDI = GDP dissociation inhibitor.

Ro = resiniferonol 9,13,14-orthophosphate.

Rx = 9,13,14-ortho-phenylacetyl-resiniferonol-20-O-homovannillate.

Rx-K = Rx-kinase.

SapA = sapinatoxin A.

SapD = sapinatoxin D.

SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

SH$_3$ = src homology region 3.

SP = substance P.

Nlei'1'1SP = norleucine-substance P.

STGPPM = starch elicited guinea pig peritoneal macrophages.

STMPM = starch elicited murine peritoneal macrophages.

TCA = trichloroacetic acid.

TLC = thin layer chromatography.

TNF = tumour necrosis factor.

TPA = 12-tetradecanoylphorbol-13-O-acetate (syn. PMA).

Tris = Tris(hydroxymethyl)amino methane.

Tx = thymeleatoxin.

Unc-13 = phorbol ester binding protein from C. elegans.
Chapter 1. Introduction

1.1 Phorbol esters.

The compounds of the phorbol ester family have proved to be valuable biochemical tools in the study of normal and pathological cellular states involving the cellular signalling molecule, protein kinase C (PKC). The name phorbol esters is misleading since the group is made up of diterpenoid derivatives of tigliane, daphnane and ingenane hydrocarbon skeletons (see figure 1.1 page 2) and only the tigliane derivatives are truly phorbols. These compounds have been isolated from a wide range of plant species all of which are members of the Euphorbiaceae and Thymelaceae families. Although these plants have been used to prepare traditional medicines, notably croton oil from Croton tigilum, Hecker has shown that they contain tumour promoting activity (Hecker 1968), which was subsequently attributed to the phorbol esters.

The phorbol esters of relevance to this project are: 12-tetradecanoylphorbol-13-O-acetate (TPA); 9,13,14-ortho-phenylacetyl-resiniferonol-20-O-homovanillate (Rx); and resiniferonol 9,13,14-orthophenylacetate (Ro) (see figure 1.2 page 3). The chemistry and biological effects of each of these are discussed below.

The most well known member of the phorbol ester family is the tumour promoter 12-tetradecanoylphorbol-13-O-acetate (TPA, also called PMA), which is a tetracyclic diterpene with a tigliane hydrocarbon skeleton. TPA is a potent tumour promoter and also causes; erythema of skin, platelet aggregation, prostaglandin secretion, lymphocyte
Figure 1.1; Tigliane, Ingenane and Daphnane hydrocarbon skeletons.
Figure 1.2: The chemical structures of 12-O-tetradecanoylphorbol 13-acetate (TPA), 9,13,14-ortho-phenylacetyl-resiniferonol-20-O-homovanillate (Rx) and 9,13,14-orthophenylacetate (Ro).
mitogenesis and superoxide production from phagocytes (Evans and Edwards 1987; Pick and Mizel 1981).

Resiniferatoxin (Rx), 9,13,14-ortho-phenylacetyl-resiniferonol-20-O-homovanillate, is a tricyclic diterpene with a daphnane hydrocarbon skeleton, it is not a tumour promoter, but is a potent inflammatory agent. Indeed Rx is 1000-fold more potent than TPA in the mouse ear erythema assay (Adolph et al. 1982). The powerful inflammatory nature of Rx has been attributed to a C20 homovanillate substitution, which resembles the polar head group of the neurotoxin capsaicin. De Vries has argued that Rx behaves purely as an ultra potent capsaicin analogue in inducing neurogenic inflammation (Vries (de) and Blumberg 1989). However, Evans has shown that in mouse ear erythema tests Rx showed a biphasic response that cannot be explained as a capsaicin response alone (Evans et al 1992). The transient phase was antagonized by capsaicin desensitization, but unaffected by hydrocortisone, suggesting a capsaicin mode of action. In contrast the latent phase was sensitive to low dose hydrocortisone treatment, but unaffected by chronic capsaicin desensitization, suggesting a phorbol ester mode of action. Therefore Rx seems capable of dual biological responses, mediated both by the C20 homovanillate substitution and the phorbol ester structure.

Resiniferonol 9,13,14-orthophenylacetate (Ro) is a tricyclic diterpene with a daphnane hydrocarbon skeleton. The structure of Ro only differs from Rx by an absence of a C20 homovanillate group and, like TPA, has a hydroxyl substitution at this position. The biological effects of Ro are very different from those of Rx, Ro is not inflammatory, but is
a tumour promoter. This shows the importance of a C20 hydroxyl group for tumour promoting activity in phorbol esters (Adolph, W. et al. 1982).

1.2 The phorbol ester receptor, Protein Kinase C (PKC).

The biological effects of the phorbol esters are thought to be mediated by the intracellular signalling protein, protein kinase C (PKC). Castagna (Castagna et al. 1982) showed that phorbol esters stimulated a calcium and phospholipid dependent protein kinase. Then Niedel (Niedel et al. 1983) and Parker (Parker et al. 1984) showed that the phorbol ester receptor co-purified to homogeneity with PKC.

PKC is not a single protein but, a multi-gene family of serine/threonine kinases that have been classified according to sequence homology. To date, screening of DNA libraries, has yielded three categories of PKC: classical (cPKC) containing four PKC isotypes α, β₁, β₂ and γ; novel (nPKC) containing a further four isotypes δ, ε, η and θ; and atypical (aPKC) containing two isotypes ζ and λ (see figure 1.3 page 6). Recently two new PKC isotypes (τ and μ) have been identified and cloned. PKC τ has initially been assigned to the atypical category, whilst PKCμ appears to fall between the novel and atypical categories (Selbie et al. 1993; Johannes et al. 1994; Johannes et al. 1995).

The three categories reflect sequence homologies based on four conserved domains (termed C1–4). The cPKC isotypes contain all four of these conserved domains. The nPKC isotypes lack the C2 domain, and the aPKC isotypes also lack the C2 domain and have a modified C1 domain containing only one of the two cysteine repeat sequences seen
Figure 1.3; The molecular structures of protein kinase C family members. The PKC isotypes can be split into three categories 'classical' (cPKC), 'novel' (nPKC) and 'atypical' (aPKC). PKC isotypes contain conserved regions (termed C1-C4) and variable regions (termed V1-V5), the quotient of these regions determines the category of PKC for each isotype.
in the other PKC categories (review: Stabel and Parker 1991).

As might be expected these structural differences are reflected in the activities of the three categories of PKC. The C-terminal C3 and C4 domains are present in all the PKC isotypes and contain sequences conserved in all protein kinases, and it has been concluded that they contain the active site of the enzymes (Hanks et al. 1988). The C1 and C2 domains are thought to regulate the catalytic domain by determining co-factor requirements for the isotypes. Although all three categories require phospholipid, there are differences in the other cofactor requirements. Thus cPKC isotypes exhibit calcium-dependent and DAG/phorbol ester dependent activity; nPKC isotypes are DAG/phorbol ester dependent, but calcium-independent; whilst aPKC isotypes are calcium and DAG/phorbol ester independent. These observations imply that the C1 domain is involved in DAG/phorbol ester binding whilst the C2 domain confers calcium-sensitivity on the cPKC isotypes. Furthermore comparison of the primary amino acid sequence of PKC with other lipid dependent and lipid-binding proteins reveals homology with the C1 and C2 regions implying that they are involved in phospholipid binding as well as DAG/phorbol ester and calcium binding (Burns and Bell 1992).

Further investigation of the C1 domain, through expression in E. coli, has shown that either of the two cysteine-rich motifs are sufficient for binding phorbol ester (Cazubon et al. 1990; Kaibuchi et al, 1989; Ono et al. 1989b). Mutation of one of the conserved cysteine residues in both of the repeats destroys binding activity (Ono et al. 1989b). It has therefore been proposed that a single cysteine repeat is enough for phorbol ester binding, which has been confirmed by the discovery of the phorbol ester binding protein chimearin,
which contains just one cysteine repeat (Areces et al. 1994). However the observation that other proteins that contain a single cysteine repeat sequence (e.g.,ζ-PKC, Vav, c-Raf), but do not support phorbol ester binding implies that residues outside the cysteine repeat may also be involved (Coppola, et al. 1991; Bonner, et al. 1986; Kazanietz, et al. 1994). The involvement of residues outside the C1 region of PKC in phorbol ester binding is supported by the observation that diacylglycerol kinase (DGK) contains two cysteine repeats, binds DAG, but not phorbol ester (Sakane et al. 1990). However, it is important to realise that residues outside the C1 region may be involved in inhibiting phorbol ester binding in Vav, c-Raf and DGK, rather than enhancing binding seen in PKC, n-chimerearin and Unc-13.

1.2.1 A model for PKC activity.

It has been suggested that PKC is a two domain enzyme with the N-terminal regulatory region linked to the C-terminal catalytic region by a flexible hinge region (variable region 3 = V3). Proteolytic cleavage of PKC generates a 30 kDa fragment which retains phorbol ester binding and a 50 kDa constitutively active kinase fragment. This implies that the regulatory region acts to inhibit the activity of the intact enzyme (Huang et al. 1989b; Lee and Bell, 1986). Consistent with the independent behaviour of these two domains, is the observation that expression of the C-terminal sequences alone in mammalian cells gives rise to functional PKC kinase activity, as assessed by an increased level of expression of a reporter gene from a phorbol-ester inducible promoter (Hata et al. 1989). Similarly, expression of the amino-terminal domain in E. coli leads to the production of a functional phorbol ester-binding protein (Ono et al. 1989a).
The inhibitory activity of the regulatory domain on the catalytic domain has been attributed to the pseudosubstrate site. The pseudosubstrate site consists of a moderately conserved sequence of amino acids with similarities to a consensus phosphorylation site in PKC substrates. However, the pseudosubstrate site lacks a serine/threonine residue that can be phosphorylated, containing an alanine instead (Gschwent et al. 1991; Bell and Burns 1991; Kemp and Pearson 1991; Hardie 1988). The pseudosubstrate site for the calcium-independent forms of PKC differs from that of the calcium-dependent forms, suggesting that substrate specificity varies between the isotypes (Schaap and Parker 1990; Makowske and Rosen 1989). A model has been proposed such that in inactive PKC the pseudosubstrate site of the regulatory domain binds to and inhibits the activity of the catalytic domain, but upon binding of cofactors the pseudosubstrate site is removed from the active site and substrate can bind and be phosphorylated (Dekker and Parker 1991). It has been recently suggested that even at saturating cofactor concentrations the pseudosubstrate site still interacts with the active site (Dekker et al. 1993). In this model the substrate competes with the pseudosubstrate site for occupancy of the active site, even in the active enzyme. Thus although histone is a poor substrate for η-PKC, mutation or removal of the pseudosubstrate site increases the level of phosphorylation of histone by η-PKC, implying that in the wild-type enzyme histone competed poorly with the pseudosubstrate site (Dekker et al. 1993).

1.2.2 Activation of PKC by phorbol esters

Experiments measuring the in vitro activation of pool-PKC (i.e. containing mixed isotypes) by phorbol esters suggested that these compounds worked in several ways and
could be divided into three groups (Ellis et al. 1987). The first group contained compounds that fully activated pool-PKC at low concentrations ($AC_{50} \leq 100$ ng/ml) and included TPA, Sap A (12-O-[2-methylaminobenzoyl]-4-deoxyphorbol-13-acetate), and Sap D ((12-O-[2-methylaminobenzoyl]-phorbol-13-acetate). The second group, containing DOPPA (12-deoxyphorbol-13-phenylacetate-20-acetate) and Rx, were only able to activate pool-PKC to 30% of the maximal levels seen with TPA. Whilst the last group contained phorbol esters that were only able to activate pool-PKC at concentrations greater than 5000 ng/ml and since it contained biologically inactive structures (e.g. $\alpha$-Sap A) probably reflected non-specific activation.

Activation of individual PKC isotypes by different phorbol esters in vitro was investigated by Ryves (Ryves et al. 1991). The members of the phorbol ester family showed a degree of selectivity for activation of the various PKC isotypes isolated from bovine brain and COS cells. Firstly the calcium-dependence of cPKC and calcium-independence of nPKC was seen with TPA and DOPP (12-deoxyphorbol-13-phenylacetate). These two phorbol esters activated $\alpha$, $\beta$- and $\gamma$-PKC (cPKC isotypes) in a calcium-dependent manner, whereas activation of $\delta$- and $\epsilon$-PKC (nPKC isotypes) was calcium independent. Secondly the observation that Rx and DOPPA were only able to stimulate pool-PKC to 30% of the maximal levels seen with TPA was explained because both DOPPA and Rx only activated $\beta_1$-PKC, in a calcium dependent manner. These phorbol esters only partially activated pool-PKC, because they only activated a subset of the pool-PKC, namely $\beta$-PKC. Finally, Ryves observed that Sap A, DOPPA, Tx (9,13,14-orthobenzoyl-6-7, epoxy-resiferonol-12-O-cinnamate) and Rx all
failed to activate δ-PKC at concentrations up to 1.6 μM, implying that this isotype has more rigid phorbol ester structure requirements than the other isotypes tested.

### 1.2.3 Cofactor dependence of phorbol ester binding to PKC

Addition of phorbol ester or diacylglycerol (DAG) to PKC *in vitro* causes an increase in affinity for phospholipid and calcium such that the enzyme is fully active at the physiological concentrations of these mediators (Takai et al. 1979; Kishimoto et al. 1980; Castagna et al. 1982). Furthermore, Konig has shown that DAG and phorbol 12,13 dibutyrate (PDBu) compete for binding at the same site(s) (Konig et al. 1985b). It has therefore been proposed that PKC forms a quaternary complex *in vivo* with phorbol ester/DAG, calcium and phospholipid, causing activation of the enzyme by relieving the inhibitory effect of the pseudosubstrate site. The relevance of phospholipid and calcium to phorbol ester interaction with PKC has been further investigated, both co-factors are discussed below.

Initial studies showed that phorbol ester binding was dependent on phospholipid (Hannun and Bell 1986; Konig et al. 1985a) and that the most effective of the naturally occurring phospholipids was phosphatidylserine, although all the anionic phospholipids showed some cofactor activity (Konig et al. 1985a). A more recent report indicates that some phorbol ester binding can occur in the absence of phospholipid, although with lower affinities than in its presence (Kazanietz, et al. 1995a). Binding studies have been further complicated because the affinity between phorbol esters and PKC changes with the level of interaction between the enzyme and phospholipids. There are two types of interaction between PKC and phospholipids: the first is reversible, associated with co-factor dependent activity and
the enzyme may be released by calcium chelators; the second is irreversible, activity is
independent of cofactors and the enzyme can only be released by detergents. Although
activity is independent of cofactors in the irreversible form, phorbol ester binding (Kd < 0.5 nM) is still observed. It is possible that in the irreversible form part of the enzyme is
inserted into the membrane, changing the conformation of the enzyme such that the
pseudosubstrate site no longer inhibits activity, but the C1 domain is still free to bind
phorbol ester. The reversibly bound form shows much weaker affinity for phorbol ester
(Kd > 1nM), but can be converted to the membrane inserted form by high concentrations
of phorbol ester (Dimitrijević, et al. 1995; Bazzi and Nelsestuen 1989). The low Kd
values (< 1 nM) observed by Kazanietz (Kazanietz, et al. 1993) for in vitro binding of
[^3]H]PDBu to 100% PS vesicles implies that measurements were being recorded for the
inserted form of the enzyme. Whilst the higher Kd values (> 1nM) observed by
Dimitrijevic (Dimitrijevic, et al. 1995), using PS/TRITON micelles, must have involved
reversible binding as such a system only supports reversible binding.

The role of calcium in the binding of phorbol ester by PKC is also complex. Calcium-
dependent activity is limited to classical PKC (cPKC), but it is not clear whether binding of
phorbol ester is also dependent on calcium in these isotypes. Kazanietz (Kazanietz, et al.
1993) found no significant difference in PDBu binding to cPKC isotypes in the presence or
absence of calcium. However, the same research showed that calcium-dependent PDBu
binding could be observed in cPKC isotypes when the PS content of the vesicles was
decreased from 100% to 20%, but concluded that the difference was due to protein-
phospholipid, rather than phorbol ester-protein, interactions. Dimitrijevic, using PS/Triton
micelles, found increased Kd values for[^3]H]PDBu binding to α-, β1- and β2-PKC with
addition of calcium, suggesting that calcium increases one or more parameters of binding (Dimitrijevic, et al. 1995). However, Dimitrijevic also found increased Kd values for the atypical δ-PKC, which does not exhibit calcium sensitive activity, and no increase in Kd for the classical γ-PKC, when calcium was added. These anomalies throw doubt on the relevance of calcium to phorbol ester binding. Thus, although calcium is required for activation of cPKC by phorbol esters it may control binding of the enzyme to phospholipid rather than the binding of phorbol ester to PKC.

1.2.4 Phorbol ester binding to PKC.

Binding studies have been conducted to investigate the interaction between [³H]phorbol-12, 13-dibutyrate ([³H]PDBu) and purified recombinant PKC isotypes that had been expressed in the baculovirus/insect cell system (Kazanietz et al. 1993; Dimitrijevic et al 1995). Both researchers found [³H]PDBu binding to all isotypes tested with the exception of ζ-PKC which showed no binding. The lack of even low affinity phorbol ester binding to ζ-PKC has been confirmed by Kazanietz (Kazanietz, et al. 1994). The inability of ζ-PKC to bind phorbol esters is thought to be due to the lack of one of the cysteine repeat sequences in the C1 region of the enzyme (Ono et al. 1989b). Presumably other members of the atypical PKC category will also be unable to bind phorbol ester.

Competition studies measuring the ability of various phorbol esters to compete for binding sites with [³H]PDBu on various PKC isotypes have also been conducted (Dimitrijevic, et al. 1995; Kazanietz, et al. 1993). No difference was observed for competition between [³H]PDBu and other phorbol esters in the presence and absence of calcium, which is more
evidence that this ion may not be involved in the actual binding of phorbol esters to PKC. The functional group at the C20 position on the phorbol ester structure was found to be critical in the ability of these compounds to compete with $[^3\text{H}]$PDBu. A free alcohol group at C20 conferred potent competition (e.g. TPA, DOPP, and Sap A) whilst an acetyl group (e.g. DOPPA) or a bulky ester linkage (e.g. Rx) significantly decreased or abolished competing activity. The cPKC isotypes $\beta_1$ and $\beta_2$, unlike the other isotypes tested, supported competition by Rx and DOPPA for $[^3\text{H}]$PDBu, suggesting that these isotypes have less rigid requirements for a free alcohol at the C20 position of the phorbol ester structure (Dimitrijevic, et al. 1995). However, Kazanietz (Kazanietz, et al. 1993) found no competition with Rx for $[^3\text{H}]$PDBu binding sites on any isotypes. This was probably due to more potent $[^3\text{H}]$PDBu binding caused by formation of the inserted form of PKC due to the use of 100% PS vesicles, which precluded effective competition by Rx (Kazanietz, et al. 1993). Furthermore, the presence of one aliphatic or aromatic ester group at the C12/C13 positions on the phorbol ester structures was found to be adequate for competition with PDBu, which contains two butyrate groups. The presence of a hydroxyl group at the C4 position was not found to interfere with the ability of phorbol esters to compete with $[^3\text{H}]$PDBu.

The calcium dependence of binding, and the displacement of $[^{3}\text{H}]$-PDBu, by Sap A to $\delta$-PKC observed by Dimitrijevic (Dimitrijevic, et al. 1995), may reflect the lack of post transcriptional modification of PKC in the insect cell line used to manufacture the isotypes for this study, but otherwise the binding data match the in vitro activation studies of Ryves (Ryves, et al. 1991).
1.2.5 Translocation of PKC.

Differences in subcellular location of various PKC isotypes have been reported. Compartmentalization appears to be dynamic and can change in a manner that depends on the activation state of the cell. For example, in IIC9 fibroblasts, stimulation with $\alpha$-thrombin caused translocation of $\alpha$-PKC, but not $\epsilon$-PKC or $\zeta$-PKC from the cytosol to the nucleus (Leach, et al. 1992).

Translocation has been defined as the movement of cytosolic PKC to the membrane fraction from extracted cells (Kawahara et al. 1980). However this definition is insufficient to fully describe the process as appearance of PKC in a membrane fraction may involve a number of distinct processes, namely: stabilisation of membrane associated PKC by DAG/phospholipid; association of cPKC isotypes with membrane due to calcium; and stabilisation of PKC-membrane association by phorbol esters. It has been argued that translocation does not simply involve movement of PKC from a cytosolic compartment to a membrane compartment, but rather a shift in a dynamic equilibrium towards membrane association. A dynamic association of PKC with the membrane would explain how the cytosolic protein initially comes into contact with hydrophobic DAG, its physiological activator.

Translocation has been used as a measure of the level of activation of PKC, thus allowing studies on which isotypes are activated by a given stimulus. A model has appeared in which PKC in the resting state is soluble (i.e. found in the cytosolic fraction of cells extracted in the presence of calcium chelators), but during the process
of activation becomes associated with the membrane (i.e. found in Triton X-100 soluble, particulate fraction of cells). Translocation of PKC has been observed with a number of receptor agonists (e.g. α-thrombin, FMLP and opsonised zymosan) and PKC effectors (e.g. calcium, DAG, phorbol esters). It has been further argued that the membrane associated form is inserted into the membrane, because detergent (i.e. Triton X-100) is required for its release. However, this may not be the case with phorbol esters which may only stabilise PKC interaction with the membrane, as removal of these effectors has been shown to release PKC from the membrane implying that the association was not of the irreversible, inserted type (Szallasi et al. 1994). Furthermore evidence that translocation does not necessarily correspond to activation has been presented by Lee and Bell (1989) and Orr and Newton (1992), therefore it may transpire that translocation is a distinct step in the activation of PKC.

Although translocation was initially associated with the appearance of PKC in the membrane fraction from cells, there are reports of PKC becoming associated with a Triton X-100 insoluble fraction from cells. These include reports of association with the cytoskeleton (e.g. Kiley and Jaken 1990; Mochly-Rosen et al. 1990; Zaleweski et al. 1990) and with the nucleus (e.g. Masmoudi et al. 1989; Goodnight et al. 1995). Associations with the nucleus may involve protein-lipid interactions, but associations with the cytoskeleton can only involve protein-protein interactions. The discovery of proteins capable of specifically binding PKC were particularly interesting in this respect (Wolf and Sahyoun 1986). Subsequently a group of proteins that bind only activated PKC have been identified and these have been termed RACKs, for receptors for
activated C-kinase (Mochly-Rosen et al. 1991). Mochly-Rosen has championed the view that RACKs determine the location and therefore affect the substrate specificity of activated PKC, by acting as specific anchoring proteins. For example, soluble RACKs, injected into *Xenopus* oocytes, inhibited insulin-induced PKC translocation and regulation of cell maturation, presumably by competing with cellular RACKs for the RACK binding site on PKC (Smith and Mochly-Rosen 1992). Dorit has discovered that a peptide derived from a region of short sequence homology between β-PKC and RACK1 induced co-factor independent activation of β-PKC *in vitro* and translocation of β-PKC *in vivo* (Dorit and Mochly-Rosen 1995a). Mochly-Rosen has therefore concluded that, in addition to the pseudosubstrate site, PKC contains another autoregulatory site, termed the pseudoanchoring site, that competes with the RACK binding site of PKC in the inactive form, inhibiting RACK binding, but this inhibition is released by activation of PKC by cofactors. Finally Dorit has shown that peptides that mimic the RACK binding site on PKC inhibit phorbol ester-induced translocation of PKC in cardiac myocytes, implying that phorbol ester-induced translocation may also be mediated by anchoring proteins (Dorit and Mochly-Rosen 1995b).

1.2.6 Degradation/down-regulation of PKC.

As outlined above partial proteolysis of PKC at the V3 region leads to two fragments, one regulatory and one catalytic. PKC has been shown to be more susceptible to proteolytic degradation *in vitro* when activated, suggesting that proteolysis may be involved in the regulation of PKC (Kishimoto et al. 1989; Mikawa, et al. 1991). This state of affairs also appears *in vivo* where sustained activation of PKC by phorbol esters results in depletion or
down-regulation of PKC in cells (Rodriguez-Pena and Rozengurt 1984; Nishizuka, 1986).
The down-regulation of α-PKC has been shown to be due to proteolytic degradation
rather than changes in mRNA production or synthetic rate, presumably the same applies to
other isotypes (Young et al. 1987). PKC is cleaved by calpain to yield a catalytically
active 46 - 50 kDa fragment called PKM (Kishimoto et al. 1983). Recently a
membrane associated neutral serine protease has been isolated from human neutrophils
which cleaves PKC leading to loss of activity via a transient calcium/phospholipid
independent form (Pontremoli et al. 1990). The apparent rapidity of this down-
regulation of PKC in neutrophils argues in favour of it being significant in PKC
directed cell biochemistry.

Physiologically PKC is only activated transiently by DAG which is soon removed either by
phosphorylation by a specific kinase or by hydrolysis at the sn-2 position. This removal
results in termination of the activation (Nishizuka 1986). Analogues of DAG that are
metabolised more rapidly than phorbol esters do not induce down-regulation, but evidence
has been presented that activation by DAG alters the steady state level of PKC, implying
that down-regulation is a physiological/pathological response and not merely an artefact of
phorbol ester treatment (Wolfman et al. 1987; Young et al. 1987).

Down-regulation is not ubiquitous to all systems tested, even with chronic phorbol ester
treatment down-regulation is poor or absent in numerous cell lines (Cooper, et al. 1989).
Furthermore, within one cell line α- and β-PKC can show different patterns of down-
regulation, perhaps indicating that different pathways/enzymes are involved with each PKC
isotype (Parker 1992).
1.2.7 *In vitro* PKC assays.

The *in vitro* activation of PKC is a complex process that involves the interaction of substrate, enzyme, and cofactors. As described above the cofactor-dependence varies amongst the isotypes of PKC. However, the *in vitro* assay conditions can also affect the cofactor dependence of PKC. The two parameters that affect the cofactor dependence of PKC are the method of phospholipid presentation and the substrate used.

The phospholipid in the *in vitro* assay can be provided in one of two forms; either vesicles (Kaibuchi et al. 1981) or detergent-mixed micelles (Hannun et al 1985), but these two systems are not equivalent. For example PS vesicles can support the activity of PKC in the absence of DAG if the calcium concentrations is high, which may be due to insertion of PKC into the vesicle. In contrast Triton-PS mixed micelles also require the presence of DAG, or phorbol ester, even at high calcium concentrations (Nelsestuen and Bazzi 1991). Also, although vesicles support both reversible and irreversible (inserted) binding of PKC, Triton-PS mixed micelles only support reversible binding (Bazzi and Nelsestuen 1989). Despite these differences, on the whole, both systems reveal similar phospholipid, DAG and calcium dependencies for PKC activation, and each has associated advantages and disadvantages (Boni 1992). For example PS/PC vesicles are more representative of the lipid content of physiological membranes, but are difficult to prepare in a well-characterized manner. In contrast Triton-PS mixed micelles do not form bilayers, but are thought to be more homogeneous than vesicle preparations (Boni 1992).

The choice of substrate also affects the cofactor requirement of PKC. Three categories of *in vitro* substrate were identified by Bazzi and Nelsestuen (1987) whilst investigating
cPKC. These categories were: category A substrates (e.g. protamine sulfate), which did not require any cofactors for phosphorylation; category B substrates (e.g. myelin basic protein) required acidic phospholipids and DAG/phorbol esters, but no calcium; category C substrates (e.g. histone) required acidic phospholipids, DAG/phorbol esters and calcium. The different cofactor dependencies were thought to reflect a requirement, not only for substrate binding to PKC, but also aggregation to deliver the substrate to the active site.

Nelsestuen and Bazzi (1991) have pointed out that in the in vitro assay substrate-phospholipid interactions may be as important as substrate-protein interactions, suggesting that the cofactor dependencies of a substrate may reflect requirements for binding to phospholipid as much as requirements for activation of the enzyme.

The substrates of PKC all contain basic residues surrounding the target serine/threonine (House et al. 1987; Marais et al. 1990). Bazzi and Nelsestuen have suggested that substrate specificity is broad, as random polymers of lysine plus serine, or arginine plus serine, are some of the best in vitro substrates for PKC (Bazzi and Nelsestuen 1987). However, since the pseudosubstrate sequence of calcium-independent forms of PKC (e.g. δ, ε, ζ, and η) differs from that of the α, β, γ-forms, it would appear that there is at least some substrate selectivity between PKC isotypes (Schaap and Parker 1990; Makowske and Rosen 1989). Indeed differential phosphorylation of some substrates by various isotypes has been demonstrated in vitro, these substrates include: glycogen synthase kinase-3β (Goode et al. 1992); GAP-43 (Sheu et al. 1991); the epidermal growth factor receptor (Ido et al 1987); and the vitamin D receptor (Hsieh et al. 1991).

Leventhal has found a possible mechanism for the ability of protamine to activate PKC in
the absence of cofactors. Leventhal found that arginine-rich proteins (e.g. protamine), but not lysine-rich proteins (e.g. histone), bind to PKC at a site distinct from the active site, in the absence of calcium, phospholipid and MgATP (Leventhal and Bertics 1993). Furthermore, poly-L-arginine was found to be 150 times more potent at inhibiting protamine phosphorylation than poly-L-lysine. Finally they found that arginine-rich proteins activated PKC autophosphorylation in the absence of cofactors. Although the \textit{in vitro} activation of PKC by protamine could be an artefact due to the formation of high order aggregates, the specific binding of poly-L-arginine to PKC and inhibition of protamine phosphorylation argues in favour of a poly-L-arginine binding site in PKC that might mediate the cofactor-independent activation of PKC.

1.3 Non-PKC receptors for phorbol esters.

The broad range of biological effects of phorbol esters may be explained, at least in part, by the fact PKC comprises a multi-gene family. A further contribution could be the tissue specificity of various PKC isotypes, since northern blot analysis with specific oligonucleotide probes and \textit{in situ} messenger RNA hybridisation has indicated a degree of tissue specificity for PKC isozymes. For example, isotypes $\alpha$-, $\delta$-, $\zeta$-PKC are widespread whilst others such as $\gamma$-, $\eta$- and $\theta$-PKC are restricted to one or a few tissues (Hug and Sarre 1993). Furthermore in most cell types only a limited subset of isotypes is present (Wetsel et al. 1992). Differences in the cofactor requirements, RACK interactions, substrate specificity, and downregulation of the individual isotypes could further contribute to the wide range of biological effects stimulated by phorbol esters.

Some non-PKC receptors for phorbol esters have been found. \textit{In vivo} scatchard analysis
of binding studies with chick embryo cells found more than one class of binding site for phorbol esters (Dunn and Blumberg 1983). Since then two non-PKC phorbol ester binding proteins have been discovered, n-chimaerin (Ahmed et al. 1990) and Unc-13 (Maruyama and Brenner 1991), and these contain a single cysteine-rich region with high homology to those found in PKC isotypes.

N-chimaerin is a brain specific protein that functions as the GTPase-activating protein (GAP) for the small GTP binding protein p21<sup>rac</sup> (Hall et al. 1990; Diekman et al. 1991). Although n-chimaerin is the only member of the family that has been shown to bind phorbol esters the other chimaerin share sequence homology for the putative phorbol ester binding site and therefore probably also show phorbol ester binding (Kazanietz et al. 1995b).

Unc-13, a novel phorbol ester/diacylglycerol binding protein has recently been cloned from the soil nematode, <i>C. elegans</i> (Maruyama and Brenner 1991). The molecular function of unc-13 is not known, but it is not a kinase as it does not contain a putative ATP-binding site or any other consensus kinase sequences. Although the molecular function is not known mutation of the <i>unc-13</i> gene causes uncoordinated movement in <i>C. elegans</i>, as does phorbol ester treatment, implying a role for Unc-13 in the regulation of movement in the nematode (Maruyama and Brenner 1991; Lew et al. 1982).

1.4 Resiniferatoxin stimulated kinase activity.

Rx-kinase (Rx-K) activity was first observed by Ryves (Ryves et al. 1989). Ryves separated a peak of histone phosphorylating kinase activity from the 15,000 x g
supernatant of human mononuclear cells (HMNC) by hydroxyapatite chromatography. This kinase activity eluted from the hydroxyapatite column at 460 mM phosphate and was stimulated by 100 ng/ml (160 nM) Rx, but only in the absence of calcium. Furthermore although other peaks of histone-kinase activity from the same cells reacted with the MC5 PKC antibody, the Rx stimulated histone-kinase did not. Ryves concluded that the Rx stimulated histone-kinase activity from HMNC was distinct from known forms of PKC on the basis of: elution from hydroxyapatite by high concentrations of phosphate; calcium-independence of the PS-dependent kinase activity; and failure to react with the MC5 antibody.

Subsequently Sharma (Sharma et al. 1995) separated Rx and PS-dependent, but calcium inhibited, histone-kinase activity of a similar nature from the 25 000 x g supernatants of human neutrophils, starch elicited murine peritoneal macrophages (StMPM) and murine alveolar macrophages. The elution profile and level of activity from each of these sources was as follows: human neutrophils, 5 x 10⁴ cells gave 10 pmoles/minute/assay, eluting at 380 mM phosphate; starch elicited murine peritoneal macrophages (StMPM), 90 mice gave 60 pmoles/minute/assay, eluting between 380 - 400 mM phosphate; and murine alveolar macrophages gave two peaks of activity both 40 pmoles/minute/assay, eluting at 365 mM and 440 mM phosphate respectively (data unpublished). These histone-kinase activities were dubbed Rx-kinase (Rx-K), on the basis of Rx and PS-dependent, but calcium inhibited, activity that was eluted from an hydroxyapatite column by > 300 mM phosphate. Rx-kinase activity seems limited to phagocytes and to date has only been detected in human mononuclear cells, human neutrophils, murine starch
elicited peritoneal macrophages, rat alveolar macrophages and bovine alveolar macrophages.

Sharma also investigated whether activity was due to a PKC isotype (Sharma et al. 1995). On the basis of SDS-PAGE and superose gel exclusion experiments Rx-kinase from StMPM was tentatively assigned a molecular weight of 70-90 Kd, which is similar to that for known PKC isotypes. However, hydroxyapatite elution fractions from StMPM that exhibited 9 units per lane of activity showed no response when screened with specific antibodies to α-, β1-, γ-, and ε-PKC. Sharma argued that the lack of response was not due to low levels of protein because 9 units per lane of α-, β1-, or γ-PKC standards were detected by their respective antibodies. Differences in cofactor dependencies and substrate specificities between Rx-k and η-PKC also excluded η-PKC as the source of activity.

The phorbol ester Rx has been described as an ultra-potent capsaicin analogue, with the similarity of action attributed to the C20 homovanilate group in Rx which shows structural similarities to capsaicin (Harvey et al. 1995). However, Sharma showed that it is not the capsaicin-like pharmacophore in Rx that stimulates Rx-kinase, as capsaicin failed to stimulate histone phosphorylation by Rx-kinase (Sharma et al. 1995). Interestingly sn-1,2-dioleoyl-glycerol also failed to stimulate histone phosphorylation by Rx-kinase in the same experiment, yet more evidence that Rx-kinase is distinct from PKC as it implies that DAG does not activate Rx-kinase. Indeed, Rx-kinase appears to have strict structural requirements for activation by Rx as the phorbol esters TPA, Sap
A, DOPP, DOPPA and Thy A all failed to stimulate histone phosphorylation by Rx-kinase. Despite this Rx probably binds at a phorbol ester binding site as \(^{[3]}\text{H}\)PDBu bound to an Rx-kinase-preparation could be inhibited by Rx. Indeed the value for 50% of inhibition of binding of \(^{[3]}\text{H}\)PDBu to Rx-kinase was 12 nM for Rx and 8.6 \(\mu\)M for TPA, suggesting that Rx binds to a phorbol ester binding site on Rx-kinase with high specificity (Sharma et al. 1995).

1.4.1 Problems with the isolation of Rx-kinase.

Ryves suffered a period of time when he could not isolate Rx-kinase activity despite using the method that had previously worked. He managed to trace the problem to low levels of calcium in the water used to prepare the buffers and when he used calcium free water the Rx-kinase activity returned. Sharma determined the dose-response for the inhibition of Rx-kinase by calcium and found that the Ki value for 50% inhibition was 0.1 - 0.5 nM free \(\text{Ca}^{2+}\) (Sharma et al. 1995). Clearly this value is well below the resting level of calcium in most cells (100 nM \(\text{Ca}^{2+}\)), implying that Rx-kinase would be inactive under physiological conditions. Of course it may be that the inhibition of Rx-kinase by calcium is an artefact of the assay rather than a physiological response of the enzyme.

Even when using calcium free water to prepare buffers Sharma experienced periods of time when the standard procedure for isolating Rx-kinase failed. Sharma noticed that unelicited murine peritoneal macrophages showed no Rx-kinase activity whereas starch elicited cells showed high levels of Rx-kinase activity and argued that cells needed to be
primed for Rx-kinase activity. Sharma found that murine alveolar macrophages incubated with plumbagin or substance P for 30 - 60 minutes prior to homogenisation contained enhanced levels of Rx-kinase activity relative to untreated cells. Sharma also showed evidence for enhanced Rx-kinase activity when white blood cells isolated from different human volunteers were mixed and incubated at 37°C for 1-2 hours prior to homogenisation, relative to cells isolated from single donors (Sharma, P. PhD. Thesis). These enhancements of Rx-kinase activity were due to increases in basal levels, rather than an induction of previously absent activity. This argues that although low levels of Rx-kinase activity may be enhanced, other signals are required to induce the expression/priming of the enzyme in vivo.

Ryves found that Rx-kinase activity isolated from HMNC was independent of blood volume over the range of 200-2000 ml from separate experiments, which implies that the enzyme was not present in all of the cells, or that it was not present at the same levels in different blood samples (Ryves, W.J. PhD. Thesis). This is more evidence in support of the theory that Rx-kinase activity is not present in resting phagocytes, but needs to be induced. The observation made by Sharma that Rx-kinase activity from StMPM increased in direct proportion to the number of mice used does not contradict this hypothesis, because these cells were elicited and presumably Rx-kinase was induced by this procedure to the same extent in each mouse.

Rx-kinase activity, even stored frozen (-20°C) in protective storage buffer (Triton/glycerol), was found to be unstable (Ryves et al. 1989). Sharma found that
when frozen at -70°C in Triton/glycerol storage buffer for as little as 24 hours Rx-kinase activity was lost after repeated freeze/thaw cycles. However, Evans A.T. (Personal communication) found that larger amounts of activity were more stable, suggesting that surface adsorption may be a factor, but a loss of 30% was still observed over 5 days.

Antibodies to Rx-kinase are not available, therefore despite the lability of the enzyme, the histone phosphorylation assay remains the only method of detecting Rx-kinase. However an alternative assay may be the *in vivo* measurement of superoxide production from phagocytes. Evans (Evans A.T. et al., 1990) showed that in starch elicited mouse peritoneal macrophages, Rx stimulated superoxide ion production to 50% of the levels seen with TPA, but only to 20% in resident mouse peritoneal macrophages. This result shows that elicitation primed the cells for Rx-stimulated superoxide production. Evans also showed that the activity of the superoxide-producing enzyme complex (NADPH-oxidase), in a cell free assay in the presence of Rx and absence of calcium, was selectively enhanced by the addition of exogenous Rx-kinase, but not PKC, (both kinases derived from the StMPM cells). These results suggest that Rx-K may be involved in activating the NADPH-oxidase complex in a PKC independent fashion. Therefore, an alternative to the histone phosphorylation assay for the detection of Rx-kinase activity may be the measurement of Rx stimulated superoxide production.

1.5 *Superoxide production and the respiratory burst.*

One biological effect of phorbol esters not fully investigated to date is that of the cyanide
Figure 1.4; Schematic representation of a phagocyte engulfing a microbe into a phagocytic vacuole. An NADPH oxidase is assembled in the wall of the vacuole, generating superoxide and hydrogen peroxide in the vacuolar lumen. Cytoplasmic granules fuse with the vacuole and release their contents (degranulation), aiding the destruction of the microbe.
insensitive production of superoxide ions ($O_2^-$) from phagocytic leukocytes (see figure 1.4 page 28). This release of highly reactive radicals and the subsequent production of hydrogen peroxide, hypochlorous acid, and other bactericides, represent a powerful weapon in the armoury of phagocytes. Since superoxide production is associated with an abrupt rise in oxygen consumption, the metabolic event that gives rise to these free radicals is called the respiratory burst. The enzyme that catalyses the one electron reduction of oxygen (see equation 1) is therefore called the respiratory burst oxidase, but since the enzyme uses NADPH as a cofactor it is also known as the NADPH-oxidase (Chanock et al. 1994).

$$2O_2 + \text{NADPH} \rightarrow 2O_2^- + \text{NADP}^+ + H^+$$  \hspace{1cm} (Equation 1)

The respiratory burst oxidase is dormant in resting cells but becomes active in cells exposed to the appropriate stimuli. In resting cells the members of the heterologous oxidase complex are in different cellular compartments, but upon stimulation the oxidase is activated by coalescence of the components at the plasma membrane.

1.5.1 Chronic granulomatous disease.

The congenital chronic granulomatous disease (CGD) is manifested clinically as a poor resistance to infection by microorganisms. The underlying cause of this poor immune response is a defective respiratory burst oxidase in phagocytes of individuals suffering from CGD (Smith and Curnutte 1991). Phagocytes from individuals with CGD are deficient in one of the components of the oxidase. Each component is a polypeptide that has been given the suffix "-phox" (abbreviated from phagocyte oxidase), and is
categorised according to molecular mass in kilodaltons (kDa) and by whether it is a protein (p) or a glycoprotein (gp). Thus gp91\textsuperscript{phox} is a glycoprotein with a molecular mass of 91 kDa which is a component of the phagocyte oxidase. The absence of these polypeptides in CGD have been traced to defects in at least four genes and each gene codes for one of the components of the respiratory burst oxidase. The most common forms of CGD are X\textsuperscript{91°} (X-chromosome-linked, gp91\textsuperscript{phox}-deficient), A22\textsuperscript{°}, A47\textsuperscript{°}, and A67\textsuperscript{°} (autosomal recessive, p22\textsuperscript{phox}-, p47\textsuperscript{phox}-, and p67\textsuperscript{phox}-deficient respectively) (Curnutte 1993). The absence of any of these gene-products results in a defective respiratory burst in CGD phagocytes. The necessity of these proteins for a normal respiratory burst response has been demonstrated by the creation of superoxide production in CGD cells simply by expressing the relevant missing protein (Maly et al. 1993; Chanock et al. 1992; Zhen et al. 1993; Sekhsaria et al. 1993).

1.5.2 The components of the respiratory burst oxidase.

The minimal requirements for an active oxidase have been defined using recombinant proteins in a cell-free system (Abo et al. 1992; Rotrosen et al. 1993). The necessary proteins are: two membrane bound components gp91\textsuperscript{phox} and p22\textsuperscript{phox}, which make up the flavohemoprotein cytochrome b\textsubscript{558}; two cytosolic components, p47\textsuperscript{phox} and p67\textsuperscript{phox}, and the cytosolic small GTP-binding protein p21\textsuperscript{ras}.

1.5.2.1 Cytochrome b\textsubscript{558}.

The cytochrome b\textsubscript{558} is so called because of the spectroscopic absorption maximum associated with its \(\alpha\)-band, but is also known as cytochrome b\textsubscript{245}, because of a mid-point potential of -245 mV. It is the terminal electron carrier of the activated oxidase,
transferring an electron from NADPH to oxygen (Segal 1989). In resting neutrophils a portion of the cytochrome b$_{558}$ is located in the membrane, with the remainder in specific granules and intracellular vesicles, but upon activation the organelles fuse with the plasma membrane, transferring the cytochrome to the cell surface (Babior 1992; Sengelov et al. 1992). The cytochrome is a 1:1 complex of gp91$^{phox}$ and p22$^{phox}$, which contains 2 heme residues and FAD, but the amount of FAD in the complex has not as yet been satisfactorily determined (Chancock et al. 1994). Dissociation of the gp91$^{phox}$-p22$^{phox}$ complex leads to loss of the hemes and destroys activity.

The $\alpha$ subunit of the cytochrome is p22$^{phox}$ which is a polypeptide of 195 residues and includes hydrophobic sequences in the N-terminal region and a basic, proline-rich C-terminus (Parkos et al. 1988). The mRNA for p22$^{phox}$ has been found in many cell types, including some of non-hematopoietic lineage, but the polypeptide is limited to cell types that also contain gp91$^{phox}$ (Babior 1992; Dinauer 1993). This last observation has relevance to CGD, because although X91$^o$ CGD phagocytes express p22$^{phox}$ mRNA the associated polypeptide is absent (Parkos et al. 1989). Similarly although A22$^o$ CGD phagocytes only contain a defect in the gene coding for p22$^{phox}$, the gp91$^{phox}$ polypeptide is also absent (Parkos et al. 1989). Cytokines do not regulate the levels of p22$^{phox}$ mRNA (Cassatella et al. 1990).

The $\beta$ subunit of the cytochrome is gp91$^{phox}$ which is a polypeptide of 570-residues containing five N-glycosylation sites, three trans-membrane helices and a C-terminal region involved in binding of the cytosolic components of the oxidase (Babior 1992; Imajoh-Ohmi et al. 1992). The mRNA coding for gp91$^{phox}$ and the actual polypeptide have only
been found in professional phagocytes and B lymphocytes (Dinauer 1993). Furthermore the level of expression of gp91phox appears to be modulated by inflammatory cytokines as γ-interferon has been shown to cause an increase and IL-4 a decrease in gp91phox mRNA levels (Cassatella et al. 1990; Abramson et al. 1990). This may well reflect a mechanism for controlling the level of production of the highly reactive superoxide ions within phagocytic cells.

1.5.2.2 The cytosolic components.

Cell-free assays revealed that a cytosolic factor was required, in addition to the cytochrome b558, in order to generate the active oxidase (e.g. Curnutte 1985). The cytosol from patients suffering from autosomal recessive CGD lacked this factor and elegant complementation studies using cytosol from patients with different forms of autosomal CGD demonstrated that at least two factors were involved (Volpp et al. 1988; Nunoí et al. 1988; Caldwell et al. 1988; Bolscher et al. 1989). A rather fortuitous experiment generated antibodies to a 47- and a 67-kDa protein, one of each of which was absent in each of the types of autosomal CGD, (Volpp et al. 1988). Those proteins p47phox and p67phox were two of the cytosolic factors required for generation of the active oxidase in cell-free assays.

The cytosolic factor p47phox is a highly basic polypeptide of 390 residues which contains two SH3 domains (SH3 = src homology region 3, found in proteins involved in association with cytoskeletal-, membrane- or small G-proteins) (Volpp et al. 1989). The C-terminus of the protein contains many serine and basic residues and shows homology to known phosphorylation target sequences, including substrate sites for PKC and PKA. Expression
of p47\textsuperscript{phox} protein and mRNA is restricted to cells of phagocytic or lymphocytic lineage (Rodaway et al. 1990). The cytokine \(\gamma\)-interferon induces the synthesis of both p47\textsuperscript{phox} mRNA and protein (Cassatella et al. 1990).

The p67\textsuperscript{phox} is a polypeptide of 526 residues which contains, a proline-rich mid-section and two SH3 domains (Leto et al. 1990). The expression pattern of p67\textsuperscript{phox} mRNA and protein is the same as p47\textsuperscript{phox} i.e. limited to cells of a phagocytic or lymphocytic lineage (Rodaway et al. 1990). The p67\textsuperscript{phox} exists as an equimolar complex with p47\textsuperscript{phox} in neutrophil cytosol (Park et al. 1992; Park et al. 1994). Neutrophils also contain uncomplexed p47\textsuperscript{phox} which behaves differently during activation (Park et al. 1992; El Benna et al. 1994).

Both p47\textsuperscript{phox} and p67\textsuperscript{phox} translocate to the membrane upon stimulation of cells (Doussière et al. 1990; Clark et al. 1990) (see figure 1.5 page 34). However, p67\textsuperscript{phox} seems incapable of translocation in the absence of p47\textsuperscript{phox}, although p47\textsuperscript{phox} can associate with the membrane in the absence of p67\textsuperscript{phox} (Heyworth et al. 1991). Translocation of the cytosolic components to the membrane is also dependent upon the cytochrome b\textsubscript{558}, since it fails to occur in X91\textsuperscript{o} and A22\textsuperscript{o} CGD neutrophils (Heyworth et al. 1991). The docking site for the cytosolic components might be located in the C-terminus of the \(\beta\) subunit of the cytochrome since both antibodies to, and peptides homologous to, this region inhibit respiratory burst activity (Kleinberg et al. 1992; Rotrosen 1990). Some researchers have found evidence for interaction between SH3 regions and proline-rich SH3 binding sites between the cytosolic components and the cytochrome, suggesting that these regions play a role in the assembly of the oxidase (Sumimoto et al. 1994; Park and Babior 1992).
Figure 1.5: Organization of the NADPH oxidase complex in neutrophils, transition from resting to active state. The resting oxidase consists of redox proteins located in the plasma membrane (p22^phox and gp91^phox), but in stimulated cells cytosolic proteins (p47^phox and p67^phox) translocate to the membrane and form an active complex with the membrane components.

Fp65K represents the FAD binding subunit of NADPH-oxidase, but is now believed to be an integral part of the gp91^phox subunit.
1.5.2.3 Small GTP-binding proteins.

Analysis of the cell-free assay, activated by arachidonic acid or sodium dodecyl sulphate, showed that p67phox and p47phox were not the only cytosolic factors required for activity. The oxidase had an absolute requirement for GTP in the cell-free assay, which appeared to be mediated, at least in part, by a cytosolic component (Peveri et al. 1992; Uhlinger et al. 1988; Gabig et al. 1987). Purification revealed that the necessary factor was a complex of a 21- and a 26-kDa protein, that turned out to be p21rac (a small GTP-binding protein) and rhoGDI (a GDP-dissociation inhibitor) respectively (Abo and Pick 1991; Abo et al. 1991). Pure recombinant p21rac could replace the complex in the cell-free assay, but only in the GTP-bound form. In the resting cell p21rac and rhoGDI exist as a complex (Abo et al. 1992). The small GTP-binding protein p21rac was initially isolated from guinea pig macrophages, but a similar protein termed p21rac2 and sharing 92% sequence homology has subsequently been isolated from human neutrophils (Knaus et al. 1991). Like the other cytosolic factors p21rac2 is translocated to the membrane upon stimulation of neutrophils (Quinn et al. 1993). Quinn also showed that p21rac2 dissociated from rhoGDI, which remains in the cytosol, and translocates with the same time course as p47phox and p67phox, but Heyworth has suggested that p21rac2 translocates independently from the other cytosolic components (Heyworth et al. 1994). For a model of the role of p21rac may play in the activation complex of NADPH oxidase, see figure 1.6 page 36.

1.5.2.4 Other components.

The components described above are the minimal requirement for the assembly of the active oxidase complex in a cell-free assay. However, two other components have been implicated in oxidase activation in vivo, they are Rap1A and p40.
Figure 1.6: A model for the role of p21\textsuperscript{rac} in the activation of NADPH oxidase (ex Segal and Abo 1993). In resting cells p21\textsuperscript{rac} is present largely in the GDP-bound form in a complex with GDI. Upon stimulation of cells, p21\textsuperscript{rac} separates from GDI and translocates with p47\textsuperscript{phox} and p67\textsuperscript{phox} to the membrane where these components associate with redox proteins to form the NADPH oxidase complex. At some point point in this process the GDP bound to p21\textsuperscript{rac} is exchanged for GTP and the complex becomes active. The bound GTP is then hydrolysed to GDP by the endogenous Rac GTPase activity, a process that, with dephosphorylation events, leads to inactivation of NADPH oxidase.
Rap1A is a GTP-binding protein that copurifies with the cytochrome b\textsuperscript{558}, and an isoprenylated form of Rap1A is abundant in neutrophil plasma membranes (Quilliam et al. 1991; Quinn et al. 1989). The involvement of Rap1A is controversial, Rap1A is not required for activation of the oxidase in the cell-free assay (Abo et al. 1992), but depletion of Rap1A from neutrophil sonicates decreased oxidase activity, which could be restored by addition of a truncated, recombinant form of Rap1A (Eklund et al. 1991). Particularly interesting was the observation that phosphorylation of Rap1A by cAMP-dependent protein kinase inhibits the association of Rap1A with cytochrome b\textsuperscript{558}. This is interesting because agents that increase intracellular cAMP concentration also inhibit superoxide production (Bokoch et al. 1991; Mueller et al. 1988).

A 40 kDa, 399-amino acid protein has been identified in the p47\textsuperscript{phox}-p67\textsuperscript{phox} complex (Someya et al. 1993). This protein shows some homology to p47\textsuperscript{phox}, but it contains only one SH3 domain (Wientjes et al. 1993). The function of this protein is not known, and it is not required for oxidase activation in the cell-free assay.

1.5.3 Activation of the Respiratory Burst Oxidase

Activation of the oxidase has been associated with the coalescence of the individual components at the plasma membrane. A portion of the Cytochrome b\textsuperscript{558} is located in the membrane of resting neutrophils, but upon activation specific granules and intracellular vesicles containing the remainder fuse with the plasma membrane, transferring more cytochrome b\textsuperscript{558} to the cell surface (Babior 1992; Sengelov et al. 1992). Concomitantly the cytosolic components translocate to the membrane, the p47\textsuperscript{phox}-p67\textsuperscript{phox} complex probably moves intact, whilst p21\textsuperscript{rac} and some uncomplexed p47\textsuperscript{phox} moves individually (El
Benna et al. 1994; Heyworth et al. 1994). The active oxidase is not only bound to the membrane, but also interacts with the cytoskeleton, an observation that may have bearing on the mechanism controlling the coalescence of the oxidase components (El Benna et al. 1994).

A plausible model for activation of the oxidase suggests that the interaction of the cytosolic components with the membrane redox carriers causes conformational changes or other modifications that initiates the flow of electrons from NADPH through the flavin and heme groups to oxygen (Dinauer 1993). The signal that initiates the formation of the active oxidase complex has not, as yet, been unambiguously assigned. However, two mechanisms that seem to be involved in the activation are release of lipids by phospholipases and phosphorylation of oxidase components by protein kinases.

1.5.3.1 Lipid-mediated activation.

Superoxide production in both intact cells and cell-free systems was found to occur in the absence of phosphorylation, leading researchers to search for other types of activation (Badwey et al. 1989; Curnutte 1987). Several saturated and unsaturated fatty acids, particularly arachidonic acid, have been shown to activate the NADPH-oxidase in intact and disrupted cells and these lipids became strong candidates as activators of the respiratory burst (Badwey et al. 1984; Curnutte 1985). However the levels of exogenously applied arachidonic acid required to activate the oxidase in vivo were greater than formed in stimulated cells, suggesting that physiological fatty acid production is not a strong enough signal, on its own, to stimulate the oxidase.
Phosphatidic acid (PA), on the other hand, has been shown to reach concentrations as high as 50-100 μM in activated cells, whilst activating the oxidase at concentrations as low as 2 μM (30 μM optimal) in cell-free assays (Agwu et al. 1989a; Agwu et al. 1991). Further evidence for the involvement of PA in the activation of the oxidase comes from studies with the chemotactic agonist FMLP in the presence of primary alcohols. Ethanol or butanol prevent formation of phosphatidic acid by forming the corresponding phosphatidyl alcohols. Neutrophils stimulated with FMLP in the presence of primary alcohols showed decreased levels of superoxide production, implying that PA was involved in normal FMLP stimulated superoxide production (Bonser et al. 1989). This conclusion was strengthened by experiments using propranolol, this compound inhibits phosphatidic acid phosphohydrolase, thereby leading to accumulation of phosphatidic acid. Propanolol was found to partially reverse ethanol mediated inhibition of FMLP-mediated oxidase activation, implying that PA is involved in respiratory burst activation (Nilson et al. 1992). The final piece of evidence is that the level of phosphatidic acid production in neutrophils stimulated by various agonists matches the observed levels of superoxide production (Korchak et al. 1988).

Other lipid products have been shown to affect superoxide production. Dilauryl PA is a particularly potent activator (Agwu et al. 1991); DAG enhances the stimulation by SDS, in a cell-free system (Burnham et al. 1990); and recently DAG has been shown to synergise with PA in the activation of the oxidase in vitro (Qualliotine-Mann et al. 1993). Finally, Wortmannin, an inhibitor of Phosphatidylinositol 3-kinase (PI 3-kinase), has been shown to inhibit chemotactic peptide stimulated respiratory burst in human neutrophils (Arcaro and Wymann 1993). This inhibition was found to be due to suppression of the production
of phosphatidylinositol-3,4,5-triphosphate (PIP₃), the latter being induced with a chemotactic peptide in a calcium independent manner. These results argue in favour of the involvement of PIP₃ in the activation of the respiratory burst.

It is not yet clear whether these lipid products directly activate the oxidase, or merely function as second messengers that mediate kinase and phosphatase activity. However, two lines of evidence argue that in some cases lipid products directly activate the oxidase. The first is that intact cells can show superoxide production in the absence of phosphorylation of oxidase components (Badwey et al. 1989). The second is the ability of lipid products and anionic amphiphiles to activate the cell-free oxidase, even in the recombinant system that lacks kinases and phosphatases (Abo et al. 1992). Miyahara (Miyahara et al. 1988) and Heyworth and Badwey (Heyworth and Badwey 1990a) have suggested a mechanism for the activation of the oxidase by anionic lipids. They suggest that these lipids are able to neutralise the positive charge of \( p47^{phox} \), thereby facilitating the interaction of that cytosolic component with the cytochrome \( b_{558} \).

1.5.3.2 Protein kinase-mediated activation.

The evidence for the involvement of phosphorylation in the activation of the respiratory burst is based on experiments that used inhibitors of protein kinases and phosphatases, and analysis of phosphorylation patterns in stimulated cells.

Superoxide production stimulated by agonists is decreased by inhibitors of protein kinases. For example, staurosporine has been shown to inhibit superoxide production stimulated by
the phorbol ester TPA, but not by the chemotactic agonist FMLP (Combadiere et al. 1993; Lu et al. 1992). Whilst the tyrosine kinase inhibitor erbstatin inhibited the superoxide production mediated by FMLP, but not by TPA (Naccache et al. 1990). These results not only show that protein kinases are involved in the activation of the oxidase, but that different signal transduction pathways are activated by different stimulants.

Analysis of phospho-proteins in stimulated cells has revealed that the various components of the oxidase become phosphorylated: p21\textsuperscript{phox} and gp91\textsuperscript{phox} (Garcia and Segal 1988); p47\textsuperscript{phox} (Heyworth and Segal 1986; Okamura et al. 1988); p67\textsuperscript{phox} (Dusi and Rossi 1993); Rap1A (Bokoch et al. 1991).

The phosphorylation of p47\textsuperscript{phox} is the most well studied of these components. The extent of p47\textsuperscript{phox} phosphorylation closely matches the intensity of the respiratory burst in human neutrophils implying a close link between the two events (Heyworth and Segal 1986; Okamura et al. 1988). Furthermore, in resting cells p47\textsuperscript{phox} is not phosphorylated, but, upon stimulation of the cell, phosphate groups are added to multiple sites on p47\textsuperscript{phox} (Rotrosen and Leto 1990). The addition of phosphate groups is sequential, the first five are added in the cytosol, whilst the final four are added at the membrane (Rotrosen and Leto 1990). Concomitant with the addition of phosphate groups is a stepwise change in the charge of p47\textsuperscript{phox}, from highly basic in the cytosol to acidic at the membrane (Rotrosen and Leto 1990). These results suggest that phosphorylation of p47\textsuperscript{phox} may result in a charge shift that is involved in the process of translocation. Curnutte has shown that translocation is dependent upon phosphorylation, he found that the protein kinase inhibitor H7 inhibited both p47\textsuperscript{phox} translocation and superoxide production in response to TPA.
(Curnutte et al. 1994). Curnutte also found that the protein phosphatase inhibitors calyculin A and okadaic acid prevented the H7 inhibition and concluded that the level of p47phox phosphorylation was controlled by a phosphorylation/dephosphorylation cycle in active cells (Curnutte et al. 1994). This idea was previously proposed by Heyworth and Badwey who found that deactivation of the respiratory burst is accompanied by the loss of $^{32}$P from p47phox (Heyworth and Badwey 1990b). These experiments demonstrate that phosphorylation, at least of p47phox, is involved in the activation of the respiratory burst. This begs the question which kinases are involved.

1.5.3.2.1 **Serine/threonine Kinases.**

The involvement of PKC in the activation of the respiratory burst has been demonstrated by several lines of evidence. TPA, a specific activator of PKC, has been shown to stimulate the superoxide production from human neutrophils (e.g. Curnutte et al. 1994). The p47phox oxidase component contains several recognition sites for PKC which become phosphorylated after stimulation of neutrophils with TPA (Rotrosen and Leto 1990). The level of phosphorylation of p47phox was found to match the level of superoxide production in TPA stimulated cells (Schneider et al. 1981). The PKC inhibitor 1-O-hexadecyl-2-O-methylglycerol, was found to block both the phosphorylation of p47phox and the production of superoxide in neutrophils and cytoplasts stimulated with TPA (Kramer et al. 1989). Finally, exogenous PKC isotypes have been shown to stimulate superoxide production in a cell-free assay, in the presence of TPA/Triton X-100/PS micelles (Sharma et al. 1991).

A number of researchers have investigated the isotypes of PKC found in human
neutrophils. PKC isotypes β1, β2, and ζ, but not δ, ε nor γ have been detected in human neutrophils, using isotype specific antibodies to PKC (Fujiki et al. 1988; Pontremoli et al. 1990; Majumdar et al. 1991; Smallwood and Malawista 1992; Balazovich et al. 1992; Dang et al. 1994; Dang et al. 1995). Some controversy exists over PKC isotype α, with some researchers finding very low levels of immunoreactivity, whilst others find none at all. Devalia, using an RNAase protection assay and antibodies to α-PKC, could find no immunoreactivity nor mRNA for α-PKC in mature neutrophils (Devalia et al. 1992). Indeed Devalia showed that HL60 cells contained both α-PKC mRNA and protein, which disappeared as cells matured to a neutrophil phenotype in response to retinoic acid and therefore concluded that PKCα is specifically down-regulated during human neutrophil terminal differentiation. The presence of ζ-PKC is also controversial as it has only been detected with a Gibco antibody which has been subsequentially shown to cross react with other isotypes of PKC (Allen et al. 1994). Majumdar has found another PKC isotype in human neutrophils which has been rather poorly termed ‘nPKC’, not to be confused with the calcium independent category, novel-PKC isotypes (nPKC), or the isotype η-PKC (Majumdar et al. 1991; Majumdar et al. 1993). This ‘nPKC’ isozyme showed PS-, DAG-dependent, but calcium-independent histone-IIIs phosphorylating activity suggesting a novel-PKC type of activity, excluding the possibility that nPKC is ζ-PKC. Thus, the consensus to date is that β1-PKC is the predominant isotype in human neutrophils with lower levels of β2-, ‘nPKC’ and possibly even lower levels of α-PKC in some preparations. It must be realised though that human neutrophils have not been screened for η-, θ-, ι-, μ-PKC nor the novel protein kinase C related protein kinases PRK 1-3 (Dekker et al. 1995).
Majumdar showed that β-PKC, but not nPKC, phosphorylated both endogenous and recombinant p47^phox, and that both phosphorylation and superoxide production were inhibited by a β-PKC pseudosubstrate peptide (Majumdar et al. 1993). However, he also found other proteins whose phosphorylation was inhibited by the pseudosubstrate peptide, including 11, 14, 22, 36, 45, 54, 58, and 80 kDa polypeptides. Therefore some or all of these, as well as p47^phox, could be involved in the activation of the oxidase by PKC. Other PKC substrates in neutrophils are known including: lipocortins I and II (now known as annexins I and II); the myristoylated protein MARKS (myristoylated, alanine-rich, C-kinase substrate); and vimentin; but the involvement of these proteins, if any, in oxidase activation is unknown (Huang 1989a; Kramer et al. 1988a; Stoehr et al. 1990; Thelen et al. 1990). Finally, PKC may not always be involved in oxidase activation, since arachidonic acid, or cytochalasin E plus concanavalin A, do not translocate β-PKC nor p47^phox, yet stimulate similar levels of superoxide production to TPA (Curnutte et al. 1994).

Another serine/threonine kinase thought to mediate oxidase activity is the cAMP dependent kinase (PKA), which has been shown to phosphorylate both p47^phox and Rap1A (Kramer et al. 1988b; Quilliam et al. 1991). However since PKA agonists inhibit the respiratory burst it is likely that phosphorylation of p47^phox and Rap1A leads to inhibition of the oxidase. Indeed phosphorylation of Rap1A by PKA has been shown to inhibit the formation of Rap1A-cytochrome b_{558} complexes and phosphorylation of p47^phox by PKA does not induce a respiratory burst (Bokoch et al. 1991; Kramer et al. 1988b).

The involvement of other serine/threonine kinases in the activation of the respiratory burst has also been suggested. High levels of calmodulin have been found in neutrophils,
suggesting a possible role for calmodulin-dependent kinases in neutrophil activation (Chafouleas et al. 1979). Huang has found another neutrophil protein kinase thought to mediate the H-7-resistant FMLP stimulated superoxide production, which he has termed H4 kinase, because of a preference for histone H4 as substrate in an *in vitro* assay (Huang and Laramee 1988). Ding found several renaturable kinases whose activity was increased in cells treated with TPA and concluded that these may be involved in the activation of superoxide production from neutrophils (Ding et al. 1993). The preferred substrate(s) and physiological action of these kinases are unknown.

### 1.5.3.2.2 Tyrosine kinases

Several studies have shown that neutrophils contain proteins phosphorylated on tyrosine residues, when stimulated by: chemoattractants (Huang 1989a; Gomez-Cambronero et al. 1991; Berkow and Dodson 1990; Grinstein and Furaya 1992); cytokines, e.g. TNFα and SP (Lloyds et al. 1995); and phorbol esters (Gaudry et al. 1993). Receptor-mediated tyrosine phosphorylation was found to depend on a pertussis-sensitive G-protein, and neutrophils activated with GTP[S] showed an increase in both tyrosine phosphorylation and the level of superoxide production (Gomez-Cambronero 1989; Nasmith et al. 1989; Grinstein and Furaya 1991). Stimulation of neutrophils with PDBu (1 μM) led to redistribution of two tyrosine kinase activities with molecular weights of 60 and 93 kDa (Gaudry et al. 1993). Incubation of neutrophils with the tyrosine kinase inhibitor erbstatin led to a decrease in both FMLP stimulated superoxide production and tyrosine-phosphorylation (Naccache et al. 1990). Whilst incubation of cells with orthovanadate, a tyrosine phosphatase inhibitor, led to increased tyrosine phosphorylation of a number of neutrophil proteins (Lloyds and Hallett 1994). The identity of the tyrosine kinases and the
mechanism involved in the activation the respiratory burst have not yet been elucidated, although the activation of Raf-1, Erk-1 and PI-3 kinase have been postulated (Lloyds and Hallett 1995b; Grinstein and Furuya 1992; Nakanishi et al. 1995).

1.5.4 **Agonist stimulated superoxide production.**

Activation of the respiratory burst oxidase *in vivo* is mediated by a wide range of soluble and particular agents. These stimuli operate through receptor-mediated signal transduction pathways (reviews: Thelen et al. 1993; Baggiolini et al. 1993) (see figure 1.7 page 47). Typical receptor-dependent stimuli include: bacterial extracts (e.g. f-met-leu-phe (FMLP), muramyl dipeptides (MDP), and lipopolysaccharides (LPS)); opsonised particles (e.g. opsonised zymosan); complement components (e.g. C5a); immune complexes; and lectins (e.g. Concanavalin A). Receptor-independent stimulants are also known and these act at the second messenger level of signal transduction. The receptor-independent stimuli include: bioactive lipids (e.g. platelet activating factor (PAF), phosphatidic acid (PA), and leukotriene-B4); PKC activators (e.g. TPA and DAG); trimeric G-protein activators (e.g. GTP[S]); and ionophores (e.g. A23187 and ionomycin).

1.5.5 **Production of second messengers responsible for the activation of the respiratory burst oxidase.**

Binding of agonist to a seven-transmembrane-domain type of receptor is thought to lead to production of an active G-protein complex, which in turn interacts with effector enzymes responsible for producing second messengers. The second messenger generating enzymes involved in the activation of the respiratory burst are phospholipase C, phospholipase D and phospholipase A2.
Figure 1.7; Receptor mediated activation of NADPH oxidase (ex Morel et al 1991). Activation of phospholipase C (PLC) and phospholipase D (PLD) is triggered by binding of agonist (A) to specific receptors (R). The signal is mediated by a trimeric G-protein. Diacylglycerol and inositol 1,4,5-triphosphate (InsP$_3$) are generated from the breakdown of phosphatidylinositol 4,5-bis phosphate (PtdInsP$_2$). Binding of InsP$_3$ to calcium stores induces release of Ca$^{2+}$ into the cytosol. Ca$^{2+}$ and DAG activate protein kinase C (PKC). Activated PKC catalyses the phosphorylation of cytosolic factors (CF) which are translocated to the membrane where they associate with redox proteins to form the active NADPH oxidase.
1.5.5.1 Phospholipase C

Investigations by a number of groups have found that agonists of the respiratory burst stimulate a phosphatidylinositol-4,5-bis phosphate-(PIP$_2$)-specific phospholipase C through a pertussis toxin sensitive trimeric G-protein (Cockcroft and Gomperts 1985; Ohta et al. 1985; Smith et al. 1985; Smith et al. 1986; Cockcroft and Stutchfield 1988; Stutchfield and Cockcroft 1991). The cleavage of PIP$_2$ by phospholipase C generates inositol trisphosphate (IP$_3$) and diacylglycerol (DAG), these second messengers activate PKC either directly through DAG or indirectly by the IP$_3$-mediated increase in intracellular calcium concentration.

Metabolism of IP$_3$ generates a number of inositol phosphate derivatives including phosphatidyl inositol 3,4,5-trisphosphate which is known to activate ζ-PKC (Nakanishi et al. 1993). Also, the fungal metabolite wortmannin inhibits both PI 3-kinase, an enzyme responsible for PI-3,4,5-trisphosphate (PIP$_3$) production, and superoxide production, which is positive evidence that PIP$_3$ is involved in oxidase activation (Baggiolini et al. 1987; Stephens et al. 1994). This suggests that phospholipase C may activate superoxide production through DAG-independent PKC isotypes as well as DAG-dependent PKC isotypes. The discovery by Ding and Badwey of a range of low molecular weight kinases, whose activation by FMLP is inhibited by wortmannin, raises the possibility of multiple phosphatidyl inositol-dependent kinases in the activation of the NADPH oxidase (Ding and Badwey 1994).

As outlined above IP$_3$ production leads to an increase in intracellular calcium levels. Kinetic studies with agonist-stimulated neutrophils show that increases in intracellular
calcium always precede the onset of superoxide production (Wymann et al. 1987; Von Tscharner et al. 1986). Further evidence for the involvement of calcium comes from experiments with cation chelators. Neutrophils depleted of calcium fail to produce superoxide in response to FMLP unless the extracellular medium contains calcium (Lew et al. 1984). In normal cells, intracellular calcium stores seem to be enough, as neutrophils respond normally when extracellular calcium is chelated (Pozzan et al. 1983). It has therefore been suggested that stimulation of phagocytes with agonists gives rise to increases in intracellular calcium levels through release from stores within, and influx from outside, the cell (Demaurex et al. 1994; Montero et al. 1991). There is controversy over whether an increase in intracellular calcium levels is enough, on its own, to activate the oxidase. Some researchers have found that ionophore induced increases in intracellular calcium concentration, to the levels observed with FMLP, do not elicit superoxide production (Sklar and Oades 1985; Korchak et al. 1984b; Pozzan et al. 1983). Whilst, Hallett has shown that rises in intracellular calcium concentration to levels greater than 250 nM activate the oxidase (Hallett et al. 1990). These experiments are complicated by the buffering capacity of calcium indicators (e.g. Quin2), which may limit the amount of calcium available for effector enzymes (Grinstein and Furuya 1988). Some researchers have shown evidence for localised accumulation of calcium stores, which can lead to localised increases in calcium concentrations, these too may be involved in oxidase activation (Stendahl et al. 1994).

The calcium-dependent activation of superoxide production is mediated, at least in part, by PKC. Increases in intracellular calcium cause PKC translocation (O'Flaherty et al. 1990) and, even in calcium-depleted cells, a response can be induced by FMLP if the cells are
first primed with sub-threshold doses of TPA (thought to translocate, but not activate PKC) (O'Flaherty et al. 1990; Grzeskowiak et al. 1986).

Investigations using phorbol esters need to be conducted with care. Sub-threshold doses of TPA synergise with calcium to give superoxide production, suggesting that calcium-mediated translocation of PKC may be involved (Robinson et al. 1984). However, higher concentrations of TPA activate superoxide production at vanishingly small cytosolic calcium levels, implying that TPA itself can cause PKC translocation at high enough concentrations (Di Virgilio et al. 1984). A plausible model is that increases in cytosolic calcium levels cause translocation of PKC which is activated by DAG in the membrane, low concentrations of TPA can substitute for DAG, but require calcium for PKC translocation, whilst high concentrations of TPA both translocate and activate PKC by substituting for calcium and DAG.

PKC may not be the only enzyme whose activity is mediated by calcium flux. The calcium-requiring thiol protease, calpain, is translocated to the membrane by increases in cytosolic calcium levels (Melloni et al. 1985). The calcium-activated protein phosphatase, calcineurin, has been implicated in TPA stimulated superoxide production (Garcia et al. 1992). Furthermore, calmodulin-dependent kinases may be involved in neutrophil activation (Chafouleas et al. 1979).

1.5.5.2 Phospholipase D.

Phospholipase D catalyses the cleavage of phosphatidylcholine to yield phosphatidic acid (PA) and choline. Phosphatidic acid can be converted into DAG by the action of PA.
phosphohydrolase. Evidence that these reactions are involved in the activation of NADPH oxidase has come from several lines of research. Firstly, metabolic studies using radiolabelled phospholipid precursors has shown that stimulation of neutrophils with cytochalasin B and FMLP leads to production of DAG from phosphatidylcholine (Billah et al. 1989; Gelas et al. 1989). Secondly, neutrophils stimulated with FMLP in the presence of ethanol or butanol showed decreased levels of superoxide production (Bonser et al. 1989). This was shown to be due to production of phosphatidyl alcohols which prevented formation of phosphatidic acid. Furthermore propranolol has been shown to partially reverse the ethanol mediated inhibition of FMLP stimulated superoxide production (Nilson et al. 1992). Since propranolol inhibits phosphatidic acid phosphohydrolase and therefore leads to accumulation of phosphatidic acid this result is good evidence for the involvement of PA in respiratory burst activation. Thirdly, the level of phosphatidic acid production in neutrophils stimulated by various agonists matches the observed levels of superoxide production (Korchak et al. 1988).

1.5.5.3 Phospholipase A2. Phosphatidic acid formed by the action of phospholipase D may be converted to lyso-phosphatidic acid and arachidonic acid by the action of phospholipase A2, this reaction has also been implicated in the activation of the NADPH oxidase. Arachidonic acid has been shown to activate superoxide production both in intact and disrupted cells (Badwey et al. 1984; Curnutte 1985) and lyso-phosphatidic acid has been shown to activate the oxidase in a cell-free assay (Agwu et al. 1991).
1.5.6 Priming.

The activation states of neutrophils do not simply fall into two discontinuous categories, namely resting or active, but form a spectrum ranging from resting to fully active. Within this scale lies a category where cells are not activated, i.e. not producing superoxide, but not resting either, since subsequent stimulation leads to a greater response than observed with resting cells. Cells in this state are described as primed and chemicals that activate superoxide production have been found to prime it at sub-threshold doses. Priming is distinct from activation in that it does not induce superoxide production, however it does reduce the lag before the onset, and amplify the production, of superoxide in response to agonists (Johnston and Kitagawa 1985; Haslett et al. 1989; Walker et al. 1990).

A wide range of agents are known to prime phagocytic cells and these include: lipopolysaccharide (LPS); muramyldipeptide (MDP); platelet activating factor (PAF); proteolytic enzymes; chemoattractants; diacylglycerol (DAG); calcium ionophores; and phorbol esters (for review see Haslett et al. 1989). Many cytokines have also been shown to mediate priming, including; IL1-α, IL-2, IL-4, IL-6, IL-8, γ-interferon, TNF-α, G-CSF and GM-CSF (Sampling and Czuprynski 1991; Borish et al. 1989; Yuo et al. 1991; Humphries et al. 1989; Berkow et al. 1987; [review] West 1990). Furthermore several substances found in the extracellular matrix (e.g. fibrinogen, fibronectin, laminin and collagen) and thought to be involved in neutrophil-adherence are also priming agents (Stanislawski et al 1990; Senior et al. 1986; Pike et al. 1989; Monboisse et al. 1987).

The mechanism of priming is unknown, but it is thought to involve: an increase in the level of cytosolic calcium (Forehand et al. 1989; Koenderman et al. 1989); an increase in the
number and affinity of chemotactic receptors (Vosbeck et al. 1990); an increase in DAG levels (Reibman et al. 1988; Agwu et al. 1989b; Dougherty et al. 1989); alterations in G-protein signalling (McColl et al. 1990); modulation of oxidase components (Della Bianca et al. 1988); activation of phospholipase D (Bauldry et al. 1991); activation of phospholipase A₂ (Dipersio et al. 1988); and tyrosine phosphorylation (Gomez-Cambronero et al. 1989; Lloyds et al. 1995; Lloyds and Hallett, 1995 a,b). These changes presumably reflect alterations in the signal transduction pathways that lead to oxidase activation.

1.5.7 Deactivation of the oxidase.

There are two dangers that phagocytes risk by activating the respiratory burst, metabolic exhaustion and damage by free radicals. Superoxide production uses large amounts of energy during the formation of NADPH by the hexose monophosphate shunt, this needs to be rigorously controlled if the cell is to survive. Phagocytes run the risk of serious damage through peroxidation of proteins, lipids and genetic material by superoxide ions or hydroxyl radicals. This danger is partially averted by the action of the detoxifying enzymes superoxide dismutase and catalase, but control of the rate of superoxide production must also be important.

The respiratory burst stimulated by agonists is short-lived lasting no longer than 2-5 minutes and ceases within seconds if the agonist is removed (Sklar et al. 1985).

Physiological termination of oxidase activation can occur by several mechanisms, including: receptor internalisation, and subsequent degradation of agonist; an increase in intracellular cAMP levels (Snyderman et al. 1986); removal of phosphate groups by
phosphatases (Garcia et al. 1991); and alteration in lipid metabolism, for example by production of 3,3'-thio-bis(didodecyl) propanoate (Eklund and Gabig 1990). However the interplay of these different mechanisms in physiological deactivation of the oxidase have not yet been elucidated.

1.6 The Objectives of the Research.

The principal aim of the project was to purify Rx-kinase to homogeneity and to further investigate its properties such as co-factor requirements, substrate specificity and tissue distribution. Given time it was hoped to determine the amino acid sequence of the this novel kinase. These objectives were not expected to be easy to meet because of the nature of Rx-kinase. Rx-kinase activity is found to be limited to phagocytes and sporadic even in those cells. Furthermore, Rx-kinase activity was present at low levels, compared to PKC, and labile even when stored in glycerol/Triton storage buffer at -70 °C. Finally, the histone-IIIs phosphorylation activity of Rx-kinase was found to be inhibited by nM concentrations of calcium, necessitating the use of calcium free buffers.

A second objective was to investigate the stimulation of the respiratory burst of phagocytes by phorbol esters. Since, in vitro, Rx has been shown to activate the respiratory burst oxidase only in the presence of Rx-kinase, it was decided to investigate the ability of Rx to stimulate superoxide production from whole cells. Biological assays for phorbol ester activity routinely conducted in the department included Mouse Ear Erythema, Tumour Promotion and Inhibition of HL-60 Leukaemia Cells, but not superoxide production. Therefore the measurement of phorbol ester stimulated
superoxide production from neutrophils required the development of a novel micro-titre plate assay (see chapter 4). The aim was to use the superoxide assay to screen compounds for ability to enhance Rx stimulated superoxide production, with the aim of finding compounds capable of inducing Rx-kinase activity in human neutrophils (see chapter 5).

The third and final objective was to investigate the types and behaviour of PKC isotypes in both resting and phorbol ester treated neutrophils. The aim was to screen neutrophils for the various PKC isotypes using specific antibodies and the phorbol ester stimulated PKC assay developed in this department. It was hoped to investigate the activity of PKC isotypes and correlate such activity with superoxide production in neutrophils stimulated with phorbol esters (see chapters 6 and 7).
2.1. Isolation of human granulocytes and monocytes.

2.1.1. Method A.

Whole blood was collected from healthy human donors by venous puncture, using citrate (3.6% trisodium citrate) or acid-citrate-dextrose (90 mM citric acid, 70 mM trisodium citrate, 100 mM glucose) as anticoagulant. Platelets were removed by centrifugation at 200 x g for 20 minutes, the platelet rich plasma was discarded and the lower, erythrocyte and white blood cell rich, layer was diluted 1:1 with Hank’s balanced salt solution (H.B.S.S.). White blood cells were separated by density gradient centrifugation. Histopaque 1077 (Sigma) was layered over an equal volume of histopaque 1119 and a layer of platelet free blood poured onto this density gradient. Centrifugation was carried out at 700 x g for 30 minutes and granulocyte cells aspirated from the 1077/1119 interface and monocytes from the plasma/1077 layer. Contaminating red blood cells were removed by osmotic shock.

The osmotic shock procedure was as follows; cells were centrifuged at 350 x g / 10 minutes, the pellet was resuspended in 5 ml distilled water (4°C) and after 30 seconds 1.65 ml of 0.6 M sodium chloride was added to make the solution isotonic. The burst erythrocytes were then removed by centrifugation (2 x 350 x g / 10 minutes) with Hank’s Balanced Salt Solution (HBSS) washes of the pellet. Cells were counted using a haemocytometer and viability was determined using Trypan blue exclusion.
2.1.2. Method B.

Whole blood was collected from healthy human donors by venous puncture and mixed 9:1 with citrate buffer (3.6% trisodium citrate). The citrate treated blood was mixed 1:1 with 6% w/v dextran (M.W. > 70,000). After being left to stand for 1 hour at room temperature the upper layer was aspirated off and centrifuged (100 x g for 12 minutes). The pellet, containing neutrophils, was subjected to an osmotic shock for 30 seconds, to remove contaminating erythrocytes and then washed by centrifugation with phosphate buffered saline (160 x g for 4 minutes). Cells were counted using a haemocytometer and viability was determined by Trypan blue exclusion.

2.2. Isolation of alveolar macrophages.

Alveolar macrophages were isolated from rat. Rat lungs were sliced into thin strips and placed in 10 ml of oxygen saturated RPMI 1640 supplemented with 10% v/v FCS, 2 mM L-glutamine and 50 μg ml⁻¹ gentamycin and agitated for 30 minutes ± 0.1 μg ml⁻¹ Rx (for priming studies). Cells were collected by filtration and subsequent centrifugation/wash cycles (400 x g for 10 minutes, twice) and finally resuspended in oxygen saturated medium and cultured in a plastic petri dish for 2 hours at 37°C. Adherent cells were washed, scraped from the dish and resuspended in homogenisation buffer.

2.3. Preparation of Bovine lung.

Bovine lung was collected from freshly slaughtered cows (Ziff meats) and transported on ice to the laboratory. The lung was cut into strips which were frozen to -80 °C using liquid nitrogen. The frozen slices were crushed to powder and 37g added to 50 ml homogenisation buffer. This was homogenised and centrifuged at 25,000 x g for 30
minutes, then the pellet was discarded and 50 ml of the supernatant added to 1.52g hydroxyapatite (0420 HTP Biorad), suspended in 21.42 ml x 1M phosphate buffer (i.e. final phosphate concentration = 300 mM). After shaking at 4 °C for 10 minutes the hydroxyapatite was sedimented by centrifugation at 400 x g for 2 minutes, then the supernatant was frozen in liquid nitrogen and the hydroxyapatite loaded into a column, ready for FPLC.

2.4. Preparation of Guinea pig lung.

The lungs of two female guinea pigs (350g) were removed and washed with P.B.S. The lungs were chopped with small scissors in 50 ml culture medium (RPMI 1060, glutamine 2mM, 10 mM EGTA, PMSF, Plumbagin (0.5 μM) was added to the chopped lungs and the tube incubated at 34 °C for 2 hours. The tube was then centrifuged at 400 x g for 10 minutes, then the pellet was homogenised in 5 ml of homogenisation buffer (20 mM Tris-HCl (pH 7.5), DTT 1 mM, leupeptin 100 μg/ml, Benzamidine 1mM, PMSF 1 mM, aprotinin 0.1 units/ml, sucrose 0.25 M, EGTA 10 mM, EDTA 5 mM). The homogenate was centrifuged at 25,000 x g for 30 minutes. The supernatant was frozen in liquid nitrogen and stored at - 80 °C. The pellet was resuspended, by ultrasonication, in 5 ml of homogenisation buffer (supplemented with 1 % Triton X-100), stored on ice for 30 minutes with regular and vigorous mixing, then centrifuged at 1538 x g for 10 minutes, the pellet was discarded and the supernatant frozen in liquid nitrogen and stored at - 80 °C.

2.5. Isolation of murine starch elicited peritoneal macrophages.

Peritoneal cells were obtained by aseptic lavage of peritoneal cavities of male CD1 mice (~20 g body weight) with 10 ml RPMI 1640 medium containing 50 μg/ml¹ gentamycin.
Macrophages had been elicited by intra peritoneal injection with 2 ml of 2\% (w/v) starch suspension three days before the cell harvesting. After washing with phosphate buffered saline by centrifugation (400 x g, for 2 minutes) the cells were then resuspended in homogenisation buffer.

2.6 Priming.

2.6.1. Priming by mixing different cell types.

Monocytes and neutrophils were incubated, apart or together (see results and discussion), in RPMI 1640 medium (Gibco) supplemented with 10 $\mu$gml$^{-1}$ leupeptin (Sigma), 1 mM PMSF (Sigma) for 2 hours at 37°C prior to homogenisation. Alternatively cells were homogenised immediately (see results and discussion).

2.6.2. Priming with plumbagin.

Cells were incubated in phosphate buffered saline ± 1 $\mu$g plumbagin at 37°C for 30 minutes. Then cells were washed with phosphate buffered saline, centrifuged (160 x g for 4 mins) and resuspended in homogenisation buffer.

2.7. Primary Cell Culture.

Cells were cultured in 500 ml culture flasks at a concentration of 10$^6$ cells ml$^{-1}$ in Dulbecco's Modified Eagles Medium (DMEM), or RPMI 1640, both with 10\% v/v heat inactivated foetal calf serum (F.C.S.) and 2 mM glutamine. The antibiotics gentamicin (50 $\mu$g ml$^{-1}$) and amphotericin B (50 $\mu$g ml$^{-1}$) were used in some experiments, as indicated. Culture flasks were incubated in 5\% CO$_2$ / 95\% air at 37 °C. In the ‘overnight’ experiments cells were incubated at room temperature in 15 ml Falcon tubes at a
concentration of ~ $1 \times 10^7$ cells ml$^{-1}$.

2.8. EGTA washes

Cells were depleted of calcium by washing with HBSS + EGTA (5 mM). Cells were pelleted by centrifugation at 350 x g / 10 minutes and resuspended in HBSS + EGTA (5 mM), then the process was repeated a second time. When superoxide production was measured from EGTA washed cells the cytochrome c solution also contained 5 mM EGTA.

2.9. Superoxide ion production microassay

Superoxide ion production was measured by an adaption of the micro-titre plate method of Pick (Pick 1986). This was based on the reduction of ferric cytochrome c to its ferrous form, which is accompanied by an increase in absorbance of light with a wavelength of 550 nm.

$$\text{Oxidized cytochrome c (Fe}^{3+}) + O_2^->\text{reduced cytochrome c (Fe}^{2+}) + O_2$$

The reduction of ferricytochrome C (160 $\mu$M) was followed by measuring its absorbance of light with a wavelength of 550 nm. Stimulants were added to batches of 320 $\mu$M ferricytochrome C, buffered to pH 7.5 prior to addition to the wells. The reaction was begun by adding to each well 100 $\mu$l of cells ($1 \times 10^6$ cells ml$^{-1}$) in HBSS, also buffered to pH 7.5. The plate was covered with a lid and incubated at 37 °C. At desired time intervals the plate was transferred to a microtitre plate reader, the instrument blanked on air then the absorbance at 550 nm measured. The $A_{550nm}$ is a measure of all the reducing species.
present, superoxide ion concentration is derived from these values by subtracting A550nm values for controls containing superoxide dismutase (SOD) \( \frac{300 \text{Uml}^{-1}}{\text{}} \). The absorbance values at 550 nm were converted to nanomoles of superoxide based on the extinction coefficient of (reduced minus oxidized) cytochrome c \( \Delta E_{550 \text{nm}} = 21 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \), according to Massey (Massey 1959). Since one mole superoxide reduces 1 mole of cytochrome c, the vertical light path passing through the microtitre plate is 4 mm long and the volume in each well is 200 \( \mu \text{l} \), the conversion factor for nmol superoxide per well is 23.81. The superoxide production due to a given stimulant = \( (A_{550 \text{nm stimulant}} - A_{550 \text{nm SOD}}) \times 23.81 \). Unless otherwise stated the data points represent means of duplicate assays ± S.E.M.

2.10. Cell fractionation.

2.10.1. Method A.

Cells were suspended in 10 ml ice cold homogenisation buffer (20 mM Tris-HCl (pH 7.5), DTT 1 mM, leupeptin 100 \( \mu \text{g/ml} \), sucrose 0.25 M, EGTA 10 mM, EDTA 2 mM) and cells lysed by ultra-sonication (3 x 10 second burst, maximum power). After centrifugation (25,000 x g for 15 minutes) the supernatant was loaded into a 10 ml superloop (Pharmacia) and the pellet discarded. In some cases the pellet was resuspended in 1 ml of homogenisation buffer, supplemented with 1 % Triton X-100, stored on ice for 30 minutes with frequent and vigorous mixing and then centrifuged at 11,000 x g for 10 minutes. The pellet from this centrifugation step was discarded and 200 \( \mu \text{l} \) of storage buffer added to the supernatant (= Triton X-100 soluble particulate fraction), which was snap frozen in liquid nitrogen and stored at - 80 °C.
2.10.2. Method B.

Cells were suspended in 1 ml ice cold homogenisation buffer (20 mM Tris-HCl (pH 7.5), DTT 1 mM, leupeptin 100 μg/ml, Benzaamidine 1mM, PMSF 1 mM, aprotinin 0.1 units/ml, sucrose 0.25 M, EGTA 10 mM, EDTA 5 mM). Cells were lysed by \( \text{freeze/thaw cycles} \) or ultrasonication (as above). For \( \text{freeze/thaw cycles} \); cells were snap frozen in liquid nitrogen (20 seconds), then thawed in 30 °C water bath, followed by a 30 second vortex. This was repeated a further two times.

A cytosolic fraction was prepared by centrifugation of the lysed cells at 100,000 x g for 30 minutes, the supernatant = cytosolic fraction (C fraction). The pellet was resuspended in 1 ml of homogenisation buffer, supplemented with 1 % Triton X-100, ultra-sonicated (3 x 10 second bursts, max.), left on ice for 30 minutes (vortexed every 10 minutes), centrifuged (6,000 x g for 10 minutes) to obtain the supernatant, designated as the Triton X-100 soluble particulate fraction (Ps fraction). In some cases the pellet from this last centrifugation step was resuspended in Laemmli buffer (Laemmli 1970) by ultra-sonication, boiled for 5 minutes and designated as the Triton X-100 insoluble particulate fraction (Pi fraction).

2.11. Protein kinase isolation.

2.11.1. Hydroxypatite FPLC.

The 25,000 x g supernatant, C and Ps fractions were further fractionated by Fast Protein Liquid Chromatography (FPLC). An hydroxypatite column (0420 HTP Biorad) was equilibrated in chromatography buffer A (20 mM, or 300mM, sodium phosphate, 10% v/v glycerol, 1.0 mM EGTA, 1 mM DTT, pH 7.5), then the cell extract was loaded. The
column was washed with chromatography buffer A to remove unbound proteins and then bound proteins eluted with various concentration gradients of potassium phosphate. Concentration gradients were achieved by mixing chromatography buffer A with chromatography buffer B (500mM, sodium phosphate, 10% v/v glycerol, 1.0 mM EGTA, 1 mM DTT, pH 7.5). In all cases the flow rate was 1 ml/min.

- **Gradient A.** 20 mM to 500 mM, over 36 minutes.
- **Gradient B.** 356 mM to 500 mM potassium phosphate, over 20 minutes.
- **Gradient C.** 300 mM to 350mM, over 1 minute; held at 350 mM for 20 minutes; 350 mM to 500 mM, over 25 minutes; held at 500 mM for 7 minutes.
- **Gradient D.** 308 mM to 500 mM, over 26 minutes.
- **Gradient E.** 308 mM to 500 mM, over 1 minute; then held at 500 mM for up to 20 minutes.

The gradients shown above show the phosphate concentration entering the column, but the concentration in a given fraction depends on the void volume of the system, here 5ml. Therefore, for example, with a 20 - 500 mM / 36 minutes gradient the actual phosphate concentration in a given fraction will have been:

\[
20 \text{ mM} + \frac{(X - 5 \times 480 \text{ mM})}{36}
\]

(Where \(X\) = fraction number.)

Fractions of 1 ml were collected on ice and immediately mixed with 200 \(\mu\)l storage buffer (glycerol and Triton X-100, ratio 16 : 1). These fractions were frozen in liquid nitrogen.
and stored at -70 °C. In some cases each fraction was split: 300 µl were mixed with 300 µl double strength Laemmli solution (Laemmli 1970), boiled for 5 minutes and stored at 4 °C; the rest stored in separate eppendorf tubes (600 µl and 300 µl), frozen in liquid nitrogen and stored at -70 °C.

2.11.2. Hydroxypatite batch elution.

The 25,000 x g supernatant of rat alveolar macrophages was added to 1ml hydroxyapatite (0420 HTP Biorad) and mixed by vortex and left to stand. After 10 minutes the supernatant was poured off (= fraction 1), 10ml x 20 mM phosphate buffer A (see above) added, the suspension mixed by vortex and left to stand. This procedure was repeated a further five times, but with phosphate buffer volumes and concentrations of; 10ml x 20 mM, 10 ml x 250 mM (twice) and 0.5 ml x 500 mM (twice), to generate fractions 2,3,4,5,6,7. Finally 2ml x 500 mM phosphate buffer was added, the tube centrifuged at 4000 x g and the supernatant taken to give fraction 8.


2.12.1. Method A.

Kinase activity was measured using an adaptation of the method of Hannun (Hannun et al. 1985). Phorbol ester (concentration 0-1600 nM) was sonicated with the micelle mixture (28.57 mM Tris-HCl, 2.4% w/v Triton X-100, 6 mg ml\(^{-1}\) phosphatidyl serine, pH 7.5). 25 µl of this micellar suspension was added to 50 µl Histone mix (6.63 mM EGTA, 28.57 mM Tris-HCl, 4 mg/ml Histone III\(\text{s}\), ± 10.2 mM CaCl\(_2\), pH 7.5) and 25 µl of the enzyme fraction from the hydroxyapatite chromatography. The assay was started with 100 µl of ATP mixture (6.63 mM EGTA, 28.5 mM Tris-HCl, 200 µM ATP, 20 mM MgCl\(_2\), \(^{32}\)P-
ATP at 300,000 cpm, pH 7.5). The assay was terminated after 10 minutes by the addition of 1 ml 25% w/v TCA, followed by 100 µl of 2 mg/ml BSA. Precipitates were filtered through Whatman GF/C filters, washed with 10 ml 10% w/v TCA and counted in a Beckman LS600 spectrometer. The activity was expressed as pmol/min/assay, and converted to give the level of phorbol ester stimulated activity over the background activity seen with phosphatidyl serine. One assay unit equalled the transfer of one picomole of phosphate, above any phosphate transfer seen with phosphatidyl serine (i.e. the phorbol ester plus phosphatidyl serine stimulated activity minus the phosphatidyl serine activity).

2.12.2. Method B

Kinase activity B was based on the same method as A, but differed in the substrate used and the method of termination. 25 µl of FPLC fraction was added to 20 µl of mix buffer (= 100 µl substrate 5 mg/ml Protamine sulfate, 200 mM HEPES pH 7.5, 2 mM EGTA, 100 µl ion buffer (3 mM CaCl₂, 50 mM MgCl₂)). The reaction was started by addition of 5 µl ATP mix (500 µM ATP + 500,000 c.p.m. ^32P-γ-ATP). Each fraction was incubated at 35 °C for 10 minutes and the reaction terminated by pipetting 30 µl of the reaction mix onto P81 paper which was dropped into 30% v/v acetic acid. 20 µl of d/s Laemmli solution (Laemmli 1970) was added to the remaining 20 µl of reaction mixture, which was then boiled for 5 minutes and stored at 4°C. The P81 papers were washed three times with 30% v/v acetic acid and then counted in a Beckman LS600 spectrometer.

The results were expressed as pmol/min/assay, and represent the total transfer of phosphate to protamine sulfate. Since this method did not contain PS/Triton micelles,
there was no background transfer stimulated by phosphatidylserine, and therefore no need to subtract the PS background.

When Histone III was the substrate the mix buffer also contained 100 ul micelle mix. PS or PS/TPA micelles were prepared by drying down 4.69 mg phosphatidyl serine (PS), or 4.69 mg PS + 2.36 µg TPA and resuspending in 500 µl micelle buffer (20 mM HEPES, 1.875% w/v Triton X-100) and sonicated until clear, in such cases the results were expressed in the same way as method A.

2.13. Translocation studies.

Human monocytes and neutrophils, isolated as described above, were incubated with various concentrations (0 - 1 µM) of TPA or 10 µM Rx (batch BH-107) for 10 minutes at 34 °C. The cells were then pelleted by centrifugation, resuspended in 1 ml of ice cold homogenisation buffer (20 mM Tris-HCl (pH 7.5), DTT 1 mM, leupeptin 100 µg/ml, Benzamidine 1mM, PMF 1 mM, aprotinin 0.1 units/ml, sucrose 0.25 M, EGTA 10 mM, EDTA 5 mM) and lysed using repeated freeze/thaw cycles and/or ultrasonication. Cells were then fractionated into cytosolic (C) and 1% Triton X-100 soluble particulate (Ps) fractions (see above), which were further separated by SDS-PAGE (see below).

2.14. SDS PAGE analysis.

Enzyme fractions from the hydroxyapatite chromatography and kinase assays were mixed 1 : 1 with double strength Laemmli buffer (125 mM Tris-HCl, 4% SDS, 2% DTT, 20% v/v glycerol, 0.0025% bromophenol blue) and boiled for 5 minutes (see above). These fractions were separated on 10 or 15 % T SDS-polyacrylamide gels with a mini-protean II
system in a running buffer of 25 mM Tris-HCl, 192 mM Glycine, 0.1% SDS, pH 8.3.
Molecular weight markers and PMN or rat brain crude PKC were included as controls.
The gels were run using a 40 mA constant current for ~1 hour.

2.15. Silver staining.
Proteins were visualised directly in the gels using silver staining. All the following
procedures were conducted at 37°C with gentle agitation. Gels were fixed for 30 minutes
with 50% methanol, 10% acetic acid followed by 2 x 15 minutes with 10% ethanol, 5%
acetic acid. Fixed gels were oxidised using 100 ml oxidiser (0.1 % K$_2$Cr$_2$O$_7$ + 0.14%
nitric acid) for 10 minutes. Then silver reagent (0.31% AgNO$_3$) was added for 30 minutes
and then discarded. Gels were developed using 3% Na$_2$CO$_3$ + 0.05% formaldehyde and
the reaction was stopped using 5% acetic acid.

2.16. Immunoblotting.
The proteins were transferred from the SDS-polyacrylamide gels to nitro-cellulose filters
by western blotting. Sheets of Hybond-c-extra were soaked in Western transfer buffer (25
mM Tris/HC1, 192 mM glycine, 20 % (w/v) methanol). The electrode of the Western
blot apparatus was wetted with western transfer buffer, then six sheets of Whatman 3 mm
chromatography paper were soaked in buffer and laid onto the electrode, any air bubbles
were squashed out, then the sheet of hybond was laid down, followed by the
polyacrylamide gels and a further six sheets of chromatography paper, which were also
soaked in buffer. Once again any air bubbles were gently squashed out, the upper
electrode connected and 11 volts passed between the electrodes for 30 minutes. After 30
minutes the chromatography paper was discarded, the nitrocellulose marked with the positions of the molecular weight markers and then the gel was discarded also. The nitrocellulose was gently agitated in blocking buffer (10% milk powder, 1% azide, 0.2% TWEEN 20, phosphate buffered saline) for 30 minutes. The nitrocellulose was then washed in washing buffer (0.2% TWEEN 20 in PBS) for five minutes, three times.

PKC isotypes were detected by incubating the blocked and washed nitrocellulose with anti-PKC antibody diluted 1:1000 in PBS plus, 0.05% Tween-20, 0.05% Azide, 0.05% Triton X-100, 3% BSA, overnight at 4°C, duplicates were incubated with anti-PKC antibody + competition epitope (kindly donated by Prof. P.J. Parker, I.C.R.F.). Immunoblots were washed 3 x 15 minutes with PBS plus, 0.05% Tween-20, 0.05% Azide, 0.05% Triton X-100 and then incubated with porcine anti-rabbit IgG linked to horseradish peroxidase at a dilution of 1:1000 for 1 hour. After incubation the membrane was washed as above (3 x 15 minutes) and developed photographically using the ECL protocol (Amersham).

2.17. Detection of Kinases by reaction with FSBA.

Detection of kinases by reaction with FSBA was based on the method of Parker (Parker 1993). Hydroxyapatite chromatography elution fractions were run through a Mono-Q column to remove any dithiothreitol, which would otherwise react with FSBA. The fractions were loaded onto a Mono-Q column which was eluted with a gradient of 0-1M NaCl over 5 minutes (elution buffer; 20 mM Tris-HCl, 2 mM EDTA, 10 mM Benzamidine, 0.1% (v/v) Triton X-100). The mono-Q elution fractions were then incubated with 10 mM Mg²⁺ and 1 mM FSBA (added from a 50 mM stock made up in DMSO), in the presence or absence
of 1 mM ATP. Samples were incubated at 30°C for 20 minutes and then terminated with Laemmli buffer containing 150 mM β-mercaptoethanol, to quench the FSBA, and boiled for 5 minutes. These samples were then separated by SDS-PAGE and transferred by Western blotting to nitrocellulose filters, as described above. FSBA bound protein was detected by anti-FSBA antibody (kindly donated by Prof. P.J. Parker, I.C.R.F.), visualised by ECL.

2.18. Chemicals.

<table>
<thead>
<tr>
<th>Compound</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid (glacial)</td>
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</tr>
<tr>
<td>Acrylamide solution (PAGE 1 protein gel mix)</td>
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<tr>
<td>Adenosine-5-[γ 32P]-Triphosphate Triethyl-ammonium salt (32P-γ-ATP)</td>
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</tr>
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<tr>
<td>horseradish peroxidase.</td>
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<td>Immunoglobulins, anti-PKC (isotype specific)</td>
<td>Prof. P.J. Parker ICRF</td>
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<td>BDH</td>
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<td>Sigma</td>
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<tr>
<td>Starch</td>
<td>Malinckrodt Chemical</td>
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<td>[Nle¹¹]-Substance P</td>
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<td>Triton-X100</td>
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<td>Tween 20</td>
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<td>Water Analar</td>
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2.19. **Apparatus used.**

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<tr>
<td>pH meter</td>
<td>Pye Unicam PW9418</td>
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<tr>
<td>Ice maker</td>
<td>Scotsman AF10</td>
</tr>
<tr>
<td>Vortex</td>
<td>Rotamixer</td>
</tr>
<tr>
<td>Bath sonicator</td>
<td>type 6442AE Ultrasonics Ltd</td>
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<tr>
<td>Centrifuge</td>
<td>small MSE microcentaur</td>
</tr>
<tr>
<td></td>
<td>medium MSE Chilspin 2</td>
</tr>
<tr>
<td></td>
<td>high speed MSE High Speed 18, Minstral, Sorval</td>
</tr>
<tr>
<td>Bag sealer</td>
<td>Calor</td>
</tr>
<tr>
<td>Bench-top shaker</td>
<td>Rotatest R100 Shaker</td>
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<td>Sterile cabinet</td>
<td>Slee, London</td>
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<td>Equipment</td>
<td>Supplier/Model</td>
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<td>-------------------------------------------------------------------------------</td>
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<td>Haemocytometer</td>
<td>Weber Scientific International Ltd.</td>
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<tr>
<td>Ultrasonicator</td>
<td>MSE probe sonicator</td>
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<tr>
<td>Scintillation counter</td>
<td>Packard Tri-carb L.S. spectrometer, model 3255</td>
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<tr>
<td>Electrophoresis</td>
<td>Biorad Mini-Protean II</td>
</tr>
<tr>
<td>Western Blotting</td>
<td>Atto electrophoresis western transfer apparatus, Genetic Research Instrumentation Ltd</td>
</tr>
<tr>
<td>Micro-titre plate reader</td>
<td>Mini-Reader II (Dynatech)</td>
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</table>
Chapter 3: Isolation of Rx-kinase activity.

3.1 Introduction.

The aim of this project was to purify and characterise Rx-kinase. Rx-kinase activity has been defined by cofactor requirements and elution profile. Thus, in phosphorylation assays Rx-kinase catalysed the transfer of $^{32}$P from $^{32}$P-$\gamma$-ATP to histone-IIIIs only in the presence of phosphatidyl serine and resiniferatoxin (Rx), this activity was inhibited by the presence of free calcium. A $K_a$ of 16 nM was observed for activation of Rx-kinase by Rx, with an $IC_{50}$ of 0.1 - 0.5 nM for free Ca$^{2+}$ (Sharma et al. 1995). Rx-kinase activity has only been isolated from the cytosol of phagocytes, specifically the supernatant of homogenates after centrifugation at 25,000 x g. Rx-kinase activity was eluted by > 300 mM phosphate when 25,000 x g supernatants were further separated by hydroxyapatite column FPLC (Sharma et al. 1995). Therefore, Rx-kinase activity has been defined as Rx/PS stimulated and Ca$^{2+}$ inhibited histone-IIIIs phosphorylating activity, isolated from the 25,000 x g supernatant of phagocytes, which elutes from hydroxyapatite FPLC columns at phosphate concentrations > 300 mM.

The first step for the purification and characterisation of Rx-kinase was to find a rich and reproducible source of the activity. The initial strategy chosen was to analyse the previously reported sources of Rx-kinase activity. These included;

(i) Human mononuclear cells (Ryves et al. 1989)
(ii) Human neutrophils (Sharma et al. 1995)
(iii) Murine alveolar macrophages. (Sharma et al. 1995)
(iv) Murine peritoneal macrophages. (Sharma et al. 1995)
It should be noted that the level of kinase activity is given in assay units (see materials and methods, chapter 2). Assay units refer to the phorbol ester stimulated transfer of $^{32}$P-labelled phosphate, above the background stimulated by phosphatidyl serine (i.e. the level of activity seen with phorbol ester/PS/Triton micelles minus the activity seen with PS/Triton micelles alone).

3.2. Human white blood cells.

Since Rx-kinase activity had previously been isolated from both human mononuclear cells and human neutrophils, the first strategy employed, aimed at increasing the yield, was to use total human white blood cells as a source. Ryves (Ryves, W.J. 1991 PhD Thesis, School of Pharmacy, London) found that Rx-kinase activity isolated from human monocytes was independent of blood volume and Sharma (Sharma et al. 1995) isolated 10 units of Rx-kinase activity in human neutrophils from 200 ml blood. However, as can be seen from table 3.1 the levels of Rx-kinase activity found in the present study were variable and low.

Table 3.1; Rx-stimulated histone-IIIis phosphorylating activity in the hydroxyapatite separated fractions of the 25,000 x g supernatant of human white blood cells.

<table>
<thead>
<tr>
<th>Date</th>
<th>Blood volume (ml)</th>
<th>Rx-kinase activity (units)</th>
<th>Rx-kinase activity (units/ml blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.10.92</td>
<td>180</td>
<td>5.04 ± 0.19 (2 donors)</td>
<td>0.028 ± 0.001</td>
</tr>
<tr>
<td>18.02.93</td>
<td>100 (2 donors)</td>
<td>1.41 ± 1.65</td>
<td>0.014 ± 0.016</td>
</tr>
<tr>
<td>03.03.93</td>
<td>280 (4 donors)</td>
<td>Negligible</td>
<td>Negligible</td>
</tr>
<tr>
<td>09.03.93</td>
<td>90 (2 donors)</td>
<td>Negligible</td>
<td>Negligible</td>
</tr>
<tr>
<td>15.04.93</td>
<td>200 (3 donors)</td>
<td>3.43 ± 0.81</td>
<td>0.017 ± 0.004</td>
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</tbody>
</table>
3.2.1. Priming of leukocytes by mixing cells from different donors.

Sharma (Sharma, P personal communication) observed an enhancement in Rx-kinase activity of 3.7 fold for neutrophils and 4.5 fold for monocytes, when white blood cells from different donors were mixed. Experiments were conducted to see whether the enhancement seen by Sharma was really due to mixing white blood cells from different donors. Various combinations of cells were tested: on 13.10.92 and 15.4.93 white blood cells were isolated from mixed donors (3 x 60 ml), but the cells were not incubated together prior to homogenisation; on 18.2.93 and 9.3.93 monocytes from different donors were mixed and incubated in culture medium at 37°C for 2 hours, granulocytes were similarly treated and then both cell types were mixed before homogenisation; on 3.3.93 both granulocytes and monocytes from different donors were mixed immediately and then incubated in culture medium for 2 hours. Table 3.1 (page 74) shows that mixing of white blood cells from different donors did not enhance Rx-kinase activity. Indeed higher levels of activity were seen when white blood cells from different donors were not incubated together (13.10.92 and 15.4.93) than when cells were incubated together (18.2.93, 3.3.93, 9.3.93). The reason for the last observation could be that Rx-kinase activity degraded during the incubation period, however protease inhibitors were included in the culture medium (see the methods section) in attempt to minimise any loss of activity due to proteolysis.

3.2.2. Plumbagin treatment of human neutrophils.

Sharma and Evans (personal communication) have suggested that the irritant plant compound plumbagin enhanced Rx-kinase levels. Therefore purified human neutrophils treated with plumbagin, were also analyzed as a source for Rx-kinase activity. These cells
were isolated by method B as described in the methods section then cultured ± plumbagin for 30 minutes at 37 °C. The control cells, not incubated with plumbagin gave negligible levels of activity, but the cells incubated with plumbagin also gave negligible levels of Rx-kinase activity. Increasing the amount of blood used did not affect the levels of Rx-kinase activity observed after plumbagin treatment. Indeed, as can be seen from table 3.2, only negligible levels of Rx-kinase activity were observed, even when 300 ml of blood from different donors was used.

<table>
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<th>Rx-kinase activity</th>
</tr>
</thead>
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<td>16.06.93</td>
<td>400 (4 donors)</td>
<td>-</td>
<td>Negligible</td>
</tr>
</tbody>
</table>

3.3 Alveolar macrophages.

The initial strategy used with alveolar macrophages was to homogenise whole lung to maximise yield of protein, but this method produced negligible levels of Rx-kinase activity (see table 3.3, experiment 13.1.93).

In subsequent experiments alveolar macrophages were isolated as described in the methods section. The experiment on 27.1.93 yielded one small peak of Rx stimulated histone kinase activity, in the absence of calcium, of 1.439 ± 0.426 units, corresponding to hydroxyapatite elution fraction 9, which eluted at 217 mM phosphate. However, fraction 9 also catalysed histone-III's phosphorylation in the presence of TPA and Rx with added
calcium suggesting that the activity was not due to Rx-kinase. In contrast Sharma reported 2 peaks of 40 units Rx-kinase activity corresponding to phosphate elution concentrations of 365 mM and 440 mM respectively (Sharma et al. 1995).

<table>
<thead>
<tr>
<th>Date</th>
<th>Number of animals</th>
<th>Primer</th>
<th>Rx-kinase activity (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.01.93</td>
<td>2 x male</td>
<td>-</td>
<td>Negligible</td>
</tr>
<tr>
<td>27.01.93</td>
<td>2 x male</td>
<td>-</td>
<td>1.439 ± 0.426</td>
</tr>
<tr>
<td>01.02.93</td>
<td>1 x male</td>
<td>-</td>
<td>1.266 ± 0.862</td>
</tr>
<tr>
<td>11.03.93</td>
<td>2 x male</td>
<td>Rx</td>
<td>1.600 ± 0.688</td>
</tr>
<tr>
<td>20.05.93</td>
<td>6 x female</td>
<td>-</td>
<td>Negligible</td>
</tr>
</tbody>
</table>

In case degradation of Rx-kinase during the FPLC procedure was responsible for the lack of activity, on 1.2.93 a more rapid batch elution procedure (see methods) was conducted on the 25,000 x g supernatant of rat alveolar macrophage homogenate. However, this method only yielded 1.266 ± 0.862 units of Rx-kinase activity (see table 3.3).

In the experiments conducted on 11.3.93 and 20.5.93 the 25,000 x g supernatants of rat alveolar macrophages were subfractionated on a hydroxyapatite column using gradient E (see methods), which should have led to coalescence of the two peaks observed by Sharma (see above) making activity easier to detect. However, little or no activity was observed, even when cells were incubated with 0.1 µg/ml (159 nM) Rx for two hours, at 37°C, prior to homogenisation, a procedure that Sharma (Sharma, P. (1995) PhD Thesis, School of Pharmacy, London.) suggested enhanced Rx-kinase activity.
On 4.5.93 and 12.5.93 the lungs of rats being used in a project studying neuropeptide production and pain became available. As part of another project these rats had been treated with capsaicin (50 mg/Kg), as neonates, in order to desensitise the efferent C-fibre neurons involved in noci-reception. As far as this project was concerned the rats represented a source of lungs from animals that were already being killed, in line with the Home Office guidelines on minimisation of animal use in research. Alveolar macrophages were isolated and treated with or without plumbagin, then the 25,000 x g supernatants of the cell homogenates were subfractionated on a hydroxyapatite column using gradient E (see methods). As can be seen from table 3.4 the levels of Rx-kinase activity were very low and the difference between capsaicin treated and control rats was reversed between the sexes. Though the last observation is interesting it is not supported by the rat alveolar macrophage experiment conducted on 20.5.93 (see table 3.3, page 77). Furthermore the plumbagin treatment made no difference to the levels of Rx-kinase activity observed. Rx-kinase activity did not correlate with cell number, or treatment and was sporadic in cell preparations treated the same way. These results imply that Rx-kinase activity from rat alveolar macrophages was inducible, but did not depend on the sex of the animal or capsaicin/plumbagin treatment.
Table 3.4; Rx-stimulated histone-III's phosphorylating activity in the hydroxyapatite separated fractions of the 25,000 x g supernatant of alveolar macrophages isolated from rats treated with capsaicin as neonates, plus controls.

<table>
<thead>
<tr>
<th>Date</th>
<th>Number of animals</th>
<th>Capsaicin treated</th>
<th>Primer</th>
<th>Rx-kinase activity. (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>04.05.93</td>
<td>2 x male</td>
<td>YES</td>
<td>Plumbagin</td>
<td>3.12 ± 0.202</td>
</tr>
<tr>
<td>04.05.93</td>
<td>1 x male</td>
<td>NO</td>
<td>Plumbagin</td>
<td>Negligible</td>
</tr>
<tr>
<td>12.05.93</td>
<td>3 x female</td>
<td>NO</td>
<td>-</td>
<td>2.59 ± 0.72</td>
</tr>
<tr>
<td>13.05.93</td>
<td>2 x female</td>
<td>YES</td>
<td>-</td>
<td>Negligible</td>
</tr>
</tbody>
</table>

3.4 Starch elicited peritoneal macrophages.

On two occasions starch elicited peritoneal macrophages were analyzed for Rx-kinase activity, but only negligible levels of activity were observed (see table 3.5).

TABLE 3.5; Rx-stimulated histone-III's phosphorylating activity in the hydroxyapatite separated fractions of the 25,000 x g supernatant of starch elicited murine peritoneal macrophages.

<table>
<thead>
<tr>
<th>Date</th>
<th>Number of animals</th>
<th>Rx-kinase activity (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.04.93</td>
<td>60</td>
<td>Negligible</td>
</tr>
<tr>
<td>21.06.93</td>
<td>60</td>
<td>Negligible</td>
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</table>

3.5 Bovine lung.

The results outlined above show that detection of Rx-kinase from known sources was sporadic and levels of activity low. It was decided to investigate bovine lung, using the logic that this represented a source of large numbers of alveolar macrophages. Frozen lung was crushed to a powder and 37 g homogenised to give a total lung extract. The 25,000 x g supernatant was subfractionated by hydroxyapatite FPLC using gradient C. This generated a fraction with 6.55 ± 2.33 units of Rx (-Ca^{2+}) stimulated histone-III's
phosphorylating activity that eluted from the hydroxyapatite column at 416 mM phosphate. This was a very low level of Rx-kinase activity considering the quantity of source material used and these results were not reproducible in other preparations.

3.6. Protamine sulfate assays.

A second type of assay was used in the hunt for Rx-kinase activity, which differed from the histone-IIIs phosphorylating assay according to substrate used and co-factor requirements. Where the first assay used histone-IIIs as a substrate and required PS and Rx the second assay used protamine sulfate as a substrate and did not require cofactors.

3.6.1. Human neutrophils.

On one occasion $2.86 \times 10^5$ neutrophils were isolated from 90 ml of blood and incubated overnight. The next day the cells were homogenised, and the C-fraction was sub-fractionated by hydroxypatite FPLC using gradient A (see chapter 2). The resulting fractions were assayed for $^{32}$P transfer activity using protamine sulfate as substrate (see figure 3.1, page 81). The early eluting peaks (fractions 9 - 18) were characteristic of PKC (see chapter 7), but the other two peaks showed elution profiles similar to Rx-kinase.

The P-fraction from these cells was also sub-fractionated by hydroxyapatite FPLC and assayed for kinase activity (see figure 3.2, page 81). Figure 3.2 shows that the Triton-X100 particulate fraction of these cells also contained late eluting protamine sulfate phosphorylating activity.
Figure 2. 32P transfer from 32P-ATP to prolamellar bodies.

Figure 4: 32P transfer (pmol/min/assay)

32P transfer (pmol/min/assay)

at pH 8.0

32P transfer (pmol/min/assay)

at pH 8.0

32P transfer (pmol/min/assay)

at pH 8.0

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32P transfer (pmol/min/assay)

at pH 8.0

32P transfer (pmol/min/assay)
Late eluting protamine sulfate phosphorylating activity, from both the C and P fractions, was only seen in the presence of calcium. This is the opposite of the case seen with the phosphorylation of Histone-IIIs by Rx-kinase where calcium potently inhibited the activity. This suggest that the late eluting protamine sulfate phosphorylating activity was not due to Rx-kinase, but Nelsestuen has argued that the cofactor requirement of kinases changes with different substrates (Nelsestuen and Bazzi 1991). It is possible that the inhibition of Rx-kinase activity by calcium seen with Histone-IIIs as substrate was actually an artefact of the assay. Furthermore, Sharma showed that the IC\textsubscript{50}, for inhibition of Rx-kinase activity by calcium was 0.1 - 0.5 nM, a value 200 fold lower than the resting level of calcium in most cells. Therefore if the inhibition of Rx-kinase activity by calcium is physiological, then the kinase would be inactive even at resting cytoplasmic calcium concentrations. It is difficult to imagine a role for a kinase that is inhibited by resting calcium concentrations, supporting the idea that calcium inhibition of Rx-kinase may be an artefact of the \textit{in vitro} assay.

The fractions containing the late eluting protamine sulfate phosphorylating activity were subsequently assayed for PS/Rx/±Ca\textsuperscript{2+} dependent histone-IIIs phosphorylating activity using the mixed micelle assay, but were found to be inactive. This result was due to degradation as the protamine sulfate controls showed no activity either (data not shown). Clearly these late eluting peaks of activity fulfill only one of the defining criteria of Rx-kinase activity, namely that elution from a hydroxyapatite column requires phosphate concentrations greater than 300 mM. The other defining criteria are cofactor requirements for histone-IIIs phosphorylation, namely Rx-, PS-dependent and calcium-independent activity. These last criteria could not be tested for the late eluting protamine sulfate
phosphorylating activity, because of loss of activity.

The late eluting protamine sulfate activity did not correlate with overnight incubation of human neutrophils. On other occasions no late eluting activity was observed in either the C- or P-fraction of neutrophils incubated overnight (see figure 3.3, page 84). Indeed this was also the case with cells homogenised on the same day as isolation, i.e. without overnight incubation, implying that the late eluting kinases described above were inducible, but not by overnight incubation.

3.6.2. Plumbagin treated human neutrophils.

The effect of plumbagin on human neutrophils was further investigated using the protamine sulfate assay. 1.98 x 10^4 neutrophils were isolated from 70 ml of blood, from a single donor. These cells were incubated in RPMI containing 1 mM PMSF and 143 μg/ml leupeptin for 30 minutes to inhibit proteolytic activity. Then the neutrophils were incubated with plumbagin (0.5 μM) at 35 °C for one hour. At the end of this time the cells were counted again and 1.75 x 10^4 neutrophils were viable, representing a loss of only 11.6%. The C fraction (see materials and methods) from these cells was subfractionated by FPLC using gradient A and the hydroxyapatite elution fractions were assayed using kinase assay B, with protamine sulfate as the substrate (see methods). This experiment generated the kinase profile shown in figure 3.4, page 84. Whilst early eluting peaks, corresponding to previous PKC activity, were observed, no late eluting peaks of activity were seen suggesting that PLM does not induce the late eluting Rx/protamine kinases.
Figure 3.3: 32P transfer from 32P-ATP to protamine sulfate, catalysed by hydroxyapatite fractions from the cytosolic and particulate portions of human PMN.

Figure 3.4: 32P transfer activity from 32P-ATP to protamine sulfate, catalysed by hydroxyapatite fractions from the cytosolic portion of human PMN, treated with plumbagin.
3.6.3. Plumbagin treated human mononuclear cells.

The effect of incubating plumbagin with human mononuclear cells was also investigated. 9.24 x 10^7 mononuclear cells were incubated with plumbagin (0.5 $\mu$M) on ice for one hour. After incubation 8.66 x 10^7 cells were still viable, representing a loss of 6.28%. The C fraction from these cells was subfractionated by hydroxyapatite FPLC using gradient A. The elution fractions were then assayed using kinase assay B, with protamine sulfate as the substrate plus added calcium (see methods). The results of that experiment are shown in figure 3.5, page 86. As before the early eluting PKC peaks were observed, but no late eluting kinases, implying that these were not induced by plumbagin treatment of human monocytes.

3.6.4. Plumbagin treated guinea pig whole lung.

The 25,000 x g supernatant and the triton-X100 soluble particulate fraction of plumbagin treated guinea pig lung homogenate were also tested for late eluting protamine sulfate phosphorylating activity (see figure 3.6, page 86). Early eluting peaks, characteristic of PKC, were observed, but no late eluting kinase activity was seen in either the C- or the P-fraction.

3.6.5. GM-CSF treated human neutrophils.

GM-CSF has been shown to prime human neutrophils for subsequent stimulation by FMLP (Roberts et al. 1993) and was therefore used in an attempt to induce late eluting kinase activity. 2 x 10^8 human neutrophils were incubated with GM-CSF (10 ng/ml) for 48 minutes, then the cells were homogenised and the homogenate separated into C and P fractions. The C and P fractions were subfractionated using hydroxyapatite FPLC and the
Figure 3.5: 32P transfer from 32P-ATP to protamine sulfate (+ calcium), catalyzed by hydroxyapatite elution fractions derived from the C fraction of human mononuclear cells treated with plumbagin.

Figure 3.6: 32P transfer activity from 32P-ATP to protamine sulfate, catalyzed by hydroxyapatite fractions from the cytosolic (C) and particulate (P) portion of guinea pig lung treated with plumbagin.
elution fractions assayed for protamine phosphorylation activity (see figure 3.7, page 88).

Early eluting PKC activity was observed, but no late eluting kinase activity, even after incubation of cells with GM-CSF.

3.7 Discussion.

3.7.1. Introduction.

Both Ryves and Sharma experienced periods of time during which they were unable to isolate Rx-kinase activity, despite following the protocol that had, at other times, been successful (personal communication). Similarly in this project experiments were conducted on cells from six different sources, but only very low levels of Rx-kinase activity were observed. Initially this could have been blamed on inexperience and unfamiliarity with the lengthy isolation procedure. However, the low levels of Rx-kinase activity persisted as the project progressed and the techniques became familiar, suggesting a more fundamental problem. The low levels or absence of activity could have been due to a number of factors;

(a) Failed assay.
(b) Degradation during isolation procedure.
(c) Insufficient source material.
(d) Absence of Rx-kinase in these preparations.

Each of these possibilities will be addressed in turn.

3.7.2. Assay failure.

Each assay was conducted with two internal standards, a crude PKC fraction from rat
Figure 3.7: 32P transfer activity from 32P-ATP to protamine sulfate, catalysed by hydroxyapatite fractions from the cytosolic portion of human PMN, treated with GMCSF.
brain and a PKC fraction from the cell type being tested. In the results shown both the PKC fractions and those being analyzed for Rx-kinase activity were assayed with Triton X-100 micelles containing either PS or PS plus TPA (or Rx), in the presence and absence of calcium. The PKC fractions showed characteristic levels of phosphorylation with TPA, indicating that the assay was functioning normally.

Although the internal standards showed that the assay worked normally with TPA and PKC, it could be argued that the Rx had lost its biological activity. TLC analysis of Rx showed a single spot with a characteristic Rf value, implying no degradation. Furthermore, similar problems in detecting Rx-kinase activity were experienced when fresh Rx was bought from Calbiochem. It therefore seems unlikely that poor quality Rx was responsible for low levels or absence of Rx-kinase activity.

3.7.3. Degradation during isolation procedure.

All the isolation procedures were carried out as rapidly as possible at 4 °C, using buffers which contained a range of protease inhibitors and had carefully adjusted pH (see methods). The procedures used by previous researchers to isolate Rx-kinase (e.g. Ryves 1989) were followed meticulously. The isolation procedure is complex and in the early stages of the project took a long time to perform. However as the techniques became familiar this time was reduced and there should have been a concomitant increase in activity if degradation was responsible, this was not observed. Typically the Rx-kinase fractions from the hydroxyapatite column were stored overnight and assayed the next day, but even when fractions were isolated and assayed on the same day there were only low levels of activity and sometimes none at all.
3.7.4. **Insufficient source material.**

Low levels of source material could have generated levels of Rx-kinase activity below the detection threshold of the assay or that was lost through non-specific binding to the containers used. A range of quantities of source materials were used in an attempt to isolate Rx-kinase. The quantities used by Ryves and Sharma (see introduction) did not produce the reported levels of activity. Furthermore there was no concomitant increase in the levels of Rx-kinase activity observed when the cell number was increased, e.g. rat alveolar macrophages (see table 3.3, page 77). Similarly only low levels of activity were observed when large quantities of bovine lung (i.e. 37 g) were used, generating 6.5 units, and this result was not reproducible. The ratio of activity to starting material reported by Ryves and Sharma could not be reproduced, despite the use of the same protocol.

3.7.5. **Absence of Rx-kinase in these preparations.**

The final possible explanation for the lack of Rx-kinase activity could be an absence of Rx-kinase in the preparations. There is strong evidence for the existence of Rx-kinase in a range of inflammatory cells, including reports of activity from four separate sources (Ryves et al. 1989 and Sharma et al. 1995), but it is possible that this protein is not expressed, or exists in an inactive form in the resting state of these cells. In this model active Rx-kinase would be expressed or released only upon specific phagocyte priming or activating conditions.

There are several lines of evidence that support this postulate. Firstly the observation of an enhancement in Rx-kinase activity in alveolar macrophages exposed to various priming agents (Sharma, unpublished). Secondly the enhancement in Rx-kinase activity observed
by Sharma in neutrophils and monocytes from mixed donors that are incubated together prior to homogenisation. Thirdly Rx-kinase activity seen in starch elicited murine peritoneal macrophages, was not seen in resident murine peritoneal macrophages (Sharma et al. 1995). Finally the late eluting protamine sulfate phosphorylating activity seen in human neutrophils was not always detected, even though cells were isolated and prepared in a constant fashion.

The immediate problem with this model is the lack of Rx-kinase activity seen in this project, even with white blood cells from mixed donors, plumbagin treated cells or starch elicited peritoneal macrophages. The results outlined in this chapter imply that these procedures alone do not reproducibly induce Rx-kinase activity. Sharma found that treatment with plumbagin or Rx, or mixing leukocytes from different donors, enhanced basal levels of Rx-kinase activity. However he did not show that these procedures induced activity in cells showing no basal Rx-kinase activity. It is therefore possible that these procedures do not actually induce Rx-kinase activity, merely increase basal activity. Alternatively these procedures may require additional factors to successfully induce activity. Such behaviour has been observed with other systems, for example the cytokine tumour necrosis factor α (TNFα) does not stimulate hydrogen peroxide production from neutrophils in suspension, but stimulates massive and immediate hydrogen peroxide production from neutrophils adhered to fibronectin (Nathan 1987). It is possible that the procedures for priming of Rx-kinase activity reported by Sharma depended on some unrecorded factor that was not reproduced in this study.

It was therefore decided to study one cell type, human neutrophils, in detail. The aim was
to find a procedure that reproducibly primed the cells for Rx-kinase activity. Furthermore, in case Rx-kinase activity was being lost during the isolation procedure, a biological response to Rx was investigated.

That biological response was superoxide production by phagocytes. Evans observed that Rx stimulated superoxide production, in starch elicitated mouse peritoneal macrophages, to 50% of the levels seen with TPA, but only to 20% in resident mouse peritoneal macrophages (Evans, A.T. et al. 1990). This result showed that starch elicitation primed the cells for Rx stimulated superoxide production. Furthermore, in a cell free assay, exogenous Rx-kinase selectively activated NADPH-oxidase in the presence of Rx, but not TPA (Evans, A.T. et al. 1990). These results show that Rx stimulated superoxide production and imply that Rx-kinase may be involved in the activation of NADPH-oxidase in phagocytes. It was therefore decided to continue the hunt for Rx-kinase by measuring the level of Rx stimulated superoxide production from human neutrophils. A search for agents that enhanced Rx stimulated superoxide production and therefore that might induce Rx-kinase, was begun using the superoxide assay described in the next chapter.
Chapter 4. Development of the superoxide anion assay for human neutrophils.

4.1 Introduction.

The assay for the production of superoxide from neutrophils was based on the method of Pick (Pick 1986). Pick developed a micro-titre plate based assay for the measurement of superoxide production by macrophages in culture, using an automatic enzyme immunoassay reader. The method of Pick was converted for use with neutrophils to give the final method outlined in the materials and methods chapter. A number of parameters had to be optimised for use with neutrophils and these are outlined below.

4.2 Cytochrome c limitations.

Pick (Pick 1986) used a concentration of 160 μM cytochrome c to measure superoxide production from guinea pig macrophages. Experiments were conducted to establish the best cytochrome c concentration to use with human neutrophils. The stimulation of superoxide production from neutrophils (1 x 10^6 cells/ml) was tested with three concentrations of cytochrome c, namely 320 μM, 160 μM and 80 μM. The 320 μM cytochrome c was too high to allow transmission of light and therefore was rejected for use in this assay. The results of the experiments using 160 μM and 80 μM cytochrome c are shown in figures 4.1 and 4.2, page 94. Figure 4.2 shows that the upper threshold for detection of superoxide using 80 μM was half that using 160 μM cytochrome c. These figures imply that cytochrome c was limiting and that the upper detection threshold of the assay is determined by the cytochrome c concentration. Therefore, 160 μM cytochrome c was selected to ensure a concentration of cytochrome c high enough to trap the superoxide before it had a chance to spontaneously dismute to oxygen or hydrogen.
Figure 4.1: Time course for TPA stimulated superoxide production, detected with 160 micromolar cytochrome c.

Figure 4.2: Time course for TPA stimulated superoxide production, detected with 80 micromolar cytochrome c.
peroxide.

4.3 \textbf{Superoxide dismutase}.

Pick (Pick 1986) did not routinely check whether cytochrome c reduction by stimulated guinea pig macrophages was SOD inhibited because there is no evidence for cytochrome c reduction by materials other than superoxide on the surface of, or released by, intact macrophages. This hypothesis was tested for human neutrophils stimulated with various concentrations of TPA, in the presence or absence of SOD. The results are shown in figures 4.3, 4.4, 4.5 and 4.6, page 96. At all concentrations of TPA tested 300 U/ml SOD abolished all cytochrome c reduction, implying that the reduction of cytochrome c by neutrophils stimulated with TPA was due entirely to superoxide. Mayo (Mayo and Curnutte 1990) has also shown that when neutrophils are stimulated with TPA, over 99% of the reduction of cytochrome c is inhibitable by superoxide dismutase. Therefore, although a SOD control was always included as a measure of cytochrome c independent light absorption, stimulant + SOD controls were not routinely used in this project.

4.4 \textbf{Cell concentration}.

The stimulation of superoxide production by TPA was measured with a range of cell concentrations (1 \times 10^4 - 1 \times 10^7 cells/ml). The time courses for these experiments are shown in figures 4.7 - 4.10, page 97. These time courses show that at cell concentrations of less than 10^5 cells/ml only minimal superoxide production was seen, even with 1 \mu M TPA (see figures 4.9 and 4.10). At 1 \times 10^7 cells/ml the superoxide production reached the upper detection threshold of the assay in under 18 minutes, with \geq 0.1 \mu M TPA (see figure 4.7), preventing calculation of the initial rate of superoxide production, because
Figure 4.3: A graph showing that the reduction of cytochrome c by human neutrophils, stimulated with 1 micromolar TPA, is completely inhibited by 300 U/ml SOD.

Figure 4.4: A graph showing that the reduction of cytochrome c by human neutrophils, stimulated with 0.1 micromolar TPA, is completely inhibited by 300 U/ml SOD.

Figure 4.5: A graph showing that the reduction of cytochrome c by human neutrophils, stimulated with 0.01 micromolar TPA, is completely inhibited by 300 U/ml SOD.

Figure 4.6: A graph showing that the reduction of cytochrome c by human neutrophils, stimulated with 0.001 micromolar TPA, is completely inhibited by 300 U/ml SOD.
Figure 4.7: Detection of TPA stimulated superoxide production from human neutrophils at a concentration of 10 million cells/ml.

Figure 4.8: Detection of TPA stimulated superoxide production from human neutrophils at a concentration of 1 million cells/ml.

Figure 4.9: Detection of TPA stimulated superoxide production from human neutrophils at a concentration of 0.1 million cells/ml.

Figure 4.10: Detection of TPA stimulated superoxide production from human neutrophils at a concentration of 0.01 million cells/ml.
readings were taken every 30 minutes. Therefore a concentration of $1 \times 10^6$ cells/ml was selected for use in the assay, as it allowed calculation of the initial rate, whilst giving a reasonable level of superoxide production (see figure 4.8, page 97). Mayo and Curnutte (1990) have shown that superoxide production from neutrophils is linear with respect to cell concentration over the range of $0.5 \times 10^6$ to $3.5 \times 10^6$ cells/ml using a microassay. Furthermore Lehrer and Cohen (1981) found that the superoxide production from neutrophils, stimulated by TPA (0.5 ng/ml = 0.811 nM), was linear with respect to cell concentration over the range of $10^4$ - $10^7$ cells/ml.

4.5 Solvent problems.

Pick (Pick 1986) used dimethyl sulfoxide (DMSO) as a solvent for stimulants and stated that the final concentration of DMSO in the assay should not exceed 0.1% (v/v). However, since acetone is routinely used as the solvent for phorbol esters in our laboratory, it was used for the superoxide microassay. When it became apparent that acetone itself affected the production of superoxide by neutrophils, a range of different solvents were tested. The solvents tested were acetone, dimethylsulfoxide (DMSO), ethanol and methanol, over a range of concentrations (10% - 0.01% v/v). They were tested for their ability to effect superoxide production, compared to a positive control (1 μM TPA) and a blank (no stimulant). The results are shown in figures 4.11 to 4.14, page 99. At 10% (v/v) all the solvents inhibited superoxide production and with concentrations ≤ 0.01 % (v/v) all the solvents stimulated superoxide production. However, at 1% (v/v) acetone and ethanol inhibited superoxide production, whilst DMSO and methanol had little or no effect.
Figure 4.11: A graph showing the effect of various concentrations of acetone on superoxide production from human neutrophils.

Figure 4.12: A graph showing the effect of various concentrations of DMSO on superoxide production from human neutrophils.

Figure 4.13: A graph showing the effect of various concentrations of ethanol on superoxide production from human neutrophils.

Figure 4.14: A graph showing the effect of various concentrations of methanol on superoxide production from human neutrophils.
The acetone effect was not constant, with some cell preparations little or no superoxide production was seen with acetone, but with other preparations acetone alone stimulated high levels of superoxide production. It was found that 50 mM EGTA washes of the cells entirely abolished acetone stimulated superoxide production, implying the involvement of calcium and/or magnesium ions (see figure 4.15, page 101). Perhaps high concentrations of acetone caused a phase transition of lipids in the cell membranes allowing calcium influx into cells. Once sterile technique was used to prepare the cells only low levels of acetone stimulated superoxide production were seen, suggesting that the acetone effect involves cell priming/activation.

An attempt was made to remove the acetone from the assay by adding the stimulant to an eppendorf tube and allowing the acetone to evaporate off before adding the cytochrome c. However, the stimulation of superoxide production from neutrophils by TPA using this method was much lower than when TPA, dissolved in acetone, was directly added to cytochrome c (see figure 4.16, page 101). This result implies that acetone is required to ensure the solubility of TPA in the aqueous reaction buffer, presumably by decreasing the non-specific binding to the tube.

Since serial dilution was being used to prepare cytochrome c containing stimulants, little advantage would accrue from changing the solvent. Therefore acetone was used as the solvent, but at concentrations < 0.1 % (v/v) and acetone controls were included in all assays. The superoxide production due to a stimulant was calculated from (A550nm + stimulant, dissolved in acetone) - (A550nm + acetone vehicle alone).
Figure 4.15: A graph showing inhibition of acetone stimulated superoxide production by 50 mM EGTA washes of the cells.

Figure 4.16: A graph showing the difference in superoxide production from human neutrophils when TPA was added with or without acetone.
4.6 Calculation of data.

The absorbency values at 550 nm were converted to nanomoles of superoxide based on the extinction coefficient of (reduced minus oxidized) cytochrome C \( \Delta E_{550\text{nm}} = 2.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \), according to Massey (Massey 1959). Since, the volume in each well was 200 \( \mu \text{l} \), the vertical light path passing through the micro titre plate was 4 \text{ mm} long, and one mole of superoxide reduces one mole of cytochrome c, the number of nanomoles of superoxide per well can be calculated from the following equation;

\[
A_{550\text{nm}} \times \frac{1}{2.1 \times 10^4 \text{M}^{-1} \text{cm}^{-1}} \times 0.2\text{ml} \times \frac{1000\text{ml}}{0.4\text{cm}}
\]

Therefore, the number of nanomoles of superoxide per well can be calculated by multiplying the \( A_{550\text{nm}} \) by 23.81. However, as explained above, the solvent itself caused some superoxide production, therefore the true value for superoxide production caused by a stimulant is given by \( (A_{550\text{nm stimulant}} - A_{550\text{nm acetone}}) \times 23.81 \).

The original cytochrome c assay for superoxide used single time points to calculate the rate of superoxide production (Babior et al. 1973). However, it has since become apparent that the production of superoxide by phagocytes is not linear with time, depending on the type of stimulus there may be a lag of between 5 seconds and 10 minutes before the production reaches maximal velocity and the duration of the response varies with type of stimulus used (Mayo and Curnutte 1990). Therefore single fixed time readings cannot be used for calculation of the rate of superoxide production. In this project multiple readings were taken allowing a time course for the production of superoxide to be observed. This meant that in some cases negative values for superoxide production were seen. These values represented cases where cells showed high levels of superoxide production with acetone alone, but that acetone stimulated superoxide production was inhibited by the treatment of the cells by, for example, phorbol ester.
superoxide to be plotted. The initial rate of superoxide production could then be calculated from these time courses. Clearly this method is not as accurate as the continuous assays developed by Cohen (Cohen and Chovaniec 1978) and others, but the disadvantage of the discontinuous nature of the microassay was made up for by its ability to assay multiple samples at once.

The superoxide assay described in this chapter was used to measure TPA and Rx stimulated superoxide production from human neutrophils. Then it was used to screen compounds for ability to induce and/or enhance Rx stimulated superoxide production. These experiments are described and discussed in chapter 5.
5.1 Introduction.

TPA (100 nM) was found to stimulate high levels of superoxide production from human neutrophils and such a response had an immediate onset (see figure 5.1, page 105). The response with Rx was more complex, in the majority of cases cells stimulated with Rx (100 nM) did not produce any superoxide, despite showing a normal TPA response, such cells were dubbed type A (see figure 5.1, page 105). However, some cells demonstrated superoxide production when stimulated with Rx (100 nM), associated with a late onset (≥ 60 minutes) and these cells were dubbed type B (see figure 5.2, page 105). Therefore, in stimulation experiments, neutrophils exhibited two separate sensitivities to Rx, despite the use of the same cell isolation and assay procedure. Rx is unable to stimulate PKC in vitro at concentrations less than 1.6 μM (Ryves et al. 1991), it therefore seemed unlikely that the response of type B cells was mediated by PKC, making Rx-kinase a more reasonable candidate. Furthermore, Rx stimulated superoxide production, like Rx-kinase activity, was sporadic, suggesting that the biological action of Rx may have been mediated by Rx-kinase. The sporadic nature of Rx-kinase activity and Rx stimulated superoxide production also suggests that both were inducible. It was decided to try and find compounds that were capable of inducing Rx stimulated superoxide production (i.e. converting cells from type A to type B), in the hope that such chemicals would also induce Rx-kinase activity.

A number of chemicals that activate superoxide production in phagocytes prime the response at sub-threshold doses. Subsequent activation occurs with lower concentrations
Figure 5.1: Stimulation of superoxide production from human neutrophils by TPA and Rx. (The Rx stimulation is type A.)

Figure 5.2: Stimulation of superoxide production from human neutrophils by TPA and Rx. (The Rx stimulation is type B.)

Graphs showing the relationship between TPA and Rx concentrations and superoxide production over time.
of activator and leads to a larger superoxide production of longer duration (Johnston and Kitagawa 1985; Haslett et al. 1989; Walker et al. 1990). A study was conducted to establish whether any of these known priming agents could enhance Rx stimulated superoxide production. The priming agents used in these experiments were: opsonised zymosan (O.Z.); Substance 'P' (SP) or Nε-Sp; lipopolysaccharide (LPS); muramyl dipeptide (MDP); platelet aggregating factor (PAF); the irritant plumbagin (PLM); and the cytokine GM-CSF. The ability of primers + Rx to stimulate superoxide production, was compared to Rx and primers alone.

5.2 Substance P.

Substance P is an eleven amino acid peptide distributed throughout the mammalian central and peripheral nervous system. As well as having a neurotransmitter role it has been implicated in inflammatory responses. Inactivation of SP by oxidation of the terminal methionine group occurs in aqueous solutions. A non-oxidisable analogue of SP, norleucine-SP (Nε-Sp), is commonly used to avoid this problem. In human neutrophils 100 nM - 400 μM SP has been shown to activate superoxide production whereas 1 - 100 nM primes PMN for subsequent activation by f-met-leu-phe (Lloyds and Hallett 1993). In human neutrophils (type A) substance P (371 nM) stimulated superoxide production, but failed to enhance Rx (100 nM) stimulated superoxide production (see figure 5.3, page 107). The stimulation seen with SP and Rx was greater than that seen with Rx alone, but no greater than seen with SP alone, implying that the response was due entirely to SP. Lloyds and Hallett (1993) described a transient response with 10 μM SP reaching a
Figure 5.3: Phorbol ester stimulated superoxide production from human neutrophils treated with SP.

- ■ 100 nM TPA
- ← 100 nM Rx
- ← 371 nM SP
- ○ 371 nM SP + 100 nM Rx

Superoxide production (nmol)

Time (minutes)
maximum by 200 seconds and decreasing to baseline with a half life of approximately 8 minutes. The superoxide assay used in this project could not detect superoxide production within the first 10 minutes, but figure 5.3 shows the response detected after 60 minutes.

A non-oxidisable analogue of SP, norleucine-SP (\(^{\text{Nle}^{11}}\text{SP}\)), is thought to avoid SP inactivation in aqueous solutions (Lloyds and Hallett 1993). In case enhancement of Rx stimulated superoxide production by SP had been prevented by inactivation of SP experiments were also conducted with \(^{\text{Nle}^{11}}\text{SP}\). There was no enhancement of Rx stimulated superoxide production by \(^{\text{Nle}^{11}}\text{SP}\) (50 nM) (data not shown). These results show that neither SP nor \(^{\text{Nle}^{11}}\text{SP}\) induced or enhanced Rx stimulated superoxide production.

5.3 Opsonised zymosan (OZ).

Zymosan is a yeast cell preparation which is readily opsonised (coated by complement component C3b) during incubation in serum. It is commonly used as an activator of superoxide production in phagocytic cells (Pick 1986).

The level of superoxide production seen with opsonised zymosan (1 mg/ml) and Rx (100 nM) was once again greater than that seen with Rx (100 nM) alone, but no greater than with OZ (1 mg/ml) alone (see figure 5.4, page 109). Thus although 1 mg/ml OZ stimulated superoxide production, no enhancement of Rx stimulated superoxide production was observed.
Figure 5.4: Stimulation of superoxide production from human neutrophils by phorbol esters and OZ.
5.4 Platelet-aggregating factor.

The bio-active lipid PAF is produced in neutrophils in response to chemotactic compounds such as f-met-leu-phe. PAF has been shown to prime neutrophils and monocytes for enhanced superoxide production with subsequent stimulation by chemotactic peptides (Ingraham et al. 1982).

PAF (1-1000 nM) showed no enhancement of Rx (100 nM) stimulated superoxide production, although TPA stimulated a response showing that the cells were alive and capable of superoxide production (e.g. see figure 5.5, page 111).

5.5 Lipopolysaccharide and Muramyl dipeptide.

Lipopolysaccharides and muramyl dipeptides are chemoattractant peptides isolated from bacteria. Incubation of PMN with 10-100 ng ml$^{-1}$ LPS for at least 15 minutes has been shown to lead to a more rapid and greater superoxide response upon subsequent stimulation with various agonists, compared to agonist alone (Forehand et al. 1989).

LPS (1-1000 ng/ml) showed no enhancement of Rx (100 nM) stimulated superoxide production (e.g. see figure 5.6, page 111). Another experiment showed that 1000 ng/ml LPS activated superoxide production implying that the LPS used was active (see figure 5.7, page 111).

MDP (1-1000 nM) did not induce Rx (100 nM) stimulated superoxide production (e.g. see figure 5.8, page 111).
Figure 5.5: stimulation of superoxide production from human neutrophils by phorbol esters and PAF.

Figure 5.6: stimulation of superoxide production from human neutrophils by phorbol esters and LPS.

Figure 5.7: stimulation of superoxide production from human neutrophils by TPA and LPS.

Figure 5.8: stimulation of superoxide production from human neutrophils by phorbol esters and MDP.
5.6 Plumbagin.


Plumbagin (100 nM) was unable to induce any Rx (100 nM) stimulated superoxide production (see figure 5.9, page 113). However, plumbagin did affect TPA stimulated superoxide production, high doses (≥ 1 μM) inhibited the TPA response (see figure 5.10, page 113). This inhibition probably reflects the innate toxicity of plumbagin. Furthermore, in some cases plumbagin (100 nM) enhanced the TPA response (see figure 5.11, page 113). The enhancing effect of PLM on the TPA response could be abolished by washing the cells twice with EGTA (5 mM) (see figure 5.12, page 113). This implies that the enhancement of TPA stimulated superoxide production required divalent cations. This type of abrogation is very similar to that observed with acetone stimulated superoxide production, described in chapter 4. It therefore seems probable that the enhancement of the TPA stimulated response was an artefact, due to extra acetone added as solvent for plumbagin.

In the above experiments plumbagin was added to the cells at the same time as the phorbol ester (TPA or Rx). In case enhancement of the Rx response required pre-priming by plumbagin, an experiment was conducted in which neutrophils were incubated with plumbagin (0.5 μM) for one hour prior to the addition of Rx. The cells were counted before and after incubation with plumbagin and no cell death was observed. No enhancement of Rx stimulated superoxide production was observed in the plumbagin
Figure 5.9: Stimulation of superoxide production from human neutrophils by TPA and Rx in the presence of plumbagin.

Figure 5.10: Stimulation of superoxide production from human neutrophils by TPA and resulferatorxin in the presence of plumbagin.

Figure 5.11: Stimulation of superoxide production by TPA (+/- plumbagin).

Figure 5.12: Stimulation of superoxide production by TPA and plumbagin in human neutrophils treated with EGTA (5 mM).
treated cells, showing that plumbagin did not induce the Rx response (data not shown).

5.7 Overnight effect.

During the priming studies it was noticed that incubating cells overnight led to enhanced Rx stimulated superoxide production. Figure 5.13, page 115, shows that 200 nM Rx failed to stimulate superoxide production on day 0, but after overnight incubation of the cells, 200 nM Rx stimulated a response (see figure 5.14, page 115). The increase in sensitivity of cells to Rx was not mirrored by an increase in sensitivity to TPA, indeed on day 1 the cells showed a reduced response to TPA. The fact that the TPA response was not enhanced by overnight incubation implied that changes involving PKC were not responsible for the increased sensitivity of cells to Rx (however, see later).

The overnight effect was a serendipitous discovery during an experiment to test the priming ability of LPS. The overnight culture conditions were tested, but LPS, human plasma, type of culture medium used and EGTA washes were not responsible for the enhancement of Rx stimulated superoxide production. In fact 5 mM EGTA treatment of the cells, after overnight culture, was found to inhibit the Rx response (see figures 5.15 and 5.16, page 115). The 200 nM Rx response was completely inhibited by addition of EGTA to the cell medium. Although the TPA stimulated response appears to be enhanced in figure 5.16, page 115, this was due to the high background production seen in the absence of EGTA (see figure 5.17, page 116). Subtraction of background levels of superoxide production gave artificially low values for the TPA response in the absence of EGTA.
Figure 5.13: stimulation of superoxide production by TPA and Rx on day 0.

Figure 5.14: stimulation of superoxide production by TPA and Rx on day 1.

Figure 5.15: stimulation of superoxide production by TPA and Rx (+/- EGTA) on day 0.

Figure 5.16: stimulation of superoxide production by TPA and Rx (+/- EGTA) on day 1.
Figure 5.17: Stimulation of superoxide production from human neutrophils by TPA and Rx (+/- EGTA).

Figure 5.18: Rx stimulated superoxide production from human neutrophils on day 0.

Figure 5.19: Rx stimulated superoxide production from human neutrophils on day 1.
The overnight effect was investigated further using a range of concentrations of Rx. With a range of 1 nM - 1 μM it was found that only 1 μM Rx stimulated a response and only on day 1 (see figures 5.18 and 5.19, page 116, data in both figures from the same preparation of cells). When a range of 10 nM - 10 μM Rx was used it was discovered that 10 μM Rx strongly stimulated superoxide production on day 0 (see figure 5.20, page 118). In this case 1 μM Rx stimulated a low response demonstrating that the sensitivity of fresh cells to Rx was variable. However, after incubation overnight these cells showed a greatly increased response with 1 μM Rx compared to day 0, whilst the 10 μM Rx response remained strong (see figure 5.21, page 118). These results suggest that overnight incubation of neutrophils produced a state of high sensitivity to Rx, perhaps due to induction of Rx-kinase.

A range of concentrations of TPA were also used to investigate the overnight effect. Stimulating the cells with 10 nM - 1 μM TPA revealed that overnight incubation not only affected the sensitivity of cells to Rx, but also to TPA (see figure 5.22 and 5.23, page 118). On day 1 (figure 5.23) the overall level of superoxide production had decreased, relative to day 0 (figure 5.22), but the sensitivity of the cells to TPA had increased. These results imply that overnight incubation, as well as enhancing the Rx response, could also enhance the TPA response. Ryves has shown that TPA was unable to activate Rx-kinase in an in vitro assay, but strongly activated all classical and novel PKC isotypes (Ryves, W.J. PhD Thesis, University of London 1991; Ryves et al. 1991). This suggests that the enhancement in TPA stimulated superoxide production after overnight incubation was due to changes in the PKC system rather than alterations involving Rx-kinase. Furthermore, using an in vitro assay, Ryves showed that at concentrations of 1.6
Figure 5.20: R1x stimulated superoxide production from human neutrophils on day 0.

Figure 5.21: R1x stimulated superoxide production from human neutrophils on day 1.

Figure 5.22: TPA stimulated superoxide production from human neutrophils on day 0.

Figure 5.23: TPA stimulated superoxide production from human neutrophils on day 1.
μM and above Rx can activate β1-PKC (Ryves et al. 1991). Therefore, since overnight incubation enhanced both Rx and TPA responses, and since Rx can stimulate PKC at high doses, it seems possible that the overnight effect was due, at least in part, to changes involving PKC.

5.8 Further investigations of the overnight effect.

On one occasion incubation of neutrophils overnight led to superoxide production in the absence of stimulant, and examination of the cultures showed microbial contamination. It seemed possible that the increased sensitivity of cells to Rx expressed overnight was due to low levels of microbial contamination and that higher levels of contamination led to activation of the cells. The bacterial extracts LPS and MDP had failed to enhance Rx stimulated superoxide production (see above), therefore it was decided to try priming by yeast. Superoxide assays were conducted on neutrophils that had been incubated with yeast or opsonised zymosan, but no enhancement in the Rx stimulated response was observed (data not shown).

In another attempt to determine whether the overnight effect was due to microbial contamination cells were prepared with and without sterile technique, but no difference was observed when the cells were assayed for Rx stimulated superoxide production on day 1 (data not shown). Furthermore some cell preparations showed high sensitivity to Rx on day 1 (i.e. superoxide production with 100 nM Rx) even when sterile technique and antibiotics were used. Unfortunately a day 0 assay was not conducted making it impossible to tell whether the high sensitivity of the cells was due to an overnight enhancement, or an innately high sensitivity to Rx.
The cells prepared using sterile technique had been cultured at lower concentrations than the previous overnight experiments, therefore experiments were conducted to determine whether cell concentration affected the overnight effect. Cells prepared using sterile technique were incubated overnight with antibiotics in a culture flask (3.16 x 10^6 cells/ml) or a 15 ml Falcon centrifuge tube (1.4 x 10^7 cells/ml) then aliquots of cells at concentrations of 1 x 10^6 cells/ml were assayed for levels of superoxide production after 240 minutes of Rx stimulation (see table 5.1). There was no difference in Rx stimulated superoxide production between the two concentrations of cells. This implies that the high cell concentration in the Falcon tube was not responsible for the overnight effect.

<table>
<thead>
<tr>
<th>Table 5.1: The ability of Rx to stimulate superoxide production in PMN cultured at different concentrations (t = 240 minutes).</th>
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<tr>
<td><strong>Rx concentration. (µM)</strong></td>
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<td>10.00</td>
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<tr>
<td>1.00</td>
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5.9 Rx-kinase and the overnight effect.

Cells incubated overnight were assayed for Rx-kinase activity. In the first experiment a mixed population of monocytes and neutrophils was incubated overnight. A superoxide assay was conducted on day one and a low dose of Rx (100 nM), previously unable to stimulate a response, was shown to stimulate a superoxide production of 3.45 ± 0.53 nmoles after 260 minutes. The cells were homogenised and protein fractions separated
Figure 5.24: Rx-kinase activity in protein fractions separated by hydroxypatite FPLC, from a mixed population of monocytes and neutrophils incubated overnight.

(Figure shows a graph with 32P transfer (assay units) on the y-axis and fraction on the x-axis. The graph includes data points for 100 nM Rx - Ca2+ and 100 nM Rx + Ca2+. Negative values represent cases where blanks were higher than tests and correspond to zero 32P incorporation into the substrate.)
by hydroxyapatite FPLC using phosphate gradient E, then each fraction was assayed for PS/Rx/±Ca\(^{2+}\) stimulated histone-III\(\alpha\)s phosphorylating activity (see figure 5.24, page 121). Fraction 4 catalysed transfer of \(^{32}\)P from \(^{32}\)P-\(\gamma\)-ATP to histone-III\(\alpha\)s in a PS- and Rx-dependent, but calcium inhibited, fashion. Both the elution profile and the cofactor requirements for the activity in fraction 4 were characteristic of Rx-kinase These results suggested that overnight incubation enhanced Rx stimulated superoxide production, which correlated with the presence of Rx-kinase activity.

In the next experiment human neutrophils were incubated overnight and on day one it was found that 100 nM Rx stimulated the production of 5.47 ± 0.48 nmoles of superoxide after 292 minutes. Once again the cells were homogenised and protein fractions separated by hydroxyapatite FPLC, but this time the fractions were assayed for protamine sulfate phosphorylating activity (see figure 5.25, page 123). Figure 5.25 shows that cells incubated overnight contained protamine sulfate kinase activity with the same elution characteristics (eluted by 367 mM phosphate) of the Rx-kinase activity previously detected in neutrophils (see chapter 3).

Human neutrophils, incubated overnight, did not reproducibly contain this activity, but its absence correlated with type A cells, i.e. those showing low sensitivity to Rx. Such differences are typified by comparison of figure 5.25 with figure 5.26 (see page 123), where the difference between the protamine sulfate phosphorylation profiles can be seen. This was reflected by the Rx stimulated superoxide production of each of the whole cell preparations (see inset graphs of figures 5.25 and 5.26). These results show that there was a correlation between the sensitivity of cells to Rx, as judged by superoxide
Figure 5.25: Protamine sulfate phosphorylating activity in protein fractions separated by hydroxyapatite FPLC, from human neutrophils incubated overnight. (Inset: resiniferatoxin stimulated superoxide production in whole cells).

Figure 5.26: Protamine sulfate phosphorylating activity in protein fractions separated by hydroxyapatite FPLC, from human neutrophils incubated overnight. (Inset: resiniferatoxin stimulated superoxide production in whole cells).
production, and the protamine sulfate phosphorylation profile. Furthermore cells that did not produce superoxide in response to 100 nM Rx were never found to contain late eluting protamine sulfate phosphorylating activity.

5.10 GM-CSF priming.

Yeast, OZ, LPS and MDP had all failed to enhance the Rx response in a manner similar to overnight incubation. Perhaps other mediators of inflammation were responsible, either released from a putative microbial infection or from the white blood cells themselves (e.g. cytokines). Cytokines were prohibitively expensive to use in this project, but Dr. Pamela Roberts of University College London kindly donated some GM-CSF free of charge. GM-CSF is a cytokine released by macrophages and bone stromal cells and known to prime human neutrophils for subsequent activation by FMLP (Roberts et al. 1993). Human neutrophils were treated with GM-CSF (10 ng/ml) or vehicle (FCS) for at least 45 minutes, then both sets of cells were assayed for TPA and Rx stimulated superoxide production. No enhancement of TPA or Rx stimulated superoxide production was observed (data not shown).

5.11 Elicited cells.

It was decided to investigate Rx stimulated superoxide production in elicited cells. An intra peritoneal injection of starch suspension, into mice, has been shown to elicit macrophages (Evans et al. 1990). Resident macrophages are thought to ingest the starch particles and release chemotactic mediators that attract other phagocytic cells into the peritoneal cavity. It was hoped that the cocktail of chemotactic chemicals released by the macrophages would prime the cells for Rx stimulated superoxide production. Both mice
and guinea pigs were used and the results are outlined below.

5.11.1 **Starch elicited murine peritoneal macrophages.**

On three separate occasions starch elicited murine peritoneal macrophages were assayed for superoxide production. The cells exhibited TPA (10-1000 nM) stimulated superoxide production (see figure 5.27, page 126), but no response was seen with Rx (1-1000 nM) (see figure 5.28, page 126). These results conflict with Evans et al. (1990) who found that starch elicited cells showed Rx stimulated superoxide production, whereas resident cells showed no response with Rx. A number of experiments were conducted in an attempt to reproduce the results of Evans et al. (1990), but with no success. It is possible that the parameters outlined by Evans et al. (1990) were not enough to induce Rx stimulated superoxide production in mouse peritoneal macrophages and that some unrecorded procedure contributed to the response. Also, 60 - 90 mice were required to generate enough macrophages for the superoxide assay, consequently the isolation procedure was lengthy. Therefore, in case the length of the isolation procedure contributed to the lack of the Rx response, it was decided to use guinea pigs, which provided enough cells per animal for the superoxide assay.

5.11.2 **Starch elicited guinea pig peritoneal macrophages.**

A guinea pig given an intra peritoneal injection of starch yielded $1.16 \times 10^7$ cells compared to the control animal which only yielded $2.64 \times 10^6$ cells. The larger number of cells in the starch treated animal implies that elicitation had taken place. Both elicited and resident cells showed superoxide production with TPA (10-1000 nM) stimulated superoxide production (see figures 5.29 and 5.30, page 126). The resident cells showed
Figure 5.27: Stimulation of superoxide production from starch elicited murine peritoneal macrophages by TPA.

Figure 5.28: Stimulation of superoxide production from starch elicited murine peritoneal macrophages by Rx.

Figure 5.29: TPA stimulated superoxide production from starch elicited guinea pig peritoneal macrophages (1.33E+6 cells/ml).

Figure 5.30: TPA stimulated superoxide production from resident guinea pig peritoneal macrophages (1.52E+5 cells/ml).
lower superoxide production compared to the elicited cells (see figure 5.30, page 126).

The lower levels of superoxide production observed with the resident cells can be explained by the lower cell concentration used in the assay; 1.52 x 10^6 cells/ml for resident and 1.53 x 10^6 cells/ml for elicited cells.

Elicited guinea pig peritoneal macrophages showed strong superoxide production with Rx at high concentrations (see figure 5.31, page 128), whilst resident cells only showed very low superoxide production (see figure 5.32, page 128). In both cases, with elicited and resident cells, low concentrations of Rx failed to stimulate the cells. Both cell types showed a weak response with 1 μM Rx, implying that starch elicitation did not enhance Rx stimulated superoxide production. The lack of superoxide production stimulated by 10 μM Rx in resident cells was probably due to toxicity of high levels of Rx and acetone, or priming, by the starch elicitation, of the PKC system of resident cells.

Since starch elicitation alone failed to enhance the Rx response, it was decided to prime elicited cells with plumbagin (PLM). In the first experiment starch elicited cells co-stimulated with Rx (1 μM) and PLM (10 nM) showed enhanced superoxide production compared to cells stimulated by Rx (1 μM) alone (see figure 5.33, page 128). Figure 5.33 also shows that PLM (10 nM) enhanced the TPA (100 nM) response, in a manner similar to that seen with human neutrophils. This result suggests PLM affected PKC stimulated superoxide production, a postulate further supported by the observation that enhancement of both the Rx and TPA response was inhibited in cells treated with EGTA (see figure 5.34, page 128). These results suggest that the enhancement of the Rx response by PLM may involve PKC and calcium, rather than Rx-kinase. The enhancement of the Rx
response by PLM could not be investigated further as it was not reproducible, another preparation of starch elicited guinea pig peritoneal macrophages showed no enhancement of Rx stimulated superoxide production by plumbagin. This observation implies that PLM alone was not enough to induce the response and that other signals must be involved.

5.14 Discussion.

During the development of the superoxide assay it became clear that the Rx response was variable, i.e. the same concentration of Rx gave different levels of superoxide production with each cell preparation. This led to a classification of neutrophils according to their sensitivity to Rx. For example the majority of neutrophils were type A, i.e. failed to respond with Rx (100 nM), however, some were type B i.e. produced high levels of superoxide in response to Rx (100 nM). Type B neutrophils were particularly interesting, since Rx-kinase is activated by 100 nM Rx, but PKC isotypes are not, and indeed fail to bind Rx at concentrations below 10 μM (Sharma et al. 1995; Ryves et al. 1991; Dimitrejevic et al. 1995). It was decided to test compounds for ability to convert cells from type A to type B.

A number of compounds known to prime human neutrophils for subsequent stimulation by agonists were tested for ability to enhance Rx stimulated superoxide production. The compounds tested were SP, Nε11SP, O.Z., PAF, LPS, MDP, PLM and GM-CSF, but none enhanced Rx stimulated superoxide production. However, during the priming study, it was noticed that overnight incubation of cells reproducibly enhanced the Rx response.

An attempt was made to determine the mechanism responsible for the overnight increase in
sensitivity of cells to Rx. The experimental conditions associated with the first observation of the overnight effect were dissected, but LPS, human plasma, type of culture medium, cell density, and microbial contamination were found not to be responsible. Washes of the cells with EGTA and subsequent determination of superoxide production in calcium free medium were found to abolish the overnight enhancement of the Rx response, implying that divalent cations were required for the overnight effect.

Phorbol ester stimulated superoxide production has been shown to be sensitive to changes in intracellular calcium concentrations (Di Virgilio 1984). Initial studies with a single, low (100 nM) concentration of TPA suggested that TPA stimulated superoxide production was not affected by overnight incubation of cells. However, analysis of a range of 1 nM - 1 μM TPA revealed that the TPA response was also enhanced in cells incubated overnight. This result suggested that the overnight effect could be due to changes involving the PKC system, rather than changes involving Rx-kinase. It was therefore decided to analyse neutrophils, that had been incubated overnight, for PKC and Rx-kinase.

Initial analysis of human neutrophils incubated overnight revealed that they contained protamine sulfate phosphorylating kinase activity with a similar elution profile to Rx-kinase. However subsequent analysis showed that human neutrophils incubated overnight did not always contain late eluting kinases, suggesting that overnight incubation did not always induce Rx-kinase activity. Analysis of the dose-response relationship for Rx stimulated superoxide production did, however, reveal a correlation between the presence of late eluting kinases and high sensitivity of cells to Rx (i.e. at concentrations of 100 nM or less, where Rx does not appear to interact with PKC isotypes (Ryves et al. 1991;
Sharma et al. 1995; Dimitrejevic et al. 1995). Cells that produced superoxide in response to 100 nM Rx did contain late eluting kinases suggesting a direct connection between these observations. These results also demonstrate that overnight incubation increased the sensitivity of cells to high (μM) concentrations of Rx, probably through a PKC-mediated mechanism, since Rx has been found to interact with some PKC isotypes at these concentrations (Ryves et al. 1991; Dimitrejevic et al. 1995). However, overnight incubation did not reproducibly enhance the sensitivity of cells to the 100 nM threshold that correlated with the presence of late eluting kinases.

The results suggest that experiments successfully inducing high Rx sensitivity in phagocytic cell superoxide production may thereby induce a family of related protamine sulfate/Rx kinases in these cells. Elucidation of such mechanisms of induction would be highly rewarding in unravelling the biochemical basis of inflammation.
Chapter 6: Investigation of phorbol ester stimulated superoxide production.

6.1 Introduction.

Other researchers have employed a number of indices to measure the dose-response relationship of superoxide production. They are: the lag time (i.e. the time between addition of the stimulus and the onset of the response (Cohen and Chovaniec 1978)); the activation time (i.e. the time between addition of stimulus and development of a linear rate of superoxide production (Cohen and Chovaniec 1978)); the time to $\frac{1}{2} V_{\text{max}}$ (i.e. the time, after addition of stimulus, to reach half the maximal rate of superoxide production (Lehrer and Cohen 1981); and the maximal rate (Mayo and Cumutte 1990). The format of the micro-titre assay developed for this project meant that the first time point could not be measured until 7 minutes after the addition of the stimulant, meaning that accurate determination of the onset time could not be made. This slight disadvantage was amply offset by the fact that the micro-titre assay allowed the measurement of superoxide production from multiple samples at once. It was therefore decided to use indices based upon the rate of response, rather than indices based on the onset time, as a measure of superoxide production.

6.2 TPA stimulated superoxide production.

Experiments using various concentrations of TPA revealed heterogeneous superoxide production in freshly prepared neutrophils. Whilst TPA was always capable of eliciting a strong superoxide burst, the concentration dependence varied, even though the assay conditions and the cell isolation procedure were kept constant. For example, figures 6.1 to 6.4, page 133, demonstrate four typical ‘cases’ of superoxide production stimulated by
TPA over a concentration range of 1-1000 nM, during a five hour period.

6.2.1. The initial rate of superoxide production.

The initial rate of superoxide production, i.e. the rate of response during the first 30 minutes after addition of stimulus, was used as a measure of phorbol ester stimulated superoxide production, since the response was linear over this time period. This calculation allowed the construction of dose-response curves for TPA stimulated superoxide production from various preparations of neutrophils. These results are shown in figure 6.5, page 135, which clearly demonstrates the heterogeneity of responses by various preparations of cells. It can be seen from figure 6.5 that cells could be divided into two categories, on the basis of the maximal rate of response achieved. The first category of cells (category 1) were capable of achieving high maximal rates of 0.426 ± 0.075 nmoles/minute (n = 10), within thirty minutes after the addition of TPA. Whilst the second category of cells (category 2) were only capable of achieving low maximal rates of 0.158 ± 0.029 nmoles/minute (n = 5), within thirty minutes after the addition of TPA. The superoxide responses associated with cells belonging to each of these categories are summarised in tables 6.1 and 6.2, and discussed in the next two sections.

<table>
<thead>
<tr>
<th>Table 6.1; A summary of the TPA stimulated superoxide production associated with category 1 cells (n = 10).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum rate of response, within 30 minutes after addition of stimulus.</td>
</tr>
<tr>
<td>Maximum level of superoxide production.</td>
</tr>
<tr>
<td>Maximum level of superoxide production, without acetone adjustment.</td>
</tr>
<tr>
<td>Initial rate ED$<em>{50%}$ TP$</em>{A\text{max}}$.</td>
</tr>
</tbody>
</table>
Figure 6.5: Dose response curves for TPA stimulated superoxide production.
6.2.2. **Cells capable of achieving high initial rates of superoxide production (category 1 cells).**

Table 6.1, page 134, shows that all the preparations of category 1 cells demonstrated similar maximal levels (12.37 ± 3.32 nmoles), and similar maximal rates (0.426 ± 0.075 nmoles/minute), of superoxide production. This suggests that the category 1 cells from different preparations had similar superoxide producing capabilities. However, in contrast, the ED_{50} values were heterogeneous, as shown by a high standard deviation (58.43 ± 69.81 nM), demonstrating that the sensitivity of individual preparations of cells to TPA varied. Thus, although high (μM) doses of TPA were always capable of potent stimulation of superoxide production in all category 1 cells, the level of TPA required to stimulate half the maximal response varied. This implies that the cells from different preparations were primed to different extents.

6.2.3. **Cells capable of achieving only low initial rates of superoxide production (category 2 cells).**

Category 2 cells demonstrated low rates of TPA-induced superoxide production compared...
with category 1 cells (see table 6.2, page 136). However, the maximal levels of superoxide production that could be produced by category 2 cells was similar to that seen with category 1 cells. These results suggest that the rate of response in the category 2 cells was limited in a physiological manner. Low cell number, or dead cells, could not be a cause, because cells were counted, with trypan blue as a measure of viability, before each assay. Furthermore, the difference between category 1 and 2 cells could not have been due to donor differences because the same donors gave both category 1 and 2 cells on different occasions. A possible explanation is that the rate of production of the respiratory burst oxidase substrate (NADPH) was lower in category 2 cells. The availability of the other respiratory burst oxidase substrate (oxygen) should have been the same in each preparation, because HBSS, the cell buffer and cytochrome c solvent, was prepared in a constant manner. Alternatively the activity of the respiratory burst oxidase could have been more stringently controlled in the category 2 cells.

Table 6.2 also shows that although different preparations of category 2 cells demonstrated similar maximal levels of superoxide production (12.61 ± 1.71 nmoles), the ED\textsubscript{50} values of the responses were again heterogeneous (15.84 ± 16.59 nM), as was observed with category 1 cells. This shows that although category 2 cells from different preparations had the same superoxide producing capability, the cells were primed to different extents.

6.2.4. Three classes of sensitivity to TPA.

Although the dose response curves showed heterogeneity, it was possible to group the cells into three general classes, termed high, medium and low sensitivity to TPA (figure
Graphs showing the three different sensitivities of cells to TPA. Cells were isolated as described in the methods section and assayed using the micro-titre superoxide assay. Graphs show dose-response curves for TPA stimulated superoxide production from various preparations of cells, data is expressed as % of TPA maximum.
6.6, page 138). Cells that showed high sensitivity to TPA yielded significant superoxide production with concentrations of TPA as low as 1 nM, a response associated with an ED$_{50}$ value of 2.44 ± 1.74 nM TPA (see figure 6.6A, page 138). Medium sensitivity cells only showed superoxide production with a TPA concentration greater than 10 nM TPA, and the response was characterised by an ED$_{50}$ value of 30.53 ± 11.82 nM TPA (see figure 6.6B, page 138). Whilst low sensitivity cells only showed superoxide production with concentrations of TPA greater than 100 nM, and such responses were associated with an ED$_{50}$ value of 189.45 ± 44.95 (see figure 6.6C, page 138). These results imply that different activation states exist for human neutrophils, exhibited here as different sensitivities to stimulation by TPA.

6.2.5. Late onset TPA stimulated superoxide production.

Analysis of the stimulation of superoxide production by TPA was further complicated because in some cases superoxide production had a late onset time (i.e. > 30 minutes). Late onset superoxide production, with TPA, was only observed at low concentrations (≤ 10 nM TPA), higher concentrations always causing immediate onset superoxide production. There appeared to be two types of late onset superoxide production, the first did not begin until after 90 minutes, whilst the second began after only 30 minutes.

6.2.5.1. Superoxide production beginning after 90 minutes.

The first type of late onset response began 90 minutes after the addition of TPA and was only observed with less than 10 nM TPA (see figure 6.7, page 140). It was only seen with cells that showed high background levels of superoxide production (i.e. stimulated by acetone vehicle alone) and the delay in onset represented the time taken for the TPA
Figure 6.7: A graph showing the late onset of TPA stimulated superoxide production in cells showing high background superoxide production with acetone alone, data adjusted for background superoxide production.
Figure 6.8: Time course of TPA stimulated superoxide production in cells showing high background superoxide production with acetone alone.
response to rise above that seen with acetone alone (see figure 6.8, page 141). To get a true expression of the TPA response, the stimulation by the acetone carrier solvent was subtracted, which generated the type of time course shown in figure 6.7, page 140. This type of data manipulation is valid if the superoxide production before 90 minutes was due only to the acetone carrier solvent, with the TPA stimulated response only occurring after 90 minutes. However, there are two cases in which the subtraction of acetone values would not be valid. The first case is where TPA and acetone stimulated superoxide production through different receptors, and these receptors lie before the rate limiting step of the pathway. Thus, acetone and TPA initially showed the same rate of superoxide production, but after 100 minutes the acetone signal was terminated, hence no more acetone stimulated superoxide was produced, but the TPA signal continued, generating the time course observed. The second case is where acetone and TPA stimulated superoxide production through the same receptor, but by different mechanisms. Thus the activation of that receptor by the two stimulants was not additive because the rate of superoxide production was limited by the amount of that receptor. Under this model the difference between stimulation of superoxide by TPA or acetone after 90 minutes occurred because the acetone signal was terminated, but the TPA signal continued. If either of these two scenarios was involved then TPA could have been stimulating a response before 90 minutes, and therefore subtraction of acetone values would not be valid. In that case the late onset time of 90 minutes would be an artefact of the calculation of the data. Further analysis of the acetone response is discussed in section 6.2.6.1.

6.2.5.2. Superoxide production beginning after 30 minutes.

The second type of late onset superoxide production showed entirely different
characteristics: it was only observed with the lowest dose of TPA (1 nM); began after 30 minutes; was seen even when acetone stimulated (background) superoxide production was low; and often rose to higher maximal levels than seen with 10 nM TPA (see figure 6.9, page 144). The second type of late onset superoxide production clearly could not be an artefact of subtraction of acetone values, since there was little or no acetone stimulated superoxide production (see figure 6.9). This type of late onset response could have been due to changes in the PKC system (e.g. reflecting the time for PKC translocation) or the superoxide generating complex (e.g. reflecting the time for activation by PKC and/or other signals). This type of late onset response seemed unaffected by the termination mechanisms that controlled the immediate response (see figure 6.4, page 133), this is discussed further in section 6.2.8.

6.2.6. Explanation of the heterogeneous sensitivities of cells to TPA.

Although the cells from different preparations were capable of generating similar total levels of superoxide, the sensitivity of those cells to TPA, and the onset time of such responses, were variable (see above). Experiments were conducted to investigate the cause of such variability.

6.2.6.1. Activation state of the cells.

As mentioned in chapter 4 in some cases acetone alone stimulated superoxide production. This background level of superoxide production was not constant from one cell preparation to the next, it could be split into three distinct categories, on the basis of the
Figure 6.9: A graph showing late onset superoxide production beginning after 30 minutes. TPA - acetone = data adjusted for acetone values. TPA - SOD = data not adjusted for acetone values.
maximum level of response stimulated by acetone. These categories were; high (8.23 ± 1.93 nmoles), medium (4.32 ± 0.71 nmoles), or low (2.25 ± 0.67 nmoles) levels of acetone stimulated superoxide production. It was thought that these categories might represent the activation state of the cells and as such correlate with the various sensitivities of cells to TPA. This was not the case, as demonstrated in table 6.3, which shows that cells categorised according to acetone sensitivity had highly variable ED₅₀ values for TPA. Thus, the sensitivity of cells to TPA did not correlate with the sensitivity of cells to acetone. This argues against these compounds stimulating superoxide production through the same receptor.

<table>
<thead>
<tr>
<th>Category of acetone stimulated response</th>
<th>Maximum level of acetone stimulated superoxide production (nmoles)</th>
<th>Mean ED₅₀ for TPA (nM).</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>8.23 ± 1.93</td>
<td>115.60 ± 80.39</td>
</tr>
<tr>
<td>Medium</td>
<td>4.32 ± 0.71</td>
<td>8.16 ± 8.90</td>
</tr>
<tr>
<td>Low</td>
<td>2.25 ± 0.67</td>
<td>24.05 ± 14.89</td>
</tr>
</tbody>
</table>

Despite the lack of correlation between the sensitivity of superoxide production to TPA and acetone, there was a correlation between low acetone stimulated superoxide production and preparation of cells using sterile technique, i.e. cells prepared using sterile technique did not exhibit medium or high levels of acetone stimulated superoxide production (data not shown). These results imply that medium and high levels of acetone stimulated production were a result of cell priming during cell isolation, perhaps due to contamination by microorganisms. Furthermore, as outlined in chapter 4, acetone
stimulated superoxide production could be inhibited by EGTA treatment of cells, implying that divalent cations were involved in the sensitizing of cells to acetone.

6.2.6.2. Overnight Incubation of Cultures (day 0 or day 1).

Cells were isolated and assayed for TPA stimulated superoxide production either on the same day (day 0) or cultured overnight and assayed the day after (day 1). It is possible that changes in the cells overnight may have led to changes in sensitivity to TPA. However, there was no correlation between the day of the assay and sensitivity of the cells to TPA (see table 6.4). Table 6.4 shows that the superoxide production of cells assayed fresh, or incubated overnight, both demonstrated a similar and wide range of ED$_{50}$ values for TPA. This shows that the sensitivity of cells to TPA did not depend on overnight incubation of the cultures.

| Table 6.4. The effect of overnight incubation of neutrophils on TPA stimulated superoxide production. |
|---|---|
| Age of culture | Mean ED$_{50}$ for TPA (nM) |
| Day 0 | 33.81 ± 46.80 (n = 7) |
| Day 1 | 53.34 ± 70.16 (n = 8) |

6.2.6.3. Sterile technique.

One possible explanation for the variable sensitivity of cells to TPA is that the cells were primed by the isolation procedure itself. For example, contamination of cell cultures with microorganisms could have primed the cells. To test this hypothesis cells were prepared using sterile technique, in order to minimise the chance of microbial contamination. As
mentioned above cells prepared using sterile technique always exhibited low levels of acetone stimulated superoxide production, implying that the acetone effect was due to microbial contamination. Cells prepared using sterile technique also demonstrated similar dose-response curves (see figure 6.10, page 148), suggesting that the variable sensitivity of cells to TPA may have been due to microbial contamination of the cell cultures.

The conclusions outlined above were derived from the initial rate of superoxide production, i.e. the rate of response within the first 30 minutes after addition of TPA, and imply that the TPA sensitivity of cells prepared by sterile technique was constant. However, late onset superoxide production was variable even in cells prepared using sterile technique. This shows that some mechanism other than priming by microbial contamination mediated the late onset response, and suggests that cells were primed to different extents within the bodies of donors.

6.2.7. EGTA treatment of cells.

The in vitro phosphorylation activity of classical PKC isotypes (α, β1, β2 and γ) has been shown to increase in the presence of calcium (Ryves et al. 1991). Since the activation of superoxide production by phorbol esters is thought to be mediated by PKC, experiments were conducted to test the effect, on the TPA response, of depleting cytosolic calcium levels by repeatedly washing cells with EGTA buffer (Di Virgilio et al. 1984). The maximum rates and maximum levels of TPA stimulated superoxide production were similar in different preparations of EGTA washed cells, but the TPA sensitivity was still variable (see figure 6.11, page 149, and table 6.5, page 151). The TPA sensitivity of cells prepared using sterile technique yielded TPA dose response curves with a mean ED$_{50}$ of 38.33 ± 6.83 (i.e. medium sensitivity cells). Cells that were isolated without the use of sterile technique generated TPA dose response curves that fell into low, medium or high sensitivities. In the absence of data on the level (or not) of microbial contamination in these cell preparations it was impossible to speculate whether microbial contamination increased or decreased the sensitivity of cells to TPA.
Figure 6.10: Dose response curves for cells prepared using sterile technique.
Figure 6.11: Dose response curves for TPA stimulated superoxide production, from cells treated with EGTA.
Figure 6.12: The variation in TPA sensitivity of cells treated with EGTA.
EGTA washed cells (see figure 6.12, page 150) varied in a similar manner to the cells untreated by EGTA (see section 6.2.1.). These results show that removal of calcium by repeated EGTA washes could not remove the variation in sensitivity of cells to TPA. This implies that the variation in sensitivity to TPA was not due to the influence of calcium ions.

<table>
<thead>
<tr>
<th>Table 6.5; A summary of the superoxide production associated with EGTA treated cells (n = 6).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum rate of response, within 30 minutes after addition of stimulus.</td>
</tr>
<tr>
<td>Maximum level of superoxide production.</td>
</tr>
<tr>
<td>Maximum level of superoxide production, without acetone adjustment.</td>
</tr>
<tr>
<td>Initial rate ED$<em>{50}$ TPA$</em>{max}$.</td>
</tr>
</tbody>
</table>

In one experiment (20.5.94) the cells showed a category 2 response (i.e. demonstrated a low initial rate of response, see figure 6.11, page 149). Cells prepared on the same day, from the same donor, but not treated with EGTA were also category 2 (see figure 6.5, page 135). This shows that treating cells with EGTA did not increase the low initial rates of response, and suggests that the cause of this behaviour was independent of calcium.

6.2.7.1. The effect of EGTA on the TPA sensitivity of individual preparations of cells.

The TPA sensitivity of cells treated with EGTA was compared with the TPA sensitivity of cells, from the same neutrophil preparation, that had not been treated with EGTA. In general EGTA treatment of cells had little effect on the sensitivity of cells to TPA (see table 6.6). However this was not always the case, EGTA treatment enhanced the sensitivity of cells to TPA on 8.6.94, and decreased the sensitivity on 21.4.94 (see table
Thus, in general, the initial rate of TPA stimulated superoxide production was not sensitive to cellular calcium depletion, but in some specific cases the response was highly sensitive to EGTA treatment. Furthermore it is interesting to note that in these EGTA sensitive cells, only the 100 nM TPA response was affected (see figure 6.13 and 6.14, page 153). These results imply that EGTA treatment only affected the sensitivity of cells to TPA under certain conditions, and in a complex way, perhaps through multiple mechanisms (e.g. translocation of PKC, downregulation of PKC by calcium dependent proteases, or modulation of the NADPH oxidase complex by calcium dependent proteins), and that the mechanism(s) only become relevant at 100 nM TPA.

### Table 6.6: Variation in TPA ED$_{50}$ upon EGTA treatment

<table>
<thead>
<tr>
<th>Date</th>
<th>ED$_{50}$ (nM)</th>
<th>Change in TPA ED$_{50}$, upon EGTA treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- EGTA</td>
<td>+ EGTA</td>
</tr>
<tr>
<td>19.5.94</td>
<td>23.40</td>
<td>14.45</td>
</tr>
<tr>
<td>16.4.94</td>
<td>43.60</td>
<td>23.99</td>
</tr>
<tr>
<td>5.7.94</td>
<td>144.50</td>
<td>158.50</td>
</tr>
<tr>
<td>29.6.94</td>
<td>7.24</td>
<td>3.80</td>
</tr>
<tr>
<td>8.6.94</td>
<td>234.40</td>
<td>47.86</td>
</tr>
<tr>
<td>21.4.94</td>
<td>40.00</td>
<td>275.42</td>
</tr>
</tbody>
</table>

6.2.8. Termination of superoxide production.

Since the reduced cytochrome c (ferrous) cannot spontaneously reconvert to the oxidised (ferric) form under the assay conditions used, the level of the plateau seen in the superoxide production time courses (e.g. see figures 6.1 to 6.4, page 133) represents the
Figure 6.13: Dose response curves for cells treated with (+) and without (-) EGTA.

Figure 6.14: Dose response curves for cells treated with (+) and without (-) EGTA.
total amount of ferrous cytochrome c present. For reasons outlined in chapter 4, the cytochrome c concentration was thought to be limiting in this assay, it was therefore concluded that the plateau in the level of response seen with high (µM) concentrations of TPA was due to exhaustion of oxidized (ferric) cytochrome c, through reduction by superoxide ions, and represented the upper detection threshold of the assay. Since the concentration of cytochrome c was 160 µM and the volume added to each well of the microtitre plate was 100 µl the upper detection threshold of the assay should have been 16 nmoles of superoxide. This was generally the case (see figure 6.15, page 155), however, since the data for the TPA response was adjusted for acetone stimulated superoxide production, the upper detection threshold was lower in cell preparations that demonstrated an acetone response (see figure 6.15, page 155). Thus the plateau formed by the curve for the 1 µM TPA response was taken as the upper detection threshold of the assay.

In some cases the response stimulated by ≤ 100 nM TPA reached a plateau at lower levels than seen with 1 µM TPA (see figure 6.1, page 133). This type of response was thought to be due to termination of the superoxide production, rather than exhaustion of ferric cytochrome c, since the maximal level observed with 100 nM TPA was significantly below that seen with 1 µM levels of TPA. This type of response was not an artefact of the adjustment for the acetone response as it was still seen when the data was not adjusted for acetone stimulated superoxide production (see figure 6.16, page 155). Furthermore superoxide induced cell death seems an unlikely explanation for the termination of the 100 nM TPA response, as cells were not killed by the higher levels of superoxide produced with 1 µM TPA, implying that neither the levels of superoxide produced by 100 nM TPA,
Figure 6.15: The upper detection threshold of the superoxide assay. TPA - SOD: values not adjusted for the acetone response, whilst TPA - acetone: values after subtraction of acetone response.

Figure 6.16: Termination of TPA stimulated superoxide production. Values are not adjusted for the acetone response.
nor that concentration of TPA, were toxic for the cells. All this argues in favour of a physiological termination mechanism that regulates the level of TPA stimulated superoxide production in neutrophils.

In some cases the superoxide production seen with high (μM) doses of TPA levelled off well below the upper detection threshold of the assay (see figure 6.4, page 133). Figure 6.4 shows that the response to 1 nM TPA rose to higher total levels than the response to 1 μM TPA, and implies that the plateau formed by the higher doses (≥ 10 nM TPA responses) was due to termination of the response rather than exhaustion of ferric cytochrome c. The adjustment of the data for acetone values can be ruled out as a cause of the lower maximal level of the 1 μM TPA response, because the maximal level of the 1 μM TPA response still occurred below that for the 1 nM TPA response when the data was not adjusted for acetone values (see figure 6.17, page 157). Once again cell death can also be ruled out, because the higher levels of superoxide production seen with 1 nM TPA were not toxic to the cells and in other experiments 1 μM TPA was not toxic. Thus the termination mechanism was far more stringent in these cells, implying variability in the termination mechanism. The cells that demonstrated stringent termination control also showed high sensitivity to TPA (i.e. ED_{50} = 2.44 ± 1.74 nM), which implies a link between the state of activation of the cells and the stringency of termination of superoxide production. Furthermore, low rates of superoxide production were not terminated within the time course of the assay, even in cells that demonstrated stringent termination of high rates of response (e.g. see 1 nM TPA response in figure 6.4, page 133). This result suggests that termination of superoxide production may be controlled by a feedback mechanism, based upon the rate or level of the response (e.g. a rapid superoxide
Figure 6.17: Stringent termination of superoxide production. Data is not adjusted for acetone values.
production is ‘sensed’ and restricted by the putative termination mechanism). Such a restriction would not be triggered by the slow rate of response, seen here with 1 nM TPA (however see also section 6.3.5). The termination mechanism could operate at the level of either PKC (the phorbol ester receptor), or the NADPH oxidase complex.

6.2.8.1. Abrogation of termination in EGTA pre-washed cells.
Termination of TPA stimulated superoxide production could have operated at the level of either PKC (e.g. by downregulation of the kinase) or the NADPH oxidase (e.g. by dephosphorylation of p47phox). However, evidence that the termination mechanism is calcium-dependent came from studies on the effect of EGTA washes of cells on superoxide production. Figure 6.18 (page 159) shows that in cells not treated with EGTA (TPA - EGTA) superoxide production was stringently controlled, the response to > 10 nM TPA being rapidly terminated. However, this termination was relieved by washing the cells with EGTA (see figure 6.18, TPA + EGTA, page 159), leading to enhancement of the maximal level of response seen with ≥ 100 nM TPA (see figure 6.18, page 159). These results suggest that a calcium-dependent control mechanism exists in neutrophils capable of terminating the production of superoxide.

6.3. Rx stimulated superoxide production.
It has already been shown that neutrophils demonstrated variable sensitivities to Rx (see chapter 5). It was shown in that chapter that two categories of cell responses to Rx were identified which were termed type A and type B. Type A cells did not produce superoxide upon stimulation with Rx (100 nM), whereas type B cells responded strongly to stimulation by Rx (100 nM). When a range of concentrations of Rx were used it was
Figure 6.18: The effect of EGTA washes on termination of TPA stimulated superoxide production. Cells were repeatedly washed with buffer + EGTA (+ EGTA) or washed with buffer alone (- EGTA) and then assayed for superoxide production.
found that 10 μM Rx always stimulated a strong response, even in type A cells (i.e. cells that did not produce superoxide in response to 100 nM Rx). The response to 1 μM Rx was more variable, but was always seen in cells incubated overnight, unlike the response to 100 nM Rx which could not be induced by overnight incubation.

The concentration dependence of Rx stimulated superoxide production was further investigated. As with TPA the rate of superoxide production stimulated by each concentration (10 μM - 1 nM) of Rx was calculated and used to construct dose-response curves (see figure 6.19, page 161). Figure 6.19 reveals that cells responded to Rx in two distinct ways: high sensitivity cells demonstrated superoxide production in response to concentrations of Rx as low as 100 nM, and at much higher rates than low sensitivity cells, which only demonstrated a response at high (μM) concentrations of Rx. The high sensitivity cells correspond to the type A cells described in chapter 5, as they demonstrated a response with 100 nM Rx. The low sensitivity cells correspond to the type B cells of chapter 5, since they failed to demonstrate a response with 100 nM Rx.

6.3.1. Cells that demonstrated a low sensitivity to Rx.

Cells demonstrating low sensitivity to Rx were able to generate high maximal levels of superoxide production, but only at a very slow rates (see table 6.7). Thus, Rx was much less potent at stimulating superoxide production than TPA, a point underscored by the high ED50 value (1.25 μM) for Rx (see table 6.7, page 162).
Figure 6.19: Dose response curves for Rx stimulated superoxide production.
Table 6.7; A summary of the superoxide production from cells that demonstrated a low sensitivity to Rx (n = 6).

<table>
<thead>
<tr>
<th>Maximum rate of response.</th>
<th>0.072 ± 0.025 nmoles/minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum level of superoxide production.</td>
<td>9.18 ± 1.26 nmoles</td>
</tr>
<tr>
<td>Maximum level of superoxide production, without acetone adjustment.</td>
<td>12.00 ± 1.54 nmoles</td>
</tr>
<tr>
<td>Initial rate ED₅₀% Rxₘₐₓ.</td>
<td>1.25 ± 0.97 μM</td>
</tr>
</tbody>
</table>

6.3.2. Cells that demonstrated a high sensitivity to Rx

A summary of the superoxide production from cells that demonstrated a high sensitivity to Rx is shown in table 6.8. The high maximal level of superoxide production stimulated by Rx in these cells was similar to that seen with TPA (see table 6.1, page 134), but the rate of the Rx stimulated response was lower (2-fold slower). This shows that Rx was less potent than TPA at stimulating superoxide production, even in cells showing a high sensitivity to Rx.

Table 6.8; A summary of the superoxide production from cells that demonstrated a high sensitivity to Rx (n = 1).

<table>
<thead>
<tr>
<th>Maximum rate of response.</th>
<th>0.214 nmoles/minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum level of superoxide production.</td>
<td>14.16 nmoles</td>
</tr>
<tr>
<td>Maximum level of superoxide production, without acetone adjustment.</td>
<td>15.35 nmoles</td>
</tr>
<tr>
<td>Initial rate ED₅₀% Rxₘₐₓ.</td>
<td>301.99 nM</td>
</tr>
</tbody>
</table>

The maximal rate of Rx stimulated superoxide production was 3 fold higher in cells that showed a high sensitivity to Rx, compared to those cells that demonstrated only a low sensitivity to Rx (see tables 6.7 and 6.8, page 162). This implies that either the levels of
Rx-receptors were higher, or that the NADPH-oxidase was more sensitive to the Rx
stimulated signal in these cells. Several researchers (Ryves et al. 1991; Dimitrejevic et al.
1995) have shown that *in vitro* high (μM) concentrations of Rx interact with/activate β-
PKC isotypes. If the high sensitivity of cells to Rx was due to upregulation of PKC, one
would expect those cells to demonstrate high sensitivity to TPA as well. However, the
cells that demonstrated a high sensitivity to Rx (ED$_{50}$ = 302 nM) only demonstrated a
medium sensitivity to TPA (ED$_{50}$ = 33 nM), whilst cells demonstrating a high sensitivity to
TPA (ED$_{50}$ = 2.44 ± 1.74 nM) did not demonstrate high sensitivity to Rx (compare figures
6.5, page 135 and 6.19, page 161). This suggests that a receptor distinct from phorbol
ester sensitive isotypes of PKC mediated the high sensitivity of cells to Rx. An increase in
sensitivity of the oxidase to the Rx signal can be ruled out, as cells showing high sensitivity
to TPA did not show high sensitivity to Rx. The most likely candidate for the PKC-
distinct receptor is Rx-kinase, which Evans (Evans et al. 1990) showed was capable of
activating NADPH-oxidase *in vitro*. Furthermore, as previously shown (chapter 5) cells
demonstrating high sensitivity to Rx (type B cells) always contained protamine kinase
activity with the same elution profile as Rx-kinase, whereas cells demonstrating low
sensitivity to Rx never contained such kinase activity.

6.3.3. Onset time of Rx stimulated superoxide production.

Unlike TPA, Rx rarely stimulated immediate onset superoxide production. The majority
of cell preparations responded to Rx with late onset superoxide production, with negligible
response until at least 30 minutes after the addition of Rx (e.g. see figure 6.20, page 164).
As described above, the late onset of the TPA response was only observed with low (nM)
concentrations of TPA. In contrast, late onset superoxide production was
Figure 6.20: Late onset of Rx stimulated superoxide production.

- ■ 1000 nM TPA
- → 10,000 nM Rx
- ○ 1,000 nM Rx
observed even with high (μM) concentrations of Rx.

Two different types of late onset response were observed with Rx. The first type was only seen with 1 μM Rx and the late onset represented the time for the Rx response to rise above that seen with acetone alone, typically onset > 150 minutes (see figure 6.21, page 166). For the reasons outlined in the TPA section this type of late onset response may have been an artefact of the data calculation.

The second type was observed with the highest doses (10 μM) of Rx used, typically had an onset time of 30 minutes, and was seen even when acetone alone caused only a low response (see figure 6.22, page 166). This type of late response could not have been an artefact of the data calculation, because the values for acetone stimulated superoxide production were low and therefore subtracting them made no difference to the onset time.

The two categories of late onset response seen with Rx match those seen with TPA. However, whereas the late onset response was seen with concentrations of Rx as high as 10 μM, it was only seen with low (1 nM) concentrations of TPA. It has already been shown that Rx was less potent at activating the respiratory burst than TPA, so it seems probable that 10 μM Rx could only activate neutrophil PKC to the same levels as 1 nM TPA, and hence the response to 1 nM TPA and 10 μM Rx both demonstrated the same onset times. Since high (μM) doses of TPA stimulated a response with an immediate onset, it can be concluded that the PKC mediated signal had to reach a threshold level before the NADPH-oxidase complex became active. Under this model low doses of TPA, or maximal doses of Rx, would take more time to reach that threshold level of
Figure 6.21: Late onset superoxide production. Data shows phorbol ester stimulated superoxide production before acetone adjustment.

Figure 6.22: Late onset superoxide production. Data shows phorbol ester stimulated superoxide production before acetone adjustment.
activation. However, it must be noted that the threshold of activation could also reflect the amount of time required for PKC translocation, the 'quality' of PKC translocation (e.g. the level of penetration of the enzyme into the membrane), or other biophysical properties (e.g. the length of time for the phorbol esters to reach a critical intracellular concentration, a reflection of the lipophilicity of the compounds).

6.3.4. EGTA effects on Rx stimulated superoxide production.

Cells were depleted of calcium by washing with EGTA and then the response to Rx was measured, the results of these experiments are summarised in table 6.9. All the cell preparations used in these experiments showed low sensitivity to Rx, and the EGTA treatment did not alter this sensitivity. The EGTA treated cells were still able to achieve high levels of superoxide production, which were of the same order as seen with the untreated cells (compare tables 6.8, page 162 and 6.9, page 167), showing that calcium depletion did not alter the superoxide producing capacity of the cells. However, EGTA washes inhibited the superoxide production stimulated by the acetone solvent, therefore, since values for the acetone response were routinely subtracted from the values for the Rx response, the maximal level of response was slightly higher in the EGTA treated cells.

Table 6.9; A summary of the Rx stimulated superoxide production from cells washed with EGTA (n = 4).

<table>
<thead>
<tr>
<th>Maximum rate of response.</th>
<th>0.132 ± 0.037 nmoles/minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum level of superoxide production.</td>
<td>12.64 ± 1.40 nmoles</td>
</tr>
<tr>
<td>Maximum level of superoxide production, without acetone adjustment.</td>
<td>13.60 ± 0.81 nmoles</td>
</tr>
<tr>
<td>Initial rate ED$<em>{50%}$ Rx$</em>{max}$.</td>
<td>2.68 ± 0.31 μM</td>
</tr>
</tbody>
</table>
The mean maximal rate of the Rx response in normal cells was $0.072 \pm 0.025$ nmoles/minute, but increased by 1.8 fold to $0.132 \pm 0.037$ nmoles/minute in EGTA washed cells. This increase could not be attributed to artefacts created by the adjustment of the data for acetone values, as it was still seen in cells that showed only low levels of acetone stimulated superoxide production (see figure 6.23, page 169). These results suggest that fresh neutrophils contain a calcium-dependent ‘braking’ mechanism that limits the rate of Rx stimulated superoxide production, and that treatment with EGTA releases that ‘brake’. It is interesting to note that the TPA stimulated response was regulated in a similar manner (see figure 6.23, page 169), but it was the maximal level, rather than the rate, of the response that was limited in normal cells.

The increase in the rate of the Rx response was only observed with high (10 μM) concentrations of Rx. The rate of the Rx response seen with lower (1 μM) concentrations was unaffected by EGTA treatment of the cells. Thus the ED 50 value for Rx stimulated superoxide production appeared to increase in EGTA washed cells (see table 6.9, page 167), as it was expressed as a percentage of maximum rate seen with Rx in each experiment. Therefore, although the rate of response with 1 μM Rx appeared to decrease, when in reality it remained constant, after EGTA treatment, since it was expressed as %Rx maximum.

6.3.5 Termination of Rx stimulated superoxide production.

As explained above, the upper detection threshold of the assay was 16 nmoles of superoxide, but was lower in cells that demonstrated an acetone response, since data was
Figure 6.23: The effect of EGTA washes of cells on phorbol ester stimulated superoxide production. Results have not been adjusted for acetone stimulated superoxide production.
adjusted for the acetone stimulated superoxide production. In general the maximum detected level of response seen with 1 μM TPA was limited by the available ferric cytochrome c. The same was true with high (10 μM) concentrations of Rx (see figure 6.24, page 171), the maximal level achieved by the Rx response was the same as the TPA response and corresponded to the upper detection threshold of the assay.

The maximal level of response with lower doses of Rx showed termination of the response, at levels below the upper limit of the assay (see figure 6.25, page 171). This was similar to the termination of the response seen with 100 nM TPA. Proof that the termination of the Rx response was not an artefact of the acetone is also shown in figure 6.25, page 171. The maximal level of the Rx response before adjustment for acetone values was still below the upper limits of the assay, implying that termination of the response, rather than exhaustion of ferric cytochrome c, was responsible. The termination of superoxide production could have been caused by cell death. There are two lines of argument against this suggestion. Firstly, the same cells survived higher levels and rates of TPA stimulated superoxide production, implying that the cells would not have been killed by the lower levels of superoxide generated by Rx. Secondly, in other experiments human neutrophils released higher levels of superoxide in response to 10 μM Rx, implying that Rx itself was not toxic to the cells at those concentrations. Therefore the termination of the Rx response must have been a physiological response.

Preparations of cells that demonstrated termination of the response to high (μM) concentrations of TPA, also showed termination of the response to high concentrations of Rx (see figure 6.26, page 171). Figure 6.26 shows that the responses to 1 μM TPA and
Figure 6.24: Termination of phorbol ester-stimulated superoxide production. Phorbol ester - SOD - data not adjusted for acetone response. Phorbol ester - acetone - data adjusted for acetone response.

Figure 6.25: Termination of phorbol ester-stimulated superoxide production. Phorbol ester - acetone - data adjusted for acetone values. Phorbol ester - SOD - data not adjusted for acetone values.

Figure 6.26: Stringent termination of phorbol ester-stimulated superoxide production.

Graphs show the superoxide production (in nanomoles) over time (in minutes) for different concentrations of TPA and Rx. The graphs compare the effects of TPA and Rx with and without SOD treatment, and with and without acetone response adjustment.
10 \mu M Rx were both terminated, the 1 nM response is included to show that the termination occurred at lower levels than the upper limit of the assay. These results imply that the signalling pathways that generate the Rx and TPA responses are both regulated by a similar termination mechanism. However, the 1 nM response seemed unaffected by this termination mechanism. It was argued above that the termination mechanism may be regulated by the rate of superoxide production, since responses which demonstrated high rates (i.e. 1 \mu M TPA) were terminated whilst those that demonstrated only low (i.e. 1 nM TPA) rates were not. However, figure 6.26 (page 171) suggests otherwise, since the response to 10 \mu M Rx in these cells was still terminated despite demonstrating only a low rate of response. Therefore the termination mechanism appears to be independent of the rate of superoxide production.

6.4 Rx contamination.

The Rx used in this project came from the chemicals supply company Calbiochem. Calbiochem Rx (batch BH-107) was used to study the stimulation of superoxide production, and generated the data outlined above. Rx (BH-107) was found to stimulate superoxide production from human neutrophils at concentrations > 1 \mu M (see above). However, a new batch of Calbiochem Rx, BH-108, did not stimulate superoxide production from human neutrophils, even at a concentration of 10 \mu M. Both batches of Calbiochem Rx and resiniferonol (Ro), the most likely degradation product of Rx, were compared by thin layer chromatography (T.L.C.) analysis. The Rx from both batches had the same Rf value of 0.55 and showed no contamination with Ro (Rf = 0.25) (see figure 6.27, page 173).
Figure 6.27: A photograph of a TLC plate showing the apparent purity of Calbiochem Rx batches BH-107 and BH-108, compared to Ro. (Mobile phase = 8:2 chloroform/acetone).
In case trace contaminants, undetectable by T.L.C., were responsible both batches were analysed by N.M.R. The analysis of Rx batch BH-108 yielded a trace completely consistent with Rx implying no degradation nor contamination (see figure 6.28, page 175). Unfortunately so little of Rx batch BH-107 remained that it was impossible to record a clear trace even when the sample was scanned overnight. However, the N.M.R. results of the analysis of Rx (BH-108) implied that the difference in activity between the two batches was due to a contaminant(s) in Rx (BH-107), rather than degradation of Rx (BH-108).

The inability of the T.L.C. analysis to detect any contaminants in Rx (BH-107) implied very low levels of contamination, it was therefore decided to perform TLC analysis on larger quantities of Rx. To this end all the remaining Rx BH-107 (~ 0.31 mg) was loaded onto a T.L.C. plate and separated using a mobile phase of 8:2 chloroform:acetone. Four bands could be seen under ultraviolet light with Rf values of 0.25, 0.49, 0.55 and 0.63. The silica corresponding to these four bands was scraped off the plate and washed using chloroform. Each fraction was then filtered, dried down and reconstituted in 55.7 µl of acetone (i.e. the same volume as the starting material). Each of these acetone fractions was then assayed for ability to stimulate superoxide production in human neutrophils, to ascertain whether there were any active impurities in the Rx batch (see table 6.10, page 176).

Only fraction 1 (Rf = 0.25) stimulated superoxide production to any significant level, yielding 28.9% TPAmax. The Rf value of 0.25 demonstrated by fraction 1 was not consistent with Rx (Rf = 0.55), but was consistent with the related compound Ro (Rf = 0.25). Furthermore the material corresponding to fraction 1 migrated with an Ro
Figure 6.28: The NMR trace for Rx batch BH-108.
standard run on the same TLC plate and looked identical to the Ro standard when the TLC plate was bathed in ultraviolet light. The lower level of stimulation seen with fraction 1 (28.9% of TPAmax compared to 55.1% seen with the crude Rx (BH-107)) probably reflects loss of material during the wash and filter steps as none of the other fractions stimulated superoxide production from the neutrophils.

Table 6.10: The production of superoxide from human neutrophils stimulated by Rx (BH-107), Rx (BH-108) and T.L.C. derived fractions of Rx (BH-107).

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Superoxide production (%TPAmax)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM Rx (BH-108)</td>
<td>0</td>
</tr>
<tr>
<td>10 μM Rx (BH-107)</td>
<td>56.80</td>
</tr>
<tr>
<td>Fraction 1 ex BH-107 (Rf = 0.35)</td>
<td>28.90</td>
</tr>
<tr>
<td>Fraction 2 ex BH-107 (Rf = 0.58)</td>
<td>1.20</td>
</tr>
<tr>
<td>Fraction 3 ex BH-107 (Rf = 0.65)</td>
<td>0.01</td>
</tr>
<tr>
<td>Fraction 4 ex BH-107 (Rf = 0.74)</td>
<td>0</td>
</tr>
</tbody>
</table>

Calbiochem sold Rx BH-107 and BH-108 with a quoted purity of 98% and 99%+ respectively, it would appear therefore that Rx (BH-107) was contaminated with Ro and that it was this component that was responsible for the stimulation of superoxide production seen with Rx (BH-107).

6.5 Resiniferonol stimulated superoxide production.

The contamination of Rx (batch BH-107) by resiniferonol (Ro) was only detected at the very end of the project, however a few experiments were conducted to investigate the stimulation of superoxide production by Ro. A range of concentrations (1 μM - 1 nM) of
Ro was tested in the superoxide assay and the results are summarised in table 6.11.

Table 6.11; A summary of Ro stimulated superoxide production (n = 5).

<table>
<thead>
<tr>
<th>Maximum rate of response.</th>
<th>0.191 ± 0.079 nmoles/minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum level of superoxide production.</td>
<td>13.79 ± 0.52 nmoles</td>
</tr>
<tr>
<td>Maximum level of superoxide production, without acetone adjustment.</td>
<td>15.46 ± 1.06 nmoles</td>
</tr>
<tr>
<td>Initial rate ED$_{50}$ Romax.</td>
<td>241.94 ± 28.73 nM</td>
</tr>
</tbody>
</table>

The maximal level of Ro response (13.79 ± 0.52 nmoles) was similar to that seen with TPA (12.37 ± 3.32 nmoles), however the rate of the Ro response (0.191 ± 0.079 nmoles/minute) was 45% slower than seen with TPA (0.426 ± 0.075 nmoles/minute). This shows that Ro was less potent than TPA at stimulating superoxide production. However the maximal rate of the Rx response was only 0.072 ± 0.025 nmoles/minute, showing that Ro was more potent than Rx. The mean ED$_{50}$ value for Ro was 241.94 ± 28.73 nM, suggesting that contamination of Rx with trace levels (< 4%) of Ro would lead to high levels of Ro stimulated superoxide production, if the Rx solution was added at a 10 μM concentration. This is further evidence that the stimulation of superoxide production by 10 μM Rx was due to contaminating Ro, rather than Rx itself. However it seems unlikely that the response seen with 100 nM Rx, in cells showing high sensitivity to Rx, was due to contaminating Ro, because a 4% contamination would equal only 4 nM Ro, in a 100 nM solution. It was therefore concluded that the response to high (μM) concentrations of Rx (batch BH-107) was due to contaminating Ro, that stimulated superoxide production through the PKC system. However, the response to 100 nM Rx (batch BH-107) was due to Rx itself, which stimulated superoxide production through
another mechanism. Furthermore cells only demonstrated a response to 100 nM Rx when the late-eluting peaks of protamine sulphate kinase activity were present.

6.6 Discussion.
The number of binding sites for phorbol esters in intact neutrophils has been found to vary. Lehrer and Cohen (1981) observed $2.1 \pm 0.6 \times 10^5$ sites/cell, whilst Tauber et al. (1982) observed $7.6 \times 10^5$ sites/cell. An explanation for this apparent discrepancy was proposed by O'Flaherty et al. (1990) who found that the number of phorbol ester binding sites in intact cells ($1.6 \times 10^5$) was less than the number found in disrupted cells ($9 \times 10^5$). They also found that the intracellular calcium levels affected the number of sites available to phorbol esters in intact cells, thus calcium depleted cells had $1 \times 10^5$ sites/cell, whilst ionomycin treated cells had $2.5 \times 10^5$ sites/cell. They concluded that calcium influx, caused by the ionophore, translocated PKC to the plasma membrane thus increasing the number of available sites for phorbol ester binding. These results suggest that the number of binding sites in intact cells is a reflection of the amount of PKC translocated to the plasma membrane and therefore sensitive to any procedures that cause movement of PKC.

The variation in the number of phorbol ester binding sites per cell has been reflected in the concentration dependence of phorbol ester stimulated superoxide production. For example, Tauber et al. (1982) found that TPA stimulated superoxide production with an $ED_{50}$ of $3.9 \pm 2.1$ nM, a value that agreed quantitatively with the binding affinity to neutrophils of TPA ($K_d = 4.9$ nM), in cells that exhibited $7.6 \times 10^5$ phorbol ester binding sites per cell. In contrast Leher and Cohen (1981) reported a binding affinity for TPA of 0.29 nM, that agreed closely with the dose-response for TPA stimulated superoxide
production, in cells that exhibited \( \sim 2.1 \pm 0.6 \times 10^5 \) phorbol ester binding sites per cell. Thus the dose-response relationship for TPA stimulated superoxide production reflected the number of available binding sites, which in turn reflected the amount of translocated PKC.

These researchers found that the binding affinity and ED\(_{50}\) of TPA could also be affected by cell density (Lehrer and Cohen 1981; Tauber et al. 1982). They found that as cell concentration increased the concentration of TPA required for activation also increased, whilst the lag time remained constant. They argued that two factors could account for this effect, first the absolute concentration of the phorbol ester receptor becomes significant at higher cell concentrations, (2.5 nM at 2 \( \times \) 10\(^6\), 125 nM at 10\(^8\) cells/ml). Secondly, non-specific binding due to partitioning of the ligand in the membrane of cells could decrease the free ligand concentration. Non-specific binding is particularly relevant for highly lipophilic molecules such as TPA.

In this project the cell concentration for the superoxide assay was kept constant at 1 \( \times \) 10\(^6\) cells/ml. Therefore the heterogeneity of the dose-response curves observed with different cell preparations could not have been due to non-specific binding of phorbol ester. Furthermore the cell isolation procedure and the conditions of the assay were kept constant and therefore could not have contributed to the observed heterogeneity. Two factors could be responsible, firstly the amount of PKC available to the added phorbol ester (see above) and secondly the superoxide producing ability of the cells. Each of these will be addressed in turn.
The binding studies outlined above demonstrate that the amount of PKC available to phorbol ester in intact neutrophils is variable and dependent upon the level of PKC translocation. The physiological mediator of PKC translocation is thought to be calcium (May et al. 1985; Wolf et al. 1985; O'Flaherty et al. 1990), but high concentrations of phorbol esters (320 nM, 1 x 10^7 cells/ml) have also been shown to translocate PKC (Curnutte et al. 1994). Therefore the amount of translocated PKC, and consequently the amount available to phorbol ester, is dependent upon both the intracellular calcium concentration and the level of phorbol ester added. This relationship is complicated further by the observation that sub-threshold doses of TPA (0.162 nM, 1 x 10^6 cells/ml) synergise with ionophore-induced increases in intracellular calcium, in the stimulation of superoxide production (Robinson et al. 1984). Whilst optimal doses of TPA (20 nM, 2.5 x 10^6 cells/ml) stimulate superoxide production even in cells depleted of calcium (Di Virgilio et al. 1984). These results show that at low phorbol ester concentrations calcium is required, but at higher concentrations TPA can stimulate a response in the absence of calcium. This may be because high doses of TPA translocate and activate PKC, whilst lower doses only activate PKC that has been translocated by calcium, or that high doses of TPA convert PKC to the membrane inserted form for a qualitatively stronger, and calcium-independent, effect on superoxide production.

The calcium dependence of phorbol ester stimulated superoxide production has also been investigated by Merritt et al. (1993). They found that TPA (250 pM), and SAPA (20 nM) stimulated superoxide production after a short lag (~ 1 minute) and Rx (1 μM) stimulated a response after a lag of 10 minutes, whilst DOPPA (1μM) did not stimulate any response. Sub-threshold doses of phorbol esters could be synergised by increases in intracellular
calcium, mediated by ionomycin. An increase in intracellular calcium concentration from 90 nM to 146 nM was sufficient to promote large responses with subthreshold doses of TPA (100 pM) and SAPA (5 nM), a further increase to 233 nM calcium was sufficient for Rx (500 nM), whilst an increase to 396 nM was required before DOPPA (1 μM) stimulated a response. These results support the involvement of calcium in the observed variable concentration dependence of phorbol ester stimulated superoxide production. Furthermore Merritt et al. could not quantify their results because of the complexity of the responses, but chose to use results that were typical of at least three experiments from different cell preparations. This shows that the heterogeneity of phorbol ester stimulated superoxide production observed in this PhD project has also been observed by other researchers.

If the heterogeneity of the TPA stimulated response observed in this project was simply due to differences in intracellular calcium concentrations between cell preparations, one would expect to observe constant initial rates of superoxide production with high concentrations of TPA. However, this was not found to be the case (see figure 6.5, page 135). Furthermore, translocation of PKC due to calcium has been shown to be reversible (e.g. May et al. 1985), therefore it was expected that EGTA treatment of cells would lead to constant dose-response effects if translocation due to calcium was responsible for the heterogeneity observed. This was not the case as shown by figure 6.12, page 150. These results suggest that although variation in the calcium levels of the cells, and therefore the amount of translocated PKC, may have partially contributed to the heterogeneity of the dose-response results for phorbol ester stimulated superoxide production, other events, down-stream from PKC in the signalling pathway, must also have contributed.
The variability of the superoxide producing ability of the cells has been clearly established through the discovery of cell priming. The priming of the respiratory burst, described in the previous chapter and the introduction, results in both a larger and more rapid response than seen in unprimed cells (review; Haslett et al. 1989). Priming is a multi-faceted event that has been shown to include: an increase in the number and affinity of chemotactic receptors (Vosbeck et al. 1990); an increase in the level of cytosolic calcium (Koenderman et al. 1989); an increase in DAG levels (Reibman et al. 1988; Agwu et al. 1989b; Dougherty et al. 1989); alterations in trimeric G-protein signalling (McColl et al. 1990); modulation of oxidase components (Della Bianca et al. 1988); activation of phospholipase D (Bauldry et al. 1991); activation of phospholipase A₂ (Dispersio et al. 1988); and tyrosine phosphorylation (Gomez-Cambronero et al. 1989; Lloyds et al. 1995; Lloyds and Hallett, 1995 a,b). However, the events mediated by priming vary according to the agent used. Thus, lipopolysaccharide (LPS) priming has been shown to increase intracellular calcium levels, whilst not affecting FMLP receptor number or affinity, or trimeric G-protein signalling (Forehand et al. 1989). Whilst, TNFα and SP do not affect intracellular calcium concentration, but do stimulate tyrosine phosphorylation (Lloyds et al. 1995). Evidence for priming of phorbol ester stimulated superoxide production has been presented by McPhail et al. (1984), who found that priming with FMLP (1 μM, 45 seconds) or A23187 (10μM, 15 minutes) led to 2-fold and 1.4-fold increases in the TPA (1.6 μM) stimulated response respectively. The priming events that could affect the concentration dependance of phorbol ester stimulated superoxide production would have to operate at the level of, or down stream of, PKC. Therefore changes at the level of chemoattractant receptors or trimeric G-protein signalling can be excluded. Alterations in the intracellular levels of calcium could operate at the level of PKC by causing
translocation (see above), or downstream from PKC through calmodulin or activation of phospholipase A₂ (Haslett et al. 1989). The activation of phospholipase enzymes is particularly interesting because of reports of activation of the respiratory burst by lipid moieties such as arachidonic acid, phosphatidic acid and diacyl glycerol (see introduction). It is conceivable that partial activation of the NADPH oxidase by lipid molecules could enhance the stimulation by phorbol esters. The involvement of tyrosine phosphorylation in respiratory burst activation and priming has been outlined in the introduction, and it is possible that modulation of this pathway could also alter the sensitivity of cells to phorbol esters. Such a hypothesis is strengthened by the observation by Gaudry et al. (1993) of increased tyrosine phosphorylation in cells stimulated with PDBu.

It was not only the rate of superoxide production that was found to vary in this project but also the lag time before the onset of a response. There were two types of late onset superoxide production stimulated by TPA, one began after 60 minutes and the other after 30 minutes. However, the former occurred with cells that gave a response with acetone and the length of the lag period was probably an artefact of subtraction of acetone values during the calculation. The second type occurred even in cells that showed little or no response with acetone and therefore could not have been an artefact of the calculation. Lehrer and Cohen (1981) investigated the lag time and found that it was inversely proportional to the concentration of added TPA. These researchers investigated the lag time with 0.162 - 1.94 nM TPA and recorded a maximal lag time of 25 minutes, at 37°C. They also found that a 1.6 fold drop in temperature (i.e 37°C to 23.5°C) led to a 3 fold increase in lag time, showing that lag time was temperature sensitive. In this PhD project the temperature of the assay was 37°C, therefore, according to the results of Lehrer and
Cohen, one would expect a lag of ~7 minutes with 1 nM TPA. Instead variable lag times were observed with 1 nM TPA, either immediate (i.e. less than the first time point at 7-10 minutes), late (i.e. ≥ 30 minutes), or no response at all. The variation in the lag time is further evidence that human neutrophils can be primed to different extents for the subsequent stimulation by TPA. Cells that showed no response with 1 nM TPA were probably unprimed, those that showed an immediate response were optimally primed, whilst those that showed a late response were only partially primed. The mechanism that governs the lag time has not been established, however systems that govern the translocation of the oxidase components, or 'activation clocks' may be involved. An example of a putative 'activation clock' is the continuous phosphorylation and dephosphorylation of p47^{phox}. Kinases that add phosphate groups to p47^{phox} may not lead to activation of the oxidase until the rate of phosphorylation exceeds the rate of dephosphorylation by protein phosphatases.

The final aspect of TPA stimulated superoxide production that was found to be variable in this project was the termination of the response. Physiological activators of the respiratory burst are known to cause a transient response, for example the FMLP response peaks by 45 seconds and declines to 50% of the maximum by 2 minutes (McPhail and Snyderman 1983). In contrast the TPA stimulated response has been shown to peak by 20 minutes and still not have declined by 90 minutes (McPhail and Snyderman 1983). Others have found no change in [3H]PDBu binding over a time course that exhausts the respiratory burst, and concluded that autoinactivation of the oxidase, rather than downregulation of PKC, was the cause of the termination of the phorbol ester response (Tauber et al. 1982). These results support the interpretation of the observations in this project, namely that the
levelling off of the rate of superoxide production stimulated by TPA was due to
termination of the oxidase activity, rather than exhaustion of cytochrome c. Investigations
by Light et al. (1981) were unable to determine the mechanism of termination, but found
that neither exhaustion of NADPH nor feedback inhibition by superoxide anions was
responsible. The enhancement of TPA stimulated superoxide production by EGTA, seen
in this project, argues in favour of divalent cations as mediators of termination, but the
complexity of the results suggests involvement at several points in the
activation/termination pathway(s).

The most plausible explanation for the heterogeneity of the concentration dependence of
phorbol ester stimulated superoxide production is that the cells from different preparations
were primed to varying extents. All solutions used in these experiments were filtered with
0.6 μm filters to remove contaminating micro-organisms in an attempt to minimise
artefactual priming. Furthermore the use of strict sterile technique during the isolation of
the cells prevented the acetone stimulated response, indicating that in some preparations
the cells became activated during the isolation procedure due to contamination by
microorganisms. However, even with strict sterile technique variation in TPA stimulated
superoxide production was observed, suggesting that contamination by microorganisms
was not solely responsible for cell priming. Although Haslett has shown that standard
methods of neutrophil isolation can prime cells, he traced the cause to LPS, a product of
contaminating bacteria (Haslett et al. 1985). It would seem therefore that the neutrophils
were primed to varying degrees within the bodies of the donors, and although further
priming could be prevented by use of rigorous sterile technique, isolation of cells with a
range of activation states was unavoidable.
Rx stimulated superoxide production showed a number of differences to the TPA stimulated response. Rx was less potent than TPA at stimulating superoxide production. Thus, although low (nM) concentrations of TPA stimulated a response, much higher (µM) concentrations of Rx were required. These results agree with those of Merritt (Merritt et al. 1993), who found that low (nM) concentrations of TPA stimulated luminol chemiluminescence (a measure of superoxide production) from human neutrophils, but higher (µM) concentrations of Rx were required to stimulate a response. However, certain preparations of cells in this project demonstrated superoxide production in response to 100 nM Rx. Although the concentration dependence of the TPA response was variable, that variability did not match the sensitivity of cells to Rx. Thus cells that exhibited a high sensitivity to Rx did not necessarily show a high sensitivity to TPA, and vice versa. These differences imply that mechanisms that governed sensitivity of cells to TPA and Rx were different. Furthermore, several researchers, using in vitro assays, have shown that Rx only interacts with isoforms of PKC at concentrations ≥ 10 µM (Ryves et al. 1991; Dimitrejevic et al. 1995), which suggests that the stimulation of superoxide production by 100 nM Rx could not have been mediated by PKC. Another candidate for a receptor capable of mediating the response to 100 nM Rx is Rx-kinase. This protein has been shown to be activated by Rx at concentrations as low as 100 nM (Sharma et al. 1995) and has been shown to specifically activate the NADPH oxidase (Evans et al. 1990). Further evidence for the involvement of Rx-kinase comes in the form of the protamine kinase activity, which eluted from hydroxyapatite columns at a similar high phosphate concentration as Rx-kinase, and was only present in cells that demonstrated a superoxide response with 100 nM Rx (see chapter 5). In contrast neutrophil PKC isotypes eluted at low phosphate concentrations and were always detected, irrespective of the presence or
absence of the Rx/protamine kinases. These arguments imply that Rx/protamine kinases can be induced within neutrophils and, once induced, confer a high superoxide generating sensitivity to Rx on them.

Although low (nM) concentrations of TPA often stimulated responses with late onset times (i.e. > 30 minutes), higher (μM) concentrations always caused an immediate response. In contrast high (μM) concentrations of Rx often stimulated a response with a late onset time. Merritt also found that Rx stimulated a response with a late onset time (Merritt et al. 1993). Since the response with low (nM) concentrations of TPA often had a late onset time and since Rx was less potent than TPA, it seems probable that late onset was a function of reduced interaction between phorbol ester and the cellular receptor (PKC). Perhaps a certain threshold of PKC activity needed to be achieved before superoxide production was initiated, or a critical level of active PKC at the membrane (e.g. adjacent to the superoxide generating complex) was required. Under these models low doses of TPA and high doses of Rx would take longer to stimulate PKC to the threshold level and therefore demonstrate a delay in the onset of superoxide production.

The discovery that the Calbiochem Rx (BH-107) was contaminated with Ro, which was responsible for the stimulation of superoxide production, meant that stimulation of superoxide production by Rx had to be re-assessed. The contamination was discovered when a fresh batch (BH-108) of Rx was bought from Calbiochem. Whereas Rx (batch BH-107) always stimulated a response at 10 μM, the new batch (BH-108) failed to stimulate a response at that concentration. Subsequent NMR analysis revealed that batch BH-108 contained pure Rx and the difference in activities was due to contamination
of batch BH-107. Rx (batch BH-108) contained trace levels of contamination. It is possible that the low sensitivity state to Rx was due to Ro, present as a minor contaminant, which was responsible for the superoxide stimulating activity of the Rx (batch BH-107). Analysis of the ability of pure Ro to stimulate superoxide production indeed revealed that this compound stimulated a response with an ED₅₀ value of 241.94 ± 28.73 nM. It therefore seems probable that the response seen with 10 μM Rx (batch BH-107) was actually due to the contaminating Ro. However, contamination with Ro cannot account for the involvement of Rx-kinase in the response to 100 nM Rx, seen in cells demonstrating a high sensitivity to Rx. If contaminating Ro was only maximally active in the 10 μM solution of Rx, the activity will be diluted out by the 1/100 dilution that was used to make the 100 nM Rx solution. The fact that the 100 nM Rx solution did not consistently stimulate a response supports this argument. Although Ro activates PKC (Ellis et al. 1987), it seems unlikely that the up-regulation of PKC was responsible for the high sensitivity to Rx, because it was not matched by a high sensitivity to TPA, implying that levels of PKC were constant. Perhaps the strongest evidence for the involvement of Rx-kinase in cells demonstrating high sensitivity to Rx, is the detection of the late eluting kinase activity. This protamine kinase activity demonstrated the same elution characteristics as Rx-kinase and was only observed in cells that demonstrated high sensitivity to Rx. All these arguments support the theory that although the response to high (μM) concentrations of Rx was due to contaminating Ro, acting through PKC, the response to low (nM) concentrations of Rx was due to the Rx itself, acting through the Rx/protamine kinases.

The fact that cells demonstrating a high sensitivity to Rx did not demonstrate a high
sensitivity to TPA implied that up-regulation of PKC was not responsible for the response to low (nM) doses of Rx (see above). However, heterogeneity in the sensitivity of cells to TPA was observed. It was therefore decided to investigate the types and levels of PKC isotypes found in human neutrophils and these results are discussed in the next chapter.
Chapter 7. PKC studies.

7.1 Introduction.

The physiological receptor for the phorbol esters is thought to be protein kinase C (PKC). Since neutrophils demonstrated heterogeneous sensitivities to TPA, when superoxide production was measured, it was decided to investigate the types of PKC isotypes found in phagocytic cells. Two approaches were used: screening cell homogenates with antibodies to PKC isotypes (immunoblotting); and measurement of the protamine sulfate and phorbol ester stimulated histone-IIIs phosphorylating activities found in cell homogentaeas.

7.2 Immunoreactivity.

Both human monocytes and human neutrophils were analysed for immunoreactivity to anti-sera raised against specific PKC isotypes. Cells were fractionated into cytosolic (C) and 1% Triton X-100 soluble particulate (Ps) fractions. Each fraction, C or Ps, was boiled in Laemmli buffer, loaded onto a 10% polyacrylamide gel and separated by SDS-PAGE. Proteins were transferred to nitrocellulose by Western blotting and immunoblotted with antibodies specific to the various isotypes of PKC. To confirm the specificity of immunoreactive bands, some of each antibody was incubated with competing epitopes, for 30 minutes at 37 °C prior to immunoblotting. Immunoreactive bands that ‘disappeared’ in the presence of competing epitope represented specific binding and were identified as PKC isotypes. The cytosol fraction of homogenised rat brain, a rich source for PKC isotypes, was used throughout as a positive internal control for the detection of PKC immunoreactivity.
7.2.1. **Monocytes.**

Monocytes were screened with antibodies to \( \alpha-, \beta_1-, \beta_2- \) and \( \zeta-\)PKC (see figure 7.1, page 192). Specific immunoreactive bands were distinguished from non-specific bands by incubating the anti-PKC antibodies with the specific epitope prior to immunoblotting of the nitrocellulose blots. In the presence of epitope the antigenic binding domain of the antibody was blocked and only non-specific binding remained. Specific immunoreactivity was absent from blots incubated with antibody plus epitope, but present in blots incubated with antibody alone. Thus protein bands that 'disappeared' in blots incubated with antibody and epitope were deemed to be due to specific binding and therefore to be due to PKC. Specific immunoreactivity was observed in the C fraction for all antibodies used, corresponding to protein bands with molecular weights of 66 - 97.4 kDa (see figure 7.1, page 192). This shows that unstimulated monocytes contained \( \alpha\)-PKC (76.8 kDa), \( \beta_1\)-PKC (76.9 kDa), \( \beta_2\)-PKC (76.8 kDa) and \( \zeta\)-PKC (67.7 kDa), which was located in the the cytosolic (C) fraction. Antibodies to \( \beta_1\)-PKC also detected low levels of \( \beta_1\)-PKC in the Ps fraction, showing that some of this isotype is associated with the cellular membranes even in unstimulated cells.

7.2.2. **Human neutrophils.**

Unstimulated neutrophils were screened using anti-sera raised against PKC \( \alpha, \beta_1, \beta_2, \delta, \varepsilon, \zeta, \eta, \) and \( \theta \). Specific immunoreactivity was observed with antibodies to \( \alpha- \) and \( \beta_1\)-PKC in the C fraction alone (see figure 7.2, page 193), but no immunoreactivity was detected for PKC isotypes \( \beta_2, \delta, \varepsilon, \zeta, \eta \) or \( \theta \) in any of the sub-cellular fractions screened (data not shown). Figure 7.2 shows that the immunoreactivity detected, corresponded to protein bands with molecular weights of 66 - 97.4 kDa for anti-PKC \( \beta_1 \) and 45-47 kDa for anti-
Figure 7.1: Detection of PKC isotypes in human monocytes using specific antibodies to various isotypes.

Protein kinase C (PKC) isoform immunoreactivity in cytosol (C) and Triton-X100 soluble particulate (Ps) fractions from unstimulated human monocytes. C and Ps fractions were prepared as described in the methods section and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose by western blotting and immunoblotted with antipeptide antibodies specific for $\alpha$-, $\beta_1$-, $\beta_2$- and $\zeta$-PKC. To confirm specificity of staining antibodies were incubated with competing epitope. The right hand lane shows a rat brain PKC fraction (Rbstd) as a positive control.
Figure 7.2: Detection of PKC isotypes in human neutrophils using specific antibodies to various isotypes.

Protein kinase C (PKC) isoform immunoreactivity in cytosol (C) and Triton-X100 soluble particulate (Ps) fractions from unstimulated human neutrophils. C and Ps fractions were prepared as described in the methods section and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose by western blotting and immunoblotted with antipeptide antibodies specific for α-, β1-, β2-, δ-, ε-, ζ-, η-, and θ-PKC (only α- and β1-PKC data shown). To confirm specificity of staining antibodies were incubated with competing epitope. The right hand lane shows a rat brain PKC fraction (Rbstd) as a positive control.
PKCα. Prior incubation of antibodies with competing epitope revealed that these bands were due to specific immunoreactivity.

PKC isotypes have an apparent molecular weight of 68-83 kDa in SDS-PAGE gels (Stabel and Parker 1991), suggesting that human neutrophils contained PKC isotype β₁ (76.9 kDa). The anti-PKCα immunoreactivity corresponded to a protein band with too low a molecular weight (apparent MW = 47 kDa) to have been PKCα (76.8 kDa). A proteolytic cleavage product of PKC called PKM does have a molecular weight of 45-47 kDa and therefore the anti-PKCα immunoreactivity could have been due to PKMα. However, this conclusion seems unlikely as no PKMβ₁ was observed. A more likely candidate is p47^{phox}, which shares a common epitope with PKCα (recognised by the anti-PKCα antibody), has a molecular weight of 47 kDa and is expressed in human neutrophils (Volpp et al. 1989; Devalia et al. 1992).

These results show that β₁ was the main PKC isotype detected in human peripheral neutrophils and was located in the cytosol of resting cells. Although specific immunoreactivity was detected with antibodies to α-PKC, this was probably due to p47^{phox}. No specific immunoreactivity was detected with antibodies to β₂, γ-, δ-, ε-, ζ, η- and θ-PKC in any of the subcellular fractions.

7.2.3. Phorbol ester induced translocation of PKC isotypes.

We have shown that treatment of HL-60 cells or platelets with phorbol esters causes translocation of PKC isotypes from the C- to the Ps-fraction (unpublished data). Therefore, monocytes and neutrophils were tested for translocation behaviour upon
treatment with phorbol esters.

7.2.3.1. **Monocytes.**

Human monocytes, isolated as described in the methods chapter, were incubated with 100 nM TPA or 10 μM Rx (batch BH-107) for 10 minutes at 37 °C. The cells were then pelleted by centrifugation, resuspended in homogenisation buffer and lysed by ultrasonication. Cells were fractionated into cytosolic (C) and 1% Triton X-100 soluble particulate (Ps) fractions, which were further separated by SDS-PAGE and the resulting protein bands transferred to nitrocellulose by Western blotting. These blots were screened for β₁-PKC in the manner described above and the results are shown in figure 7.3, page 196. Translocation of β₁-PKC, from the C to the Ps fraction, was clearly demonstrated with 100 nM TPA and shows that TPA was capable of translocating β₁-PKC.

Translocation of β₁-PKC was also seen with 10 μM Rx (batch BH-107), which shows that 10 μM Rx was capable of interacting with β₁-PKC. However, Rx (batch BH-107) was found to be contaminated with Ro (see chapter 6) and it was concluded that the Ro was responsible for the stimulation of superoxide production seen with 10 μM Rx (batch BH-107). It seems probable therefore that the translocation of β₁-PKC seen with 10 μM Rx (BH-107) was due to the contaminating Ro.

7.2.3.2. **Neutrophils.**

Human neutrophils were also tested for phorbol ester stimulated translocation of PKC. Cells were incubated with various concentrations of TPA for 10 minutes at 37 °C and then treated in the same way as the monocytes (see above). The results of the translocation study with TPA and neutrophils are shown in figure 7.4, page 197. Figure 7.4 shows
Figure 7.3: Translocation of \( \beta_1 \)-PKC from C to Ps fraction of human monocytes after treatment with 100 nM TPA or 10 \( \mu \)M Rx (batch BH-107).

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<tr>
<th></th>
<th>10 ( \mu )M Rx</th>
<th>100 nM TPA</th>
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<tr>
<td>C</td>
<td>Ps</td>
<td>C</td>
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<td>Ps</td>
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Cells were incubated with 100 nM TPA or 10 \( \mu \)M Rx (batch BH-107) for 10 minutes at 34°C, pelleted by centrifugation and resuspended in homogenisation buffer. Cells were then disrupted by ultrasonication and C and Ps fractions prepared as described in the methods section. C and Ps fractions were separated by SDS-PAGE and the resulting proteins transferred to nitrocellulose by Western blotting. The \( \beta_1 \) isotype of PKC was detected by immunoblotting with antibodies to \( \beta_1 \)-PKC.
Figure 7.4: Downregulation of $\beta_1$-PKC in human neutrophils after treatment with various concentrations of TPA.

<table>
<thead>
<tr>
<th>TPA (nM)</th>
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<td>Fraction</td>
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<td>C</td>
<td>C</td>
<td>Ps</td>
<td>Ps</td>
<td>Ps</td>
<td>Ps</td>
<td>RbStd</td>
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</table>

Cells were incubated with various concentrations of TPA for 10 minutes at 34°C, pelleted by centrifugation and resuspended in homogenisation buffer. Cells were then disrupted by ultrasonication and C and Ps fractions prepared as described in the methods section. C and Ps fractions were separated by SDS-PAGE and the resulting proteins transferred to nitrocellulose by Western blotting. The $\beta_1$ isotype of PKC was detected by immunoblotting with antibodies to $\beta_1$-PKC. [N.B. the protein band seen in the Ps fraction did not disappear in lanes blotted with $\beta_1$-PKC antibody that had been previously incubated with competing epitope and therefore could not have been $\beta_1$-PKC]. The right hand lane shows a rat brain PKC fraction (RbStd) as a positive control.
that $\beta_1$-PKC disappeared from the cytosol of the cells after treatment with $\geq 100$ nM TPA, without concomittant appearance of $\beta_1$-PKC in the membrane fraction (Ps). These results show that TPA did not cause translocation of $\beta_1$-PKC from the cytosol (C) to the membrane (Ps) of neutrophils, and suggest that $\beta_1$-PKC was downregulated instead. The downregulation of $\beta_1$-PKC seen with $\leq 100$ nM TPA must have occurred within 10 minutes, since no $\beta_1$-PKC remained, in either subcellular fraction, after 10 minutes of incubation with TPA.

Since EGTA washes of neutrophils affected superoxide production (see chapter 6), the effect of EGTA treatment on the subcellular location of PKC was also investigated. A control batch of cells was prepared without incubation with TPA, half were washed twice with HBSS containing EGTA (5mM) and the other half washed twice with HBSS containing no EGTA. Since the homogenisation buffer normally contained EGTA and EDTA a special batch was prepared without these chelators and used to homogenise the untreated cells. $\beta_1$-PKC was only observed in the C-fraction from the EGTA treated cells (see figure 7.5, page 199). The loss of $\beta_1$-PKC from the C fraction of the cells that were not treated with EGTA was recognised as an artefact, since other neutrophils not treated with EGTA, but disrupted with homogenisation buffer containing EGTA/EDTA contained $\beta_1$-PKC in the C fraction (see figure 7.2, page 193). This result suggests that removal of EGTA/EDTA from the homogenisation buffer led to degradation of PKC, presumably by calcium dependent proteases such as calpain. Therefore EGTA/EDTA was included in the homogenisation buffer for the rest of the experiments designed to measure the effect of EGTA washes of cells on the location of $\beta_1$-PKC in TPA stimulated cells.
Figure 7.5: The effect of removing EGTA from the homogenisation buffer on detection of $\beta_1$-PKC in subcellular fractions from unstimulated neutrophils.

Unstimulated neutrophils were washed with HBSS buffer containing EGTA (EGTA wash +), or HBSS buffer without EGTA (EGTA wash -), then pelleted by centrifugation. The cells were resuspended in homogenisation buffer (HB), containing EGTA (HB EGTA +) or without EGTA (HB EGTA -) and then were lysed by ultrasonication. Subcellular fractions (C and Ps) were prepared as described in the methods section and then separated by SDS-PAGE. The resulting protein bands were transferred to nitrocellulose by Western blotting and $\beta_1$-PKC was detected by immunoblotting with anti-$\beta_1$-PKC antibodies. The right hand lane shows a rat brain PKC fraction (RbStd) as a positive control.
Cells were washed twice with HBSS buffer containing EGTA (5 mM) and then stimulated with various concentrations of TPA for 10 minutes at 34 °C. Subcellular fractions were prepared and the location of \( \beta_1 \)-PKC determined by immunoblotting as described above (see methods chapter for details). Figure 7.6 (page 201) shows the effect of TPA on the subcellular location of \( \beta_1 \)-PKC in EGTA washed cells. Although \( \beta_1 \)-PKC disappeared from the cytosol of the cells after treatment with \( \geq 100 \) nM TPA, no concomittant appearance of \( \beta_1 \)-PKC was observed in the membrane fraction (Ps). This is the same result as seen with cells that were not washed with EGTA buffer. This shows that EGTA washes of the cells did not alter the subcellular location of \( \beta_1 \)-PKC in resting or TPA stimulated cells.

7.3. Separation of Neutrophil PKC with hydroxyapatite FPLC.

The immunoblot experiments described above, revealed that the predominant isotype of PKC found in human neutrophils was \( \beta_1 \)-PKC, which was located in the cytosol (C fraction) of resting cells. It was decided to separate the crude cytosolic (C) and membrane (Ps) fractions further, using hydroxyapatite FPLC, in order to determine the levels of \( \beta_1 \)-PKC present. The C and Ps fractions were prepared as before (see methods chapter) and loaded onto hydroxyapatite FPLC columns. These columns were washed with 20 mM phosphate buffer to remove any unbound proteins and then a gradient of increasing phosphate concentration was used to elute the bound proteins from the columns. The eluted proteins were collected as 1 ml fractions and each fraction was split in two, half was stored at -80 °C for subsequent kinase assay and the other half boiled with an equal volume of Laemmli buffer for subsequent immunoblot analysis.
Figure 7.6: The effect of TPA on the subcellular location of β₁-PKC in EGTA washed neutrophils.

<table>
<thead>
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<th>TPA (nM):</th>
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<tr>
<td>Fraction:</td>
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<td>C</td>
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<td>Ps</td>
<td>Ps</td>
<td>Ps</td>
<td>Ps</td>
<td>RbStd</td>
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Cells were isolated as described in the methods section and washed twice with HBSS buffer containing EGTA (5 mM). The EGTA washed cells were then incubated with various concentrations of TPA for 10 minutes at 34°C, pelleted by centrifugation and resuspended in homogenisation buffer. Cells were then disrupted by ultrasonication and C and Ps fractions prepared as described in the methods section. C and Ps fractions were separated by SDS-PAGE and the resulting proteins transferred to nitrocellulose by Western blotting. The β₁ isotype of PKC was detected by immunoblotting with antibodies to β₁-PKC. [N.B. the protein band seen in the Ps fraction did not disappear in lanes blotted with β₁-PKC antibody that had been previously incubated with competing epitope and therefore could not have been β₁-PKC]. The right hand lane shows a rat brain PKC fraction (RbStd) as a positive control.
7.3.1. Protamine sulfate phosphorylation assay.

The cytosolic (C) and the triton soluble membrane (Ps) fractions were separated by hydroxyapatite FPLC as described above. Then the elution fractions from the FPLC experiments were assayed for kinase activity. The assay measured the transfer of radiolabelled phosphate from $^{32}$P-$\gamma$-ATP to protamine sulfate (see methods chapter for details). Protamine phosphorylating activity was detected in the cytosol (C fraction), but not in the triton soluble (Ps) fraction of the unstimulated neutrophils (see figure 7.7, page 203). These results matched the immunoblot data from the crude C and Ps fractions, which demonstrated that PKC was located only in the C fraction of unstimulated cells.

Two peaks of protamine phosphorylating activity could be distinguished in the FPLC elution fractions from the cytosol of the neutrophils (see figure 7.7, page 203). These peaks of activity eluted with: 60 - 113 mM phosphate, corresponding to elution fractions 8 to 12 (peak 1); and 127 - 180 mM phosphate, corresponding to fractions 13 to 17 (peak 2). These fractions were assayed for phorbol ester stimulated histone-IIIs phosphorylating activity, to determine whether any of the protamine phosphorylating activity was due to PKC (see below).

7.3.2. Histone-IIIs phosphorylating assay.

An alternative method of detecting PKC isotypes is the mixed micelle PKC assay first developed by Hannun (Hannun et al. 1985). This assay measures the phosphorylation of histone-IIIs by PKC in the presence of phosphatidyl serine, Triton-X100 and phorbol ester mixed micelles. Unlike protamine sulfate, which is phosphorylated by PKC in the absence of other cofactors, histone requires the presence of phospholipid and an activator
Figure 7.7: Protamine sulfate phosphorylating activity in C and Ps fractions of neutrophils.
of the enzyme (e.g. phorbol ester). Therefore when the micelles contain phorbol ester, stimulation of kinase activity is seen with classical and novel PKC isotypes, but not the phorbol ester insensitive PKC (i.e. atypical isotypes).

Elution fractions 7-18 from the cytosol of neutrophils were therefore assayed for histone-IIIIs phosphorylation activity in the presence of phosphatidyl serine (PS) and triton mixed micelles that contained either TPA, Ro or Rx (see figure 7.8, page 205). The data in figure 7.8 shows the phorbol ester stimulated activity, i.e. after subtraction of the activity stimulated by PS alone. Only one peak of stimulated activity (fractions 9 - 12) was observed, corresponding to the first peak of protamine phosphorylating activity (peak 1) described above (see figure 7.7, page 203). Both 1 μM TPA and 10 μM Ro stimulated the histone phosphorylation to the same levels, but 100 nM Rx failed to stimulate any activity. Thus, although the kinase responsible for peak 1 in figure 7.7 (page 203) was activated in vitro by high (μM) concentrations of TPA and Ro, low (nM) concentrations of Rx had no effect on that isotype.

Elution fractions 13 - 18 did not catalyse any phorbol ester stimulated histone-IIIIs phosphorylation (see figure 7.8, page 205), which demonstrates that the kinase responsible for the second peak of protamine phosphorylating activity (peak 2) (see figure 7.7, page 203) was phorbol ester insensitive. Thus the second peak of protamine phosphorylating activity could be due to either an atypical (i.e. phorbol ester insensitive PKC isotype, e.g. ζ-PKC), or a kinase which is not a member of the PKC family at all. An alternative candidate is the as yet uncharacterised ‘n-PKC’ described by Majumdar (Majumdar et al. 1991; Majumdar et al. 1993). This ‘n-PKC’ has been described as a calcium-independent,
Figure 7.8: Phorbol ester stimulated histone-III's phosphorylating activity.
but phosphatidyl serine (PS) and diglyceride (DAG) dependent PKC isotype and was detected, in addition to $\beta_1$-PKC, in the cytosol of unstimulated neutrophils. Although this kinase was found to be DAG-dependent it may yet prove to be phorbol ester insensitive, another protein DAG kinase (DGK) has been shown to bind DAG, but not phorbol ester, supporting this possibility (Sakane et al. 1990). These observations make 'nPKC' a strong candidate for the kinase responsible for the second peak of protamine phosphorylating activity.

7.3.3. Detection of kinases using FSBA.

The most active fraction (fraction 10) in the first peak of protamine phosphorylating activity was analysed for total kinase activity using the FSBA method of Parker (Parker, P.J. et al. 1993). In this technique fluorylsulfonylbenzoyladenosine (FSBA) covalently binds to conserved lysine residues found in the ATP binding site of all kinase proteins. ATP in the control reaction prevents FSBA from binding to active site lysine residues, thus distinguishing between exposed lysine residues within ATP binding sites of kinases and lysine residues in other protein motifs. Screening of proteins separated by SDS-PAGE with antibodies to FSBA detects proteins containing lysine residues and immunoreactivity that 'disappears' in the ATP treated fraction denotes the presence of a kinase protein.

The results of the FSBA experiment on fraction 10 are shown in figure 7.9 (page 207). Figure 7.9 shows that elution fraction 10 contained multiple protein kinases. Five distinct protein kinases were detected, migrating at approximately 110, 97, 74, 49 and 45 kDa. The 74 kDa molecular weight protein bands probably correspond to PKC, but the lower
Figure 7.9: Screening with antibodies to FSBA for kinases in hydroxyapatite elution fraction 10 from the C fraction of human neutrophils.

Fraction 10 from the neutrophils associated with figure 7.7 was loaded onto a Mono-Q column, which was washed with a NaCl buffer gradient (see methods). The elution fractions were then incubated with FSBA (1 mM) in the presence and absence of ATP (1 mM), for 20 minutes at 30°C. The FSBA reaction was quenched with Laemmli buffer containing β-mercaptoethanol (150 mM). The samples were then separated by SDS-PAGE, with the resulting protein bands being transferred to nitrocellulose by Western blotting. FSBA-bound protein was detected using anti-FSBA antibodies kindly donated by Prof. P.J. Parker (ICRF, London).
molecular weight protein bands are distinct from PKC isotypes on the basis of size (Stabel and Parker 1991). Therefore although PKC contributed to the protamine phosphorylation activity seen in FPLC elution fractions 8 - 12, other kinases were present, which may have been capable of utilizing protamine sulphate as a kinase substrate.

7.3.4. Immunoblotting of hydroxyapatite separated PKC.

The immunoblot fractions were separated by SDS-PAGE and the resulting proteins transferred to nitrocellulose by Western blotting, which was in turn screened for \( \beta_1 \)-PKC using specific antisera. Specific immunoreactivity with antibodies to \( \beta_1 \)-PKC was only observed in elution fractions 9 - 12 (see figure 7.10, page 209). This immunoreactivity correlates exactly with the phorbol ester stimulated histone phosphorylating activity and the first peak of protamine phosphorylating activity (peak 1) described above (see figure 7.7, page 203). Thus \( \beta_1 \)-PKC from unstimulated neutrophils was located exclusively in the cytosol (C fraction) and eluted from hydroxyapatite columns with 73 - 113 nM phosphate (9 - 12). The correlation of the \( \beta_1 \)-PKC immunoreactivity and the phorbol ester stimulated histone phosphorylating activity also argues in favour of \( \beta_1 \)-PKC being the predominant phorbol ester sensitive isotype of PKC found in neutrophils.

The results of the phorbol ester stimulated histone phosphorylation assay revealed that the second peak of protamine phosphorylating activity was not due to a phorbol ester sensitive kinase. The relevant fractions were screened with antibodies to \( \zeta \)-PKC, which is an atypical PKC isotype and therefore phorbol ester insensitive. No immunoreactivity was detected with antibodies to \( \zeta \)-PKC, a result that agrees with the immunoblotting data
A cytosolic (C) fraction was prepared from human neutrophils (1.54 x 10^9 cells) as described in the methods section. The C fraction was separated hydroxyapatite FPLC and the resulting fractions boiled with an equal volume of Laemmli buffer. The Laemmli treated fractions were then separated by SDS-PAGE and the resulting protein bands transferred to nitrocellulose by Western blotting. β₁-PKC was detected by immunoblotting with antibodies to β₁-PKC. To confirm specificity of staining, the immunoblotting was repeated with antibody that had previously been incubated with competing epitope (β₁-PKC + competing epitope). The right hand lane shows a crude PKC fraction from neutrophils (PMNstd) as positive control.
for crude neutrophil subcellular fractions. Although the second peak of protamine kinase activity could not be due to \( \zeta \)-PK, the ‘n-PKC’ described by Majumdar (Majumdar et al. 1991) may be responsible. Unfortunately the monoclonal PKC consensus antibodies used by Majumdar to detect ‘nPKC’ were not available in the present study.

7.3.5. Silver staining of proteins separated by hydroxyapatite FPLC.

FPLC elution fractions 6 - 21 from the cytosol of resting cells were further separated by SDS-PAGE using 10% polyacrylamide gels, and the protein bands were then silver stained in situ, rather than being transferred to nitrocellulose. Figure 7.11 (page 211) shows that each FPLC elution fractions that catalysed protamine phosphorylation (i.e. fractions 8 - 13) contained a number of distinct proteins. The silver stain experiment detected bands of protein within the molecular weight range of \( \beta_1 \)-PKC. The putative \( \beta_1 \)-PKC protein band first appeared in fraction 9, peaked in fraction 10, and rapidly diminished to undetectable levels by fraction 16. Comparison of the histone phosphorylating activity with the 76 kDa silver stained protein band showed that there was a marked correlation between the intensity of this band and the phorbol ester stimulated kinase activity. Furthermore, the \( \beta_1 \)-PKC immunoreactivity also peaked in fraction 10, Therefore, from these three sets of data it can be concluded that the first peak of protamine kinase activity was due to \( \beta_1 \)-PKC.

7.4. Kinase profile of neutrophils demonstrating high sensitivity to Rx.

The results outlined above demonstrate that the predominant PKC isotype in neutrophils was \( \beta_1 \)-PKC, which was located in the cytosol of unstimulated cells. A second phorbol ester-insensitive kinase capable of phosphorylating protamine sulfate was also located in
The C fraction from unstimulated neutrophils was separated by hydroxyapatite FPLC and the resulting fractions further separated by SDS-PAGE. The protein bands were then silver stained (see methods). The left hand lane shows the molecular weight markers (MW), whilst the right hand lane shows a rat brain PKC fraction (RbStd).
the cytosol of unstimulated cells and, although not conclusively identified, could have been 'n-PKC'.

The results discussed in chapter 5 and 6 revealed that neutrophils could be split into two categories on the basis of superoxide production in response to Rx. The majority of cells demonstrated low sensitivity to Rx (i.e. showing no superoxide production with 100 nM Rx). However, some cells demonstrated high sensitivity to Rx (i.e. showing high levels of superoxide production with 100 nM Rx). It was decided to compare the kinase profile of neutrophils that demonstrated these different sensitivities to Rx.

PMN exhibiting $5.47 \pm 0.48$ nmoles of superoxide production after stimulation with 100 nM Rx for 292 minutes (i.e. high sensitivity to Rx) were fractionated and analysed for kinase activity. The neutrophils ($2.86 \times 10^8$ cells) were homogenised and cytosolic (C) and triton soluble membrane (Ps) fractions prepared as described in the methods chapter. The C and Ps fractions were then separated by hydroxyapatite FPLC and each elution fraction split in two, half was stored at -80 °C ready for subsequent kinase assay, whilst the other half was boiled with an equal volume of Laemmli buffer ready for subsequent immunoblot analysis.

7.4.1. Protamine sulfate phosphorylation assay on fractions from cells demonstrating high sensitivity to Rx.

The elution fractions from the cytosol of neutrophils contained five peaks of protamine phosphorylating activity (see figure 7.12a, page 213). Three of these peaks eluted from the hydroxyapatite FPLC column with low (< 200 mM) concentrations of phosphate and
Figure 7.12a: Protamine phosphorlating activity from the cytosol (C fraction) of neutrophils that demonstrated high sensitivity to Rx.

Figure 7.12b: Protamine phosphorlating activity from the triton washed membranes (Ps fraction) of neutrophils that demonstrated high sensitivity to Rx.
corresponded to elution fractions 9 - 10 (peak 1), 10 - 13 (peak 2) and 13 - 18 (peak 3).

Whilst two of the peaks were eluted by high (> 270 mM) concentrations of phosphate and corresponded to elution fractions 24 - 26 (peak 4) and 31 - 32 (peak 5). These results contrast with the experiments described above, those neutrophils contained only two clear peaks of protamine phosphorylating activity corresponding to elution fractions 8 to 12 (peak 1) and 13 to 17 (peak 2).

The elution fractions from the triton washed membranes (Ps fraction) of neutrophils that demonstrated high sensitivity to Rx contained only one peak of protamine phosphorylating activity. This peak corresponded to elution fractions 29 - 30 and was eluted from the hydroxyapatite column by high (> 340 mM) concentrations of phosphate (see figure 7.12b, page 213).

7.4.2. Immunoblotting of hydroxyapatite separated PKC in fractions from cells demonstrating high sensitivity to Rx.

The elution fractions that had been boiled with an equal volume of Laemmli buffer (see above) were separated by SDS-PAGE and the resulting protein bands transferred to nitrocellulose by Western blotting. The nitrocellulose blots were then screened with specific antibodies raised against the various PKC isotypes.

The blots derived from the C fraction of the neutrophils revealed that β₁-PKC immunoreactivity was limited to elution fractions 9 and 10 (see figure 7.13, page 215). These fractions correspond to peak 1 and suggest that the protamine phosphorylating activity detected in fractions 9 and 10 was due to β₁-PKC, as observed above (see section
Figure 7.13: Detection of $\beta_1$-PKC in C fraction of neutrophils that demonstrated high sensitivity to Rx.

Neutrophils that demonstrated high sensitivity to Rx (i.e. 100 nM Rx stimulated 5.47 ± 0.48 nmoles of superoxide production) were homogenised and a C fraction prepared as described in the methods section. The C fraction was then separated by hydroxyapatite FPLC and each elution fraction boiled with an equal volume of Laemmli buffer. These Laemmli treated fractions were then separated by SDS-PAGE and the resulting protein bands transferred to nitrocellulose by Western blotting. $\beta_1$-PKC was detected by immunoblotting with anti-$\beta_1$PKC antibodies. The right hand lane shows a crude neutrophil PKC fraction as a positive control.
7.3.5. The elution fractions were also screened with antibodies to \( \alpha \)-, \( \beta_2 \)- and \( \zeta \)-PKC, but no immunoreactivity was detected in any of the fractions (data not shown).

The protamine phosphorylating activity that was eluted from the hydroxyapatite column by low concentrations of phosphate (i.e. peaks 1 - 3) was characteristic of PKC. However, only the elution fractions corresponding to peak 1 contained any PKC immunoreactivity with antibodies to \( \alpha \)-, \( \beta_1 \)-, \( \beta_2 \)- and \( \zeta \)-PKC and only to \( \beta_1 \)-PKC antibodies. It was therefore decided to assay the elution fractions corresponding to peaks 1, 2 and 3 for PKC activity using the mixed micellar assay of Hannun et al. (Hannun et al. 1985).

7.4.3. Histone-III phosphorylating assay.

The early eluting protamine phosphorylating activity from the C fraction of the cells consisted of three peaks of activity (see figure 7.12a, page 213). Two of these peaks were clearly distinguishable, although not discrete, corresponding to fractions 10 - 13 (peak 2) and 13 - 18 (peak 3), whilst the third existed as a ‘shoulder’ to peak 2 (fractions 9 - 10, i.e. peak 1). Since these peaks were not discrete there was bound to be some overlap of kinase activity in each of these fractions. To minimise this overlap it was decided to analyse the fractions that contained the highest levels of kinase activity from each peak (i.e. fractions 10, 12 and 14).

Each of these fractions was assayed for ability to catalyse the phosphorylation of histone-III, in the presence of triton mixed micelles containing phosphatidyl serine (PS), TPA, and \( \pm \) calcium (see figure 7.14, page 217). Each fraction was also assayed for protamine phosphorylating activity at the same time, as a positive control. The levels of protamine
Figure 7.14; Histone-IIIs phosphorylating activity from neutrophils that demonstrated high sensitivity to Rx. Hydroxyapatite FPLC elution fractions were assayed in a mixed micellar assay, for histone-IIIs phosphorylating activity. The Triton-X100 micelles contained various combinations of the following: phosphatidylserine (PS), TPA, and calcium (Ca^{2+}). Each fraction was also assayed for protamine phosphorylating activity, in a micelle free assay, as a positive control.
phosphorylation (see figure 7.14d, page 217) were similar to those observed in figure 7.12a (page 213), implying that no significant degradation of activity had occurred. Although all three fractions showed PS stimulated histone-III\(\text{S}\) phosphorylation, only fraction 10 showed phorbol ester sensitive activity (see figure 7.14, page 217). This is more evidence that the activity in fraction 10 was due to \(\beta_1\)-PKC, since this is a classical PKC isotype and therefore sensitive to phorbol ester. The kinase activity in fractions 12 and 14 was not sensitive to TPA and therefore could not have been due to classical or novel PKC, since these isotypes are phorbol ester sensitive. A possible candidate is 'n-PKC', alternatively the activity may not have been due to members of the PKC family at all.

Representative fractions from peaks 4 and 5 from the cytosol (C fraction) and peak 1 from the triton wash of the membranes (Ps fraction) of neutrophils were also assayed, but failed to show any activity, even with the protamine controls (data not shown). These results show that the activity had degraded, despite being stored at -80 °C in a glycerol storage buffer.

7.4.4. Summary of kinase profile in cells demonstrating high sensitivity to Rx.

Although the three peaks of protamine phosphorylating activity that eluted from the hydroxyapatite column with low concentrations of PKC were characteristic of PKC, only peak 1 demonstrated immunoreactivity with antibodies to specific isotypes of PKC. The kinase responsible for the peak 1 activity was identified as \(\beta_1\)-PKC by immunoblotting. The elution fractions that corresponded to the other two peaks of activity (peak 2 and 3) failed to demonstrate any phorbol ester sensitive histone-III\(\text{S}\) phosphorylation. This result
implies that the kinases responsible for the peak 2 and 3 activity were either atypical and therefore phorbol ester insensitive isotypes of PKC or not members of the PKC family at all.

Some peaks of protamine phosphorylating activity (i.e. peaks 4 and 5 from the C fraction and peak 1 from the Ps fraction) eluted from the hydroxyapatite column with high phosphate concentrations. Known isotypes of PKC elute from hydroxyapatite columns with low concentrations of phosphate, so these ‘late eluting’ peaks of activity could not be due to PKC activity. The ‘late eluting’ peaks of activity demonstrated similar elution characteristics to Rx-kinase isolated from neutrophils and rat alveolar macrophages (Ryves et al. 1989; Sharma 1995). Since these peaks of activity were only observed in cells that demonstrated superoxide production with 100 nM Rx and since Rx-kinase has been implicated in the activation of NADPH oxidase (Evans et al. 1990), it was concluded that the high sensitivity of these cells to Rx was due to the induction of these Rx-kinase(s).

The ‘early eluting’ peaks of activity could not have contributed to the high sensitivity of the cells to Rx, because only peak 1 was sensitive to phorbol ester. This peak 1 activity was due to β1-PKC, which is the predominant PKC isotype in neutrophils. If upregulation of β1-PKC was responsible for enhanced Rx stimulated superoxide production then the cells would be expected to also demonstrate high sensitivity to TPA, which was not the case.

7.5. Kinase profile of neutrophils that demonstrated low sensitivity to Rx.

The results outlined above show that the cytosol of neutrophils that demonstrated high
sensitivity to Rx contained multiple peaks of protamine phosphorylating activity (see figure 7.12a, page 213). It was decided to compare the kinase profile of those neutrophils with cells that demonstrated only low sensitivity to Rx. Neutrophils (1.1 x 10⁶ cells), that did not produce any superoxide in response to 100 nM Rx, were homogenised and the cytosol (C) and triton washed membrane (Ps) fractions separated using hydroxyapatite FPLC. The resulting elution fractions were assayed for protamine phosphorylating activity (see figure 7.15, page 221). Figure 7.15 shows that the protamine phosphorylating activity from neutrophils demonstrating low sensitivity to Rx was separated into only two peaks of activity by hydroxyapatite FPLC. Furthermore, both these peaks of activity were limited to the cytosol (C fraction) of the neutrophils. The peaks of activity were eluted by 73 mM - 113 mM phosphate, which corresponded to elution fractions 9 - 12 (peak 1) and 113 mM - 180 mM phosphate, which corresponded to elution fractions 12 - 17 (peak 2). The concentrations of phosphate that eluted these peaks of protamine kinase activity were the same as seen for the kinases seen in figure 7.7(page 203), implying that the same kinases were seen in both preparations of cells. For the reasons outlined above, peak 1 was thought to be due to β1-PKC, whilst peak 2 was tentatively designated as ‘n-PKC’.

The absence of the ‘late eluting’ (i.e eluting with ≥ 270 mM phosphate) kinases (see figure 7.15, page 221) was particularly interesting, because these cells demonstrated no superoxide production with 100 nM Rx (i.e. low sensitivity cells). In contrast cells that produced high levels of superoxide with 100 nM Rx (i.e. high sensitivity cells) contained these ‘late eluting’ kinases (see figure 7.12, page 213). Since the ‘late eluting’ kinases showed the same elution characteristics as Rx-kinase, and since Rx-kinase has been implicated in specific activation of NADPH-oxidase by Rx in vitro, the ‘late eluting’
Figure 7.15: Protamine phosphorylating activity from the cytosol (C fraction) and the triton washed membranes (Ps fraction) of neutrophils that demonstrated low sensitivity to Rx.
kinases were thought to be involved in the superoxide response of cells stimulated with Rx.

7.6. **Kinase profiles of cells that demonstrated different sensitivities to TPA.**

The results outlined in chapter 6 showed that the concentration dependence of TPA stimulated superoxide production was heterogeneous, one possible explanation being variation in the levels of the phorbol ester receptor PKC. It was therefore decided to compare the levels of PKC in cells that demonstrated different superoxide responses when stimulated with the same doses of TPA. Figure 7.16 (page 223) shows the protamine phosphorylating activity, separated by hydroxyapatite FPLC, from two different preparations of neutrophils. The inset figure for each kinase profile shows the TPA stimulated superoxide production determined for each preparation of cells assayed under standard conditions (i.e. 160 µM cytochrome C, 1 x 10^6 cells/ml, 1 nM - 1 µM TPA, 37°C), demonstrated different superoxide responses (see insets of figure 7.16a and 7.16b, page 223). TPA stimulated superoxide production with a high maximal rate (0.297 nmoles/minute) and an ED_{50} value of 20 nM, was observed with the cells associated with figure 7.16a, whilst a maximal rate of superoxide production of only 0.159 nmoles/minute and an ED_{50} value of 8 nM was measured from the cells associated with figure 7.16b.

In the previous sections it was demonstrated that only peak 1 of each protamine phosphorylation profile was due to a phorbol ester sensitive kinase, which was immunologically identified as β₁-PKC. Therefore the level of activity associated with peak 1 was calculated by measuring the area of peak 1 for each kinase profile. The ratio of the activity of peak 1 from figure 7.16a (page 223) to peak 1 from figure 7.16b (page 223)
Figure 7.1a. Protein kinase phosphotransferase activity from the cytosol of neutrophils separated by hydroxyapatite FPLC. Inset shows the TPA stimulated enzyme product from the same cell.

Figure 7.1b. Protein kinase phosphotransferase activity from flow through the hydroxyapatite FPLC. Rate shows the TPA stimulated enzyme product from the same cell.
were approximately 2 : 1, which implies that there was twice as much of the peak 1 kinase in the figure 7.16a cells than in the figure 7.16b cells. However the kinase profile shown in figure 7.16a was derived from 2.8 x 10^6 cells, whilst the kinase profile shown in figure 7.16b was derived from 1.1 x 10^6 cells. Therefore if the number of cells is taken into account, then it becomes apparent that both preparations of cells contained similar levels of the peak 1 kinase. Since previous experiments identified the kinase responsible for peak 1 as β₁-PKC, these results suggest that both neutrophil preparations contained similar levels of β₁-PKC. Thus although the two preparations of cells demonstrated differences in TPA stimulated superoxide production, the level of β₁-PKC was not responsible. Other possible explanations are: lower levels of NADPH-oxidase substrates (i.e. NADPH and oxygen); or stringent feedback control leading to a lower rate of response; in the cells associated with figure 7.16b (page 223). It is unlikely that the late 'eluting peaks' were responsible, because these appear to be due to Rx-kinase, which is not activated by TPA (Sharma PhD Thesis 1995).

7.7. Discussion

A number of researchers have investigated the isotypes of PKC found in human neutrophils. The current consensus, based on the use of isotype specific anti-PKC sera, is that human neutrophils express high levels of β₁- and lower levels of β₂-, but no δ-, ε- nor γ-PKC (Fujiki et al. 1988; Pontremoli et al. 1990; Majumdar et al. 1991; Smallwood and Malawista 1992; Balazovich et al. 1992; Dang et al. 1994; Dang et al. 1995). Controversy exists over PKC isotype α, with some researchers finding very low levels of immunoreactivity, whilst others finding none at all. Devalia, using an RNase protection assay and antibodies to α-PKC, could find no immunoreactivity nor mRNA for α-PKC in
mature neutrophils (Devalia et al. 1992). Indeed Devalia showed that HL60 cells contained both α-PKC mRNA and protein, which disappeared as cells matured to a neutrophil phenotype in response to retinoic acid and therefore concluded that PKCα is specifically down-regulated during human neutrophil terminal differentiation. Furthermore Dang (Dang et al. 1994) showed that PKCα immunoreactivity was weak in PMN and strong in platelets, implying that detection of PKCα immunoreactivity in PMN may be due to platelet contamination. In the present study a platelet removing step was included to avoid this problem.

Controversy also exists over the presence of ζ-PKC in human neutrophils. Only Dang has detected ζ-PKC in human neutrophils using antibodies, and he found that ζ-immunoreactivity translocated from the cytosol to the membrane fraction in response to TPA (Dang et al. 1994). This result is suspicious since ζ-PKC is phorbol ester insensitive and therefore unlikely to translocate in response to TPA. It has now been recognised that certain antibodies to ζ-PKC (notably from Gibco-BRL, Cergy Pontoise) cross react with other isoforms of PKC (Allen et al. 1994). This suggests that the detection of ζ-immunoreactivity by Dang was due to cross reactivity of the antibody with another isotype of PKC.

Majumdar has found another PKC isotype in human neutrophils which has been rather poorly termed 'nPKC', not to be confused with the calcium independent category, novel-PKC isotypes (nPKC), or the isotype η-PKC (Majumdar et al. 1991; Majumdar et al. 1993). This 'nPKC' isozyme showed PS-, DAG-dependent, but calcium-independent histone-III phosphorylating activity suggesting a novel-PKC type of activity, and although
it showed immunoreactivity with a polyclonal consensus antibody to PKC, no immunoreactivity was observed with antibodies to \( \alpha \), \( \beta \), \( \gamma \), \( \delta \), \( \varepsilon \), or \( \zeta \)-PKC.

Therefore, the consensus to date is that \( \beta_1 \)-PKC is the predominant isotype in human neutrophils with lower levels of \( \beta_2 \), 'nPKC' and possibly even lower levels of \( \alpha \)-PKC in some preparations. It must be realised though that human neutrophils have not been screened for \( \eta \), \( \theta \), \( \tau \), \( \mu \)-PKC nor the novel protein kinase C related protein kinases PRK 1-3 (Dekker et al. 1995).

The results of this project agree with this consensus, in that \( \beta_1 \) was found to be the main PKC isozyme in human peripheral neutrophils. No \( \zeta \)-PKC was detected which is unsurprising as the antibody used was not from Gibco and does not demonstrate cross-reactivity with other PKC isotypes. No immunoreactivity was detected in the present study with antibodies to \( \beta_2 \), \( \gamma \), \( \delta \), \( \varepsilon \), \( \eta \) or \( \theta \)-PKC. The absence of \( \eta \) and \( \theta \)-PKC is a new finding, but the lack of detectable \( \theta \)-PKC must be treated with caution as antibodies to \( \theta \)-PKC react poorly with human protein (Prof. P.J. Parker, personal communication). The absence of immunoreactivity with antibodies to \( \beta_2 \)-PKC suggests that the detection threshold of the methodology used was not as low as that used by other researchers. The sensitivity of this assay could be improved by including antipain, chymostatin, pepstatin and diisopropyl fluorophosphate in the homogenisation buffer, and by using larger cell numbers.

Although specific immunoreactivity was observed with antibodies to \( \alpha \)-PKC, it was associated with a protein band with a molecular weight of 47 kDa. This molecular weight
was too low to be $\alpha$-PKC, and the absence of any $\beta_1$-PKM implies that the antibody was not detecting $\alpha$-PKM. The protein responsible was probably p47$^{\text{PAC}}$, which was also detected by Devalia in HL-60 cells, using antibodies to $\alpha$-PKC from the same source as this project (Devalia et al. 1992).

The translocation of neutrophil PKC from a soluble (cytosolic) fraction to another cellular compartment, upon stimulation by TPA or ionophores, has been reported by a number of groups (O’Flaherty et al. 1990; Majumdar et al. 1991; Curnutte et al. 1994; Dang et al. 1995). The original work, conducted by O’Flaherty, measured the increase in $[^{3}H]$PDBu binding in intact human neutrophils exposed to an ionophore. The involvement of PKC was then confirmed by Majumdar, who found that $\beta_1$-PKC bound to a membrane fraction when neutrophil cytoplasts were homogenised in calcium containing medium. Majumdar also showed that treatment of neutrophil cytoplasts with 1.6 $\mu$M TPA (5 minutes, 37°C) caused complete translocation of $\beta_1$-PKC from cytosol to the membrane. It should be noted that these experiments were conducted using neutrophil cytoplasts and not whole cells. Neutrophil cytoplasts are plasma membrane vesicles filled with cytoplasm, that lack nuclei, granules and mitochondria. They are prepared by using differential density gradient centrifugation, which under specific conditions, cause neutrophils to split into two structures, a cytoplast and a karyoplast, without spilling of cell contents into the gradient solution (Roos and Voetman 1986). Although neutrophil cytoplasts can phagocytose and kill bacteria, produce superoxide in response to stimuli and aggregate, they lack other functions seen in whole neutrophils, (e.g. casein stimulated chemotaxis and C5a stimulated superoxide production). Therefore results of experiments using cytoplasts may not give a true reflection of physiological events.
The results of this project suggest that TPA treatment of whole neutrophils caused rapid
downregulation, rather than stable translocation, of $\beta_1$-PKC. Stimulation with $\geq 100$ nM
TPA caused a loss of PKC immunoreactivity in the cytosolic (C) fraction without a
concomitant increase in PKC immunoreactivity in the particulate (Ps) fraction, or
appearance of $\beta_1$-PKM in the C fraction. The method seemed valid as TPA stimulated
translocation of PKC from a C to a Ps fraction was observed with monocytes. Down
regulation of $\beta_1$-PKC in neutrophils may be due to the presence of powerful proteases
released during the preparation of subcellular fractions from whole cells. However, a
recent report by Curnutte (Curnutte et al. 1994) suggests that TPA causes translocation of
$\beta_1$-PKC to a 1% triton insoluble, possibly cytoskeletal, compartment. In this study
translocation of PKC by TPA was only investigated in the triton-X100 soluble Ps fraction
and not the triton-X100 insoluble Pi fraction. It is therefore possible that rather than being
down regulated the $\beta_1$-PKC was in fact being translocated to the Pi fraction, a movement
that would not have been observed in this study.

Dang has reported TPA stimulated translocation of neutrophil $\beta_1$- and $\beta_2$-PKC from a
cytosolic to a membrane fraction (Dang et al. 1995). Dang’s results contrast with those of
this project, despite the use of similar conditions (8 - 1.6 $\mu$M TPA, 5 minutes, 37°C).
However, the methodology of Dang differed from that used in this project in one respect,
the preparation of subcellular fractions. In the present study neutrophil homogenates were
centrifuged at 100,000 x g to generate a C fraction (supernatant) and a Ps fraction
(pellet). Dang used a discontinuous sucrose gradient (50% sucrose, 30% sucrose). This
procedure may have caused separation of the membrane fraction from the protease laden
granules, and avoided PKC proteolysis. Therefore, an alternative to translocation of PKC
to a triton insoluble fraction, as an explanation of the lack of PKC in the P fraction of TPA treated cells, could be that translocated PKC was concentrated in the P fraction with neutrophil granules. Lysis of the granules by the second sonication step may have released proteases that could not be completely inactivated by the protease inhibitors used in the homogenisation buffers. Further evidence for this model comes from the observation of TPA stimulated translocation of PKC from the cytosol to the membrane in cytoplasts (Majumdar et al. 1991) and monocytes (this project), since both lack protease laden granules. Any future translocation studies should therefore use the gradient method of Dang to prepare subcellular fractions (Dang et al. 1995).

The results of the kinase assays supported the hypothesis that \( \beta_1 \)-PKC is the predominant PKC isotype in neutrophils. Neutrophil 100,000 x g supernatants, separated by hydroxyapatite chromatography, contained a single peak of TPA/PS/calcium stimulated histone-IIIs phosphorylating activity, which corresponded to anti-\( \beta_1 \)-PKC immunoreactivity and the appearance of a 76 kDa protein band in silver staining experiments. This peak of kinase activity eluted from hydroxyapatite FPLC columns at 73 - 113 mM phosphate. This is in agreement with the results of other researchers who found \( \beta \)-PKC to be the predominant PKC isotype in human neutrophils, although the concentration of phosphate required to elute \( \beta \)-PKC varied from 41mM to 140 mM phosphate, possibly reflecting the use of different grades of hydroxyapatite (Pontremoli et al. 1990; Majumdar et al. 1991; Smallwood and Malawista 1992; Balazovich et al. 1992).

The above results refer to histone-III\( s \) phosphorylating activity, but the use of protamine sulfate as the substrate in the kinase assay revealed the presence of a second peak of
activity in the cytosol of unstimulated cells. This second peak of protamine sulfate phosphorylating activity (i.e. peak 2 in figure 7.7, page 203, peak 2 in figure 7.15, page 221, and peak 3 in figure 7.12a, page 213) was observed in all the neutrophil preparations. It was distinct from α-, β1- and ζ-PKC on the basis of reaction with isotype specific antisera and therefore may have been ‘n-PKC’. The kinase responsible for this activity could not have contributed directly to phorbol ester stimulated superoxide production, because it was found to be phorbol ester insensitive in an in vitro histone-III phosphorylating assay. This conclusion is supported by the observation of Majumdar that β-PKC, but not nPKC, phosphorylated p47^phox (a component of the NADPH-oxidase complex). Thus if the second peak of protamine phosphorylating activity was ‘n-PKC’, then it may share some characteristics of δ- and ε-PKC, in that histone-III is an unacceptable substrate. Use of a range of PKC substrates will be required to elucidate whether peak 2 is a phorbol ester sensitive kinase.

Neutrophils that demonstrated a high sensitivity to Rx contained many peaks of protamine phosphorylating activity. Of these, only peak 1 (figure 7.12a, page 213) was sensitive to TPA and the responsible kinase was identified as β1-PKC on the basis of immunoreactivity to β1-PKC antibodies. The other two ‘early eluting’ peaks of activity (i.e. peaks 2 and 3) were not sensitive to TPA and therefore could not have contributed to phorbol ester stimulated superoxide production. Peak 3 (see figure 7.12a, page 213) was eluted at the same phosphate concentration as peak 2 in figure 7.7 (page 203). Therefore, for the reasons outlined above, peak 3 in figure 7.12a (page 213) may have been due to ‘n-PKC’ activity. Peak 2 from figure 7.12a (page 213) was not seen in any of the other kinase profiles and was phorbol ester insensitive as demonstrated in the in vitro histone-III phosphorylating assay.
phosphorylation assay.

Three other peaks of activity (i.e. peaks 4 and 5 from the C fraction and peak 1 from the Ps fraction) were detected exclusively in the Rx sensitive neutrophils. Unfortunately this activity degraded before a mixed micellular histone kinase assay could be conducted. However, these kinases showed the same elution behaviour as Rx-kinase (Ryves et al. 1990; Sharma et al. 1995) and were only present in cells that demonstrated high sensitivity to Rx. Since Rx-kinase has been shown to specifically activate NADPH-oxidase (Evans et al. 1989), it was concluded that induction of the Rx/protamine kinases conferred sensitivity to Rx upon neutrophils. This conclusion was supported by the absence of these late eluting kinases in cells that demonstrated only low sensitivity to Rx.

Since the sensitivity of neutrophils to TPA was heterogeneous the levels of phorbol ester sensitive kinases from different preparations of cells was measured. The histone-IIIIs phosphorylation assays showed that neutrophils contained only one peak of phorbol ester sensitive activity, which corresponded to the first peak of protamine phosphorylating activity. Since this activity correlated exactly with immunoreactivity to $\beta_1$-PKC antibodies, it was concluded to be due to $\beta_1$-PKC. The levels of $\beta_1$-PKC activity were therefore calculated from the area of the first peak protamine phosphorylating activity. Although two preparations of neutrophils demonstrated different TPA stimulated superoxide responses, they were found to contain similar levels of $\beta_1$-PKC activity. These results suggest that factors other than the level of $\beta_1$-PKC governed the heterogeneous sensitivity of neutrophils to TPA.
Chapter 8. Discussion.

8.1. Introduction.

The aim of this project was to purify and characterise the novel Rx-kinase (Rx-k), first described by Ryves (Ryves et al. 1989). Rx-kinase has been found exclusively in phagocytic cells, and defined, using an in vitro assay, as a histone-III phosphorylating kinase which is dependent on phospatidylserine, inhibited by calcium (nM range) and activated by the phorbol ester resiniferatoxin (Rx) (Sharma et al. 1995). Both Ryves and Sharma had problems working with Rx-kinase due to the sporadic nature of the activity. Therefore the initial strategy in the present study was to search for a reproducible source of Rx-kinase. Human monocytes, human neutrophils, starch elicited murine peritoneal macrophages and rat alveolar macrophages were all screened, but Rx-kinase activity was only detected sporadically. It was concluded that Rx-kinase was an inducible enzyme and a search for procedures or compounds that could induce the activity was begun. The techniques used by Sharma to enhance levels of Rx-kinase activity were tested. However, mixing of leukocytes from different donors, plumbagin treatment and starch elicitation all failed to induce Rx-kinase activity.

Evans, using a cell-free assay, showed that exogenous Rx-kinase specifically activated NADPH-oxidase in the presence of Rx, but not TPA and concluded that Rx-kinase may play a physiological role in the activation of the respiratory burst of phagocytes (Evans et al. 1990). It was therefore decided to develop a microtitre based superoxide assay to measure phorbol ester stimulated superoxide production. The aim was to find procedures that enhanced Rx stimulated superoxide production and determine whether those
procedures induced Rx-kinase activity.

The TPA stimulated superoxide response was not constant amongst different preparations of neutrophils, despite the use of constant conditions for cell isolation and assay. Reports in the literature have failed to reach a consensus on the ED₅₀ of TPA stimulated superoxide production, implying that the variation observed in the present study was not an artefact of the methodology, but a physiological characteristic of neutrophils. The most plausible explanation of the variable sensitivities of neutrophils to TPA is that the cells were primed to different extents. The activation of cells by microorganisms during the isolation procedure was found to be partly responsible for the variation in sensitivity of the cells to TPA. However, cells prepared using strict sterile technique also demonstrated variable rates and onset times for the TPA response, although administration of sufficient TPA doses elicited a constant response. This implied that the range of activation states of cells was not due simply to artefacts of the isolation procedure.

Analysis of human neutrophils revealed that they contained only one TPA sensitive kinase, namely β₁-PKC, the level of which was constant in cells that demonstrated different superoxide sensitivities to TPA. It therefore seemed unlikely that the different sensitivities of cells to TPA was due to different levels of β₁-PKC. However, it may be that the level of PKC associated with the membrane of cells governs the sensitivity of neutrophils to TPA. This hypothesis is supported by reports that demonstrate that the level of binding of phorbol ester to whole cells (a measure of PKC associated with the membrane) matches the dose-response of phorbol ester stimulated superoxide production. However, priming of the respiratory burst has been shown to involve a number of biochemical events, it is
therefore possible that mechanisms distinct from the PKC system were responsible for the variable sensitivities of neutrophils to TPA.

The results of the present study revealed that a termination mechanism, was involved in the regulation of phorbol ester stimulated superoxide production. The maximum level of superoxide production was limited by the upper detection threshold of the assay, but in some cases the response was terminated below that threshold. This termination was relieved in cells that had been repeatedly washed with EGTA, implying that the termination mechanism was calcium-dependent. The activity of this termination system varied, demonstrating stringent control of superoxide response in some cell preparations, and no detectable control in others. It was concluded that neutrophils not only regulate superoxide production by increasing the sensitivity of cells to stimuli (i.e. priming), but also by regulation of the level of the response via a calcium-dependent termination mechanism.

The aim of developing the superoxide assay was to find procedures or compounds that affected Rx stimulated superoxide production. High (μM) concentrations of Rx always stimulated a response, whilst stimulation of superoxide production by low (nM) concentrations of Rx was more sporadic. The Rx used was found to be contaminated with low levels of Ro, a compound related to Rx and a potent activator of PKC. It was therefore concluded that stimulation of superoxide production by high (μM) concentrations of Rx was due to contaminating Ro activating NADPH-oxidase through the PKC system.
The level of Ro contamination in the 100 nM Rx stock was thought to be too low to activate PKC, but 100 nM was still capable of activating the respiratory burst in some cell preparations (termed type B). Neutrophils that demonstrated high sensitivity to Rx did not demonstrate high sensitivity to TPA, implying that upregulation of PKC was not responsible for the increased sensitivity of cells to Rx. Indeed neutrophils demonstrating high or low sensitivity to Rx were found to contain similar levels of \( \beta_1 \)-PKC. Another receptor for Rx was thought to be responsible for the high sensitivity of cells to Rx, which was confirmed by the observation of protamine kinases that eluted from an hydroxyapatite column with the same phosphate concentration as Rx-kinase. These Rx/protamine kinases were only present in type B cells, i.e. those that demonstrated superoxide production with 100 nM Rx, and were therefore concluded to be responsible for the high sensitivity of those cells to Rx.

An investigation was conducted to find compounds capable of inducing the type B state in human neutrophils. Chemicals known to prime the respiratory burst were tested, but substance P, opsonized zymosan, lipopolysaccharide, muramyldipeptide, platelet aggregating factor, plumbagin and granulocyte-monocyte-colony stimulation factor all failed to enhance 100 nM Rx stimulated superoxide production. Overnight incubation of neutrophils reproducibly enhanced the superoxide response stimulated by high (\( \mu \)M) concentrations of Rx, but it also enhanced the TPA response, implying that PKC rather than Rx-kinase was involved. Analysis of the protamine kinase profile of cells incubated overnight revealed no late eluting peaks, implying that Rx-kinase was absent from these preparations and therefore not involved in the overnight incubation enhanced sensitivity of the cells to Rx.
The results suggest that experiments that successfully induce high Rx sensitivity in phagocytic cell superoxide production, also induce a family of related Rx/protamine kinases in these cells. Elucidation of such mechanisms of induction and therefore isolation, purification and characterisation of the Rx/protamine kinases, would be highly rewarding in unravelling the biochemical basis of the activation of the respiratory burst.

8.2. Absence of Rx-kinase activity.

In the beginning of the present study a reproducible source of Rx-kinase activity was sought, using a mixed micelle histone-IIIIs phosphorylation assay. Although the procedures outlined by Ryves and Sharma were used, Rx-kinase activity was low and sporadic. It was concluded that Rx-kinase was an inducible enzyme, with activity only present in ‘primed’ cells. This conclusion was reached only after the other possible explanations, namely assay failure, Rx-kinase degradation, insufficient source material had been analysed and rejected, as discussed below.

The assay contained two internal standards (rat brain PKC and PKC from the tissue being tested) which showed that the assay was functional. The internal standards were activated by TPA rather than Rx, because Rx is a poor activator of PKC, but the Rx used demonstrated a characteristic Rf value in TLC analysis, implying that the Rx itself was not at fault. Furthermore the protamine phosphorylating assay required no cofactors, and although detecting PKC activity in all preparations of cells, the detection of protamine kinase activity that eluted from hydroxyapatite with the same phosphate concentration as Rx-kinase was only sporadic. This suggests that some factor other than inactive Rx was responsible for the sporadic detection of Rx-kinase activity.
All procedures were conducted at 4 °C, using buffers which contained a range of protease inhibitors and had carefully adjusted pH. Furthermore the level of Rx-kinase activity did not increase with increase in the amount of source material or as the length of time taken for the isolation decreased. Therefore it seems unlikely that degradation of the enzyme during isolation was responsible for the low levels and sporadic nature of Rx-kinase activity.

The same amounts of tissues, as described by Ryves and Sharma, were used, but the same yields could not be achieved. Increasing the amount of source material failed to increase the level of Rx-kinase activity. These points suggest that an insufficient amount of source material was not responsible for the low yields of Rx-kinase activity.

Having considered the above arguments it was concluded that Rx-kinase was absent from some tissue preparations. However, since Rx-kinase and related ‘late eluting’ protamine kinase activity was detected in some tissue preparations, it was concluded that Rx-kinase was an inducible enzyme. That is to say that either this protein was not expressed, or existed in an inactive form in the resting cells. In this model Rx-kinase activity would only be detected after transcription of the Rx-kinase gene or modification of the inactive apoenzyme.

The immediate problem with this model is the lack of Rx-kinase activity seen in this project, even with white blood cells from mixed donors, plumbagin treated cells or starch elicited peritoneal macrophages. The results obtained in this present study imply that these procedures alone do not reproducibly induce Rx-kinase activity. Sharma found that
treatment with plumbagin or Rx, or mixing leukocytes from different donors, enhanced basal levels of Rx-kinase activity. However he did not show that these procedures induced activity in cells showing no basal Rx-kinase activity. It is therefore possible that these procedures do not actually induce Rx-kinase activity, merely increase basal activity. Alternatively these procedures may require additional factors to successfully induce activity. Such behaviour has been observed with other systems, for example the cytokine tumour necrosis factor α (TNFα) does not stimulate hydrogen peroxide production from neutrophils in suspension, but stimulates massive and immediate hydrogen peroxide production from neutrophils adhered to fibronectin (Nathan 1987). It is possible that the procedures for priming of Rx-kinase activity reported by Sharma depended on some unrecorded factor that was not reproduced in the present study.

8.3. The superoxide production assay

Evans, using a cell free assay, observed that exogenous Rx-kinase selectively activated NADPH-oxidase in the presence of Rx, but not TPA, and concluded that Rx-kinase may be involved in the physiological activation of the respiratory burst (Evans, A.T. et al. 1990). It was therefore decided to continue the hunt for Rx-kinase by using a superoxide assay. The aim was to search for compounds or procedures that reproducibly enhanced Rx stimulated superoxide production.

In order to screen a wide range of compounds a micro-titre based assay was developed. This assay was based on the micro-titre assay that Pick used to measure superoxide production from macrophages. It measured the reduction of cytochrome C by superoxide anions, by following the change in absorbance by cytochrome c of 550 nm light. The
assay was optimised for use with neutrophils and then used to measure phorbol ester
stimulated superoxide production.

8.3.1. TPA stimulated superoxide production.

TPA stimulated superoxide production was found to vary despite assay conditions being
kept constant. The rate, level and onset time of the TPA response were all found to vary.
However, neutrophils could be split into three categories according to their sensitivity to
TPA. High sensitivity cells responded to ≤ 1 nM TPA with an ED_{50} value of 2.44 ± 1.74
nM. Medium sensitivity cells responded to ≤ 10 nM TPA with an ED_{50} value of 30.53 ±
11.82 nM TPA. Low sensitivity cells responded to ≥ 100 nM TPA with an ED_{50} value of
189.45 ± 44.95 nM TPA.

The published literature also points to variation in sensitivity of neutrophils to TPA.
Researchers have failed to reach a consensus on the ED_{50} value for TPA stimulated
superoxide production (3.9 nM (Tauber et al. 1982), 0.29 nM (Lehrer and Cohen 1981),
19.4 nM (Della Bianca et al. 1986)), implying that the sensitivity of neutrophils to TPA is
variable. Furthermore Merritt (Merritt et al. 1993) reported being unable to quantify
phorbol ester stimulated respiratory burst results, because of variability of responses.
These reports suggest that the variation in TPA stimulated superoxide production
observed in the present study was not an artefact of the methodology used, but a
physiological characteristic of neutrophils.

The variation in sensitivity to TPA implies that a number of activation states exist for
neutrophils. This has been well documented and most clearly demonstrated in the
phenomenon of priming (Johnston and Kitagawa 1985; Haslett et al. 1989; Walker et al. 1990). Primed neutrophils demonstrate a larger and more rapid response to a variety of stimuli, than resting cells. Therefore, the most plausible explanation for the heterogeneity of the concentration dependence of phorbol ester stimulated superoxide production is that the cells from different preparations were primed to varying extents. All solutions used in these experiments were filtered with 0.6 μm filters to remove contaminating microorganisms in an attempt to minimise artefactual priming. However, the use of strict sterile technique during the isolation of some cells prevented the acetone stimulated response, indicating that in some preparations the cells became activated during the isolation procedure due to contamination by microorganisms. However, even with strict sterile technique variation in TPA stimulated superoxide production was observed, suggesting that contamination by microorganisms was not the sole cause of neutrophil priming. Although Haslett has shown that standard methods of neutrophil isolation can prime cells, he traced the cause to LPS, a product of contaminating bacteria (Haslett et al. 1985). These results imply that neutrophils with a range of activation states existed in the bodies of donors, and although further, artefactual, priming could be prevented by use of rigorous sterile technique, isolation of cells with a range of activation states was unavoidable.
8.4. Neutrophil PKC and activation of the respiratory burst.

The only isotype of PKC, detected by immunoblotting of neutrophils homogenates, was \( \beta_1 \)-PKC and this was located exclusively in the cytosol of unstimulated cells. Separation of the neutrophil homogenates by hydroxyapatite FPLC, followed by a PKC assay on the elution fractions revealed only one peak of phorbol ester sensitive kinase activity. This peak of histone-III phosphorylating activity correlated with immunoreactivity to \( \beta_1 \)-PKC antibodies. On the basis of this data and the observation of a 76 kDa silver staining protein band that was only present in the active fractions, it was concluded that \( \beta_1 \)-PKC was the predominant phorbol ester sensitive PKC isotype in human neutrophils. This result agrees with the current consensus found in the literature, that \( \beta_1 \)-PKC is the predominant PKC isotype found in neutrophils (Fujiki et al. 1988; Pontremoli et al. 1990; Majumdar et al. 1991; Smallwood and Malawista 1992; Balazovich et al. 1992; Dang et al. 1994; Dang et al. 1995). Some researchers have also found low levels of \( \beta_2 \)-PKC in neutrophils (Smallwood et al. 1992; Devalia et al. 1992; Dang et al. 1995), but none was detected in the present study. It is possible that \( \beta_2 \)-PKC was present at levels below the detection threshold of the antibodies used in the present study. The reports of detection of \( \alpha \)- and \( \zeta \)-PKC in neutrophils remain unconvincing as those studies: (1) failed to use competing epitope, to establish specificity of antibody binding; (2) used cross-reacting antibodies; or (3) failed to ensure absence of platelets, which contain high levels of \( \alpha \)- and \( \zeta \)-PKC (Dang et al. 1994; Dang et al. 1995).

A second peak of protamine phosphorylating activity was detected in the cytosol of unstimulated neutrophils, which was eluted from an hydroxyapatite FPLC column by a concentration of phosphate characteristic of PKC. This activity was not TPA sensitive and
failed to react with antibodies to α-, β₁-, or ζ-PKC. This second peak of protamine phosphorylating activity was tentatively identified as ‘nPKC’ (see Majumdar et al. 1991). Whatever, the true identity of this protamine kinase, it was found to be phorbol ester insensitive in a mixed micellular histone-IIIIs phosphorylating assay. This may indicate that it was either (1) TPA insensitive or (2) similar to δ- and ε-PKC and therefore unable to phosphorylate histone-IIIIs even in the presence of phorbol ester.

The involvement of PKC in the activation of the respiratory burst has been demonstrated by several lines of evidence. TPA, a specific activator of PKC, has been shown to stimulate the superoxide production from human neutrophils (e.g. Curnutte et al. 1994). The p47phox oxidase component contains several recognition sites for PKC which become phosphorylated after stimulation of neutrophils with TPA (Rotrosen and Leto 1990). The level of phosphorylation of p47phox was found to match the level of superoxide production in TPA stimulated cells (Schneider et al. 1981). The PKC inhibitor 1-O-hexadecyl-2-O-methylglycerol, was found to block both the phosphorylation of p47phox and the production of superoxide in neutrophils and cytoplasts stimulated with TPA (Kramer et al. 1989). Finally, exogenous PKC isotypes have been shown to stimulate superoxide production in a cell-free assay, in the presence of TPA/Triton X-100/PS micelles (Sharma et al. 1991).

The TPA stimulated superoxide production observed in the present study must have been mediated, at least in part, by β₁-PKC, since this was the only TPA sensitive kinase detected in the neutrophils. The involvement of β isotype of PKC in the phosphorylation of p47phox, has been demonstrated by Majumdar (Majumdar et al. 1993). Two PKC
isotypes were isolated from neutrophils, namely β-PKC and 'nPKC', but only β-PKC was able to phosphorylate $p47^{phox}$ in vitro. Furthermore a specific peptide inhibitor of PKC (pseudosubstrate(19-36)) inhibited the in vitro phosphorylation of $p47^{phox}$ and the in vivo phosphorylation of membrane associated $p47^{phox}$. Majumdar concluded that β-PKC may play a role in the phosphorylation of membrane associated $p47^{phox}$ and the assembly and maintenance of an active NADPH oxidase. Furthermore a peptide derived from the carboxy terminus of $p47^{phox}$ has been shown to inhibit the TPA stimulated phosphorylation of $p47^{phox}$ and the oxidative burst, in vivo (Labadia et al. 1996). However it is important to realise that renaturation assays have detected several $p47^{phox}$ phosphorylating kinases, in addition to β-PKC, in cells that have been stimulated with TPA (Ding et al. 1993). It is therefore possible that β-PKC is only one member of a cascade of kinases that phosphorylate $p47^{phox}$, thereby activating NADPH-oxidase in cells stimulated with TPA.

The first peak of protamine phosphorylating activity in the elution profile of neutrophil cytosol separated by hydroxyapatite FPLC was identified as being due to β₁-PKC, on the basis of immunoreactivity to anti-β₁-PKC antibodies, TPA sensitive histone-IIIs phosphorylation and the presence of a silver staining protein band of the correct molecular weight only in the active fractions. The activity of the first peak of protamine phosphorylating activity was therefore used as an estimate of the level of β₁-PKC in the cytosol of unstimulated neutrophils. Using this method, the level of β₁-PKC activity in neutrophils that demonstrated different sensitivities to TPA was compared, and found to be similar. This implies that the level of β₁-PKC in neutrophils did not determine the sensitivity of the superoxide production of those cells to TPA. However, it is possible that the level of β₁-PKC associated with the membrane of the neutrophils, rather than the total
level of β₁-PKC in the cells, determined the sensitivity of neutrophils to TPA. Evidence that supports this hypothesis is discussed below.

Phorbol esters are highly lipophilic molecules and therefore likely to partition into the membrane of cells. This means that the level of PKC associated with the membrane may govern the level of PKC available to phorbol ester, therefore the level of PKC activated by phorbol ester, and hence the sensitivity of neutrophils to TPA. This hypothesis is supported by whole cell binding studies, which measured the binding of radiolabelled phorbol ester ([³H]-PdBu) to neutrophils. The number of phorbol ester binding sites in intact neutrophils was found to vary. Lehrer and Cohen (1981) observed ~ 2.1 ± 0.6 x 10⁵ sites/cell, whilst Tauber et al. (1982) observed 7.6 x 10⁵ sites/cell. Evidence that this variation was due to the level of PKC associated with the membrane came from studies conducted by O'Flaherty et al. (1990). The number of phorbol ester binding sites in intact cells (1.6 x 10⁵) was less than the number found in disrupted cells (9 x 10⁵) and the intracellular calcium levels affected the number of sites available to phorbol esters in intact cells. Thus calcium depleted cells had 1 x 10⁵ sites/cell, whilst ionomycin treated cells had 2.5 x 10⁵ sites/cell. O'Flaherty concluded that calcium influx, caused by the ionophore, translocated PKC to the plasma membrane thus increasing the number of available sites for phorbol ester binding. Since the level of specific phorbol ester binding is a reflection of the level of activation of PKC, it is possible that the variation in the sensitivity of neutrophils to TPA was due to variation in the level of PKC associated with the membrane of cells. Under this model the number of binding sites in intact cells would be a reflection of the amount of PKC translocated to the plasma membrane and therefore sensitive to any procedures that caused movement of PKC.
This model is further supported by the correlation between the sensitivity of neutrophils to TPA and the binding affinity of phorbol ester to those neutrophils. For example, Tauber et al. (1982) found that TPA stimulated superoxide production with an ED$_{50}$ of 3.9 ± 2.1 nM, a value that agreed quantitatively with the binding affinity to neutrophils of TPA (K_d = 4.9 nM), in cells that exhibited 7.6 x 10^5 phorbol ester binding sites per cell. Whilst Leher and Cohen (1981) reported a very different binding affinity for TPA of 0.29 nM, but it agreed closely with the dose-response for TPA stimulated superoxide production, in cells that exhibited ~ 2.1 ± 0.6 x 10^5 phorbol ester binding sites per cell. Thus the dose-response relationship for TPA stimulated superoxide production reflected the number of available binding sites, which in turn reflected the amount of PKC associated with the membrane of cells (i.e. translocated PKC).

The physiological mediator of PKC translocation is thought to be calcium (May 1985; Wolf et al. 1985; O'Flaherty et al. 1990). It was shown above that increases in cytosolic calcium levels, after exposure of neutrophils to ionomycin in a calcium rich buffer, led to an increase in the number of binding sites for phorbol ester in whole cells (O'Flaherty et al. 1990). It has been shown by Merritt (Merritt et al. 1993) that ionomycin mediated increases in cytosol calcium levels also increased the sensitivity of neutrophils to phorbol ester stimulated superoxide production. It seems therefore that calcium mediated translocation of PKC increases the sensitivity of cells to phorbol ester. In the present study, overnight incubation of neutrophils increased the sensitivity of their superoxide production to phorbol ester. This increase in sensitivity could be abrogated by repeated washes of the cells with EGTA, implying that calcium was involved in the increased sensitivity. These results support the hypothesis that calcium mediated translocation of
PKC increases the sensitivity of neutrophil superoxide production to phorbol esters.

Other biochemical events, distinct from PKC translocation, could also contribute to the variable sensitivity of neutrophil superoxide production to TPA. TPA is thought to activate NADPH oxidase through the phosphorylation of p47phox (see above), but other kinases are known to phosphorylate p47phox. Renaturation assays have detected a number of kinases that phosphorylate p47phox-derived peptide in vitro, and these kinases may play a role in the physiological activation of the respiratory burst (Ding et al. 1993). Furthermore PKA mediated phosphorylation of p47phox is thought to inhibit the respiratory burst (Kramer et al. 1988b). It is possible that these other kinases could modulate the superoxide response stimulated by TPA by altering the phosphorylation pattern of p47phox, thereby altering the level of PKC mediated phosphorylation required for activation of NADPH-oxidase. By the same token the level of activity of protein phosphatases, which have been implicated in the dephosphorylation of p47phox (e.g. Curnutte et al. 1994), could contribute to the level of PKC mediated phosphorylation required for NADPH-oxidase activation. Furthermore, activation of the respiratory burst by lipid moieties such as arachidonic acid, phosphatidic acid and diacyl glycerol has been reported (Miyahara et al. 1988; Heyworth and Badwey 1990a). It is therefore conceivable that partial activation of the NADPH oxidase by lipid molecules could enhance the stimulation by phorbol esters.

The results of the present study revealed that a termination system was also involved in the regulation of phorbol ester stimulated superoxide production. The upper detection limit of the superoxide assay was limited by the level of cytochrome c added to each well of the microtitre plate. Since 100 µl of 160 µM cytochrome c were added to each well and since
one mole of superoxide reduces one mole of cytochrome C, the maximum amount of superoxide that could be detected was 16 nmoles. Therefore if the curve in the graph of the time course for superoxide production reached a plateau at 16 nmoles of superoxide, the cause was taken to be exhaustion of cytochrome C. However in some cases the curve reached a plateau at a level of superoxide production below the upper detection threshold of the assay. This was not an artefact of the adjustment of the data for the vehicle solvent (acetone) stimulated response, because the plateau was still lower than the limit of the assay in graphs showing the phorbol ester stimulated response before acetone adjustment. The plateau at levels below the limit of the assay was not due to cell death, because in the same experiment cells continued to produce higher levels of superoxide with higher concentrations of TPA, suggesting that higher levels of TPA could override the termination mechanism and implying that neither the superoxide nor the TPA was toxic to the cells at those levels. It was therefore concluded that a termination system was involved, which terminated the TPA stimulated superoxide production in some preparations of cells. This hypothesis was strengthened by the observation that repeated washes of the cells with EGTA relieved the termination of the TPA stimulated response. This result also demonstrated that the termination system was calcium dependent.

Physiological activators of the respiratory burst are known to cause a transient response, for example the FMLP response peaks by 45 seconds and declines to 50% of the maximum by 2 minutes, implying that the respiratory burst stimulated by physiological activators is terminated after a short period of time (McPhail and Snyderman 1983). In contrast the TPA stimulated response has been shown to peak by 20 minutes and still not have declined by 90 minutes (McPhail and Snyderman 1983). Phorbol ester stimulated
superoxide production has therefore been found to be long lasting and since most researchers have only measured the initial response (i.e. first 10 minutes after stimulation), the long term response to phorbol esters has not been fully investigated. However, Tauber measured $[^3H]$PDBu binding to neutrophils, over a time course that exhausted the respiratory burst. He found no change in $[^3H]$PDBu binding and concluded that autoinactivation of the oxidase, rather than downregulation of PKC, was the cause of the termination of the phorbol ester response (Tauber et al. 1982). These results support the interpretation of the observations in the present study, namely that the levelling off of the rate of superoxide production stimulated by TPA was due to termination of the oxidase activity, before exhaustion of cytochrome C. Light (Light et al. 1981) observed termination of superoxide production in a cell-free system. Light was unable to determine the cause of the 'autoinactivation', but termination occurred rapidly ($t_1/2 = 24$ min) and was not due to substrate exhaustion nor feedback inhibition by superoxide anions. Termination could be mediated by a number of possible systems, for example phosphorylation of p47$^{phox}$ and/or Rap1A by PKA or dephosphorylation of p47$^{phox}$ by protein phosphatases. However, the relief of termination by repeated EGTA washes of cells, observed in the present study, argues that the termination system was calcium-dependent.

### 8.5 Rx stimulated superoxide production

A strong superoxide response was always observed with high (μM) concentrations of Rx (BH-107), but only sporadically with low (nM) concentrations of Rx. This led to the classification of neutrophils into two categories. Low sensitivity cells demonstrated a response only with high (μM) concentrations of Rx (BH-107), whilst high sensitivity cells also demonstrated a response with low (μM) concentrations of Rx (BH-107). Several
researchers, using *in vitro* assays, have shown that Rx only interacts with isotypes of PKC at concentrations > 10 μM (Ryves et al. 1991; Dimitrejevic et al. 1995). It was therefore concluded that the superoxide response with high (μM) concentrations of Rx was mediated by PKC, but the response to low (nM) concentrations of Rx was due to another receptor, possibly Rx-kinase. However, when a new batch of Rx (BH-108) was used no stimulation of superoxide production was seen with high (μM) concentrations. The different potencies of Rx (BH-107) and Rx (BH-108) was found to be due to contaminated of Rx (BH-107) with low (≤ 4%) levels of Ro, a derivative of Rx and a potent activator of PKC (Ellis et al. 1987). Analysis of the ability of pure Ro to stimulate superoxide production revealed that this compound stimulated a response with an ED₅₀ value of 241.94 ± 28.73 nM. It therefore seems probable that the response seen with 10 μM Rx (batch BH-107) was actually due to the contaminating Ro.

It seems unlikely that the contaminating Ro contributed to the superoxide response stimulated by low (nM) concentrations of Rx, for a number of reasons. The level of contamination was low, i.e. the Rx was sold by Calbiochem as 98% pure and the contaminating Ro was not detectable by TLC analysis. Furthermore the contaminating Ro was maximally active in the 10 μM solution of Rx, thus the 100-fold dilution used to make the 100 nM Rx solution would have diluted out the Ro. The fact that the 100 nM Rx (BH-107) solution did not consistently stimulate a response supports this argument. It therefore seemed possible that the superoxide response with 100 nM Rx (BH-107), i.e. in cells demonstrating high sensitivity to Rx, was mediated by Rx, rather than the contaminating Ro. Rx only interacts with PKC at high (μM) concentrations (see above), it therefore seems improbable that PKC was responsible. Furthermore the fact that cells
demonstrating a high sensitivity to Rx did not demonstrate a high sensitivity to TPA implied that up-regulation of PKC was not responsible for the response to low (nM) doses of Rx. It therefore seems possible that a receptor with a high affinity for Rx (e.g. Rx-kinase) was involved in the superoxide response seen with 100 nM Rx.

8.6. Rx-kinase and superoxide production

It has already been shown that the stimulation of superoxide production by high (μM) concentrations of Rx (BH-107) was probably due to contaminating Ro, but that the response seen with low (nM) concentrations of Rx (BH-107) may have involved Rx-kinase. The cytosol from neutrophils that demonstrated high sensitivity to Rx were separated by hydroxypatite chromatography, assay of the resultant fractions revealed a kinase profile with multiple peaks of protamine phosphorylating activity. The peaks of activity that eluted with low (< 200 mM) concentrations of phosphate were characteristic of PKC, but only one of them was phorbol ester sensitive in an *in vitro* histone-IIIs phosphorylation assay and was identified as β₁-PKC by immunoblotting. In contrast, two peaks of protamine phosphorylating activity eluted with high (> 270 mM) concentrations of phosphate. This elution pattern is uncharacteristic of PKC, but was the same as observed with Rx-kinase (Sharma et al. 1995). The kinases responsible for the ‘late eluting’ peaks of activity could not be positively identified as Rx-kinase, because the activity was labile and degraded before a histone-IIIs phosphorylation assay could be conducted. However, the elution pattern was the same as previously seen for Rx-kinase and the ‘late eluting’ peaks of activity were only seen in neutrophils that demonstrated a high sensitivity to Rx. This last point implies a link between the ‘late eluting’ kinase activity and the sensitivity of the cells to Rx. Cells that did not demonstrate a superoxide
response with 100 nM Rx (i.e. low sensitivity to Rx cells) contained similar levels of \( \beta_1 \)-PKC to cells that demonstrated high sensitivity to Rx, but did not contain any 'late eluting' protamine phosphorylating activity.

The fact that the late eluting Rx/protamine kinases were only observed in neutrophils that demonstrated high sensitivity to Rx, shows that this type of activity was not present in all preparations of cells. This suggests that the responsible kinases were inducible, i.e. not expressed or present as inactive apo-enzymes in cells that did not demonstrate 'late eluting' kinase activity. Degradation of activity seems an unlikely explanation for the lack of the 'late eluting' activity in some cells, because the same conditions were used to isolate and assay the proteins in each experiment and similar levels of \( \beta_1 \)-PKC activity were observed in each preparation of cells.

The correlation between 'late eluting' kinase activity and high sensitivity to Rx, implies that induction of these kinases conferred a high sensitivity to Rx state upon human neutrophils. This would explain why only some preparations of cells demonstrated high sensitivity to Rx. Cells that lacked the 'late eluting' kinases, lacked a high affinity receptor for Rx, namely Rx-kinase. Thus, although such cells contained the \( \beta_1 \)-isotype of PKC, they could not respond to 100 nM Rx, because this concentration of Rx does not interact with \( \beta_1 \)-PKC (Ryves et al. 1991; Dimitrejevic et al. 1995).

8.7. Induction of the high sensitivity to Rx state.

An investigation was conducted to find compounds capable of inducing the high sensitivity to Rx state in human neutrophils. Chemicals known to prime the respiratory burst were
tested, but substance P, opsonized zymosan, lipopolysaccharide, muramyl dipeptide, platelet aggregating factor, plumbagin and granulocyte/monocyte-colony stimulation factor all failed to enhance 100 nM Rx stimulated superoxide production. These results show that the priming events mediated by these compounds are not involved in altering the sensitivity of neutrophil superoxide production to phorbol esters. If the response of neutrophils to 100 nM Rx is mediated by Rx-kinase, then these priming agents cannot be involved in the induction of that enzyme under the conditions tested.

One procedure, namely overnight incubation of neutrophils, did enhance the Rx stimulated superoxide production. However, overnight incubation only reproducibly enhanced the response to high (μM) concentrations of Rx (BH-107), which was thought to mediated by the contaminating Ro acting through PKC. Furthermore this procedure also enhanced the response to TPA, implying that the PKC system, rather than Rx-kinase, was involved. Protamine phosphorylation profiles from neutrophils incubated overnight did not reproducibly contain ‘late eluting’ kinase activity, whereas they always contained β1-PKC, showing that this procedure did not induce the Rx/protamine kinases.

The search for compounds or procedures that induce neutrophils to respond to 100 nM Rx must continue. The analysis of compounds which operate through cell-surface receptors has failed, therefore compounds that operate downstream of these receptors should be tested. For example compounds that interfere with protein phosphatases, calcium metabolism, tyrosine phosphorylation or gene expression should be screened.
8.8. Conclusion.

Purification of Rx-kinase proved impossible, due to the sporadic nature of the activity. Attempts to find a reproducible source of Rx-kinase activity following the procedures outlined by previous research also proved unsuccessful. Evans (Evans et al. 1990) showed that Rx-kinase may be physiologically involved in the activation of the respiratory burst. A superoxide assay was therefore developed with the aim of finding compounds or procedures that enhanced the Rx stimulated response. The superoxide response of cells to low (nM) concentrations of Rx was also sporadic, thus demonstrating a similarity to Rx-kinase activity. Analysis of cells that demonstrated a superoxide response with low (nM) concentrations of Rx, revealed the presence of protamine kinases that eluted from an hydroxyapatite column with the same phosphate concentration required to elute Rx-kinase. These ‘late eluting’ kinases could not be positively identified as Rx-kinase, but were only detected in cells that responded to low concentrations of Rx with high levels of superoxide production.

The superoxide assay developed in the present study represents a tool for identifying neutrophils that contain the ‘late eluting’ kinases. It was used to screen compounds for ability to induce a superoxide response to low (nM) concentrations of Rx, but the compounds tested failed to induce that state. This assay can accommodate larger numbers of test compounds than the mixed micellar histone-III's assay, that has traditionally been used to hunt for procedures that induce Rx-kinase. It therefore represents a logical step forward in the hunt for Rx-kinase and will prove to be a powerful tool in future studies.

The results of the present study suggest a link between the ‘late eluting’ kinases and Rx
stimulated superoxide production. Since these kinases appear distinct from PKC their isolation and characterisation will be highly rewarding in the study of the activation of the respiratory burst.
Bibliography.


superoxide and on change in shape by human neutrophils: reversibility by albumin.


Bell, R.M. and Burns, D.J. (1991) J. Biol. Chem. 266; 4661-4664. Lipid activation of Protein Kinase C.


Bokoch, G.M. Quilliam, L.A. Bohl, B P. (1991) Science 254; 1793-1796. Inhibition of Rap1A binding to cytochrome b538 of NADPH oxidase by phosphorylation of Rap1A.


Diradyl glycerol synergises with an anionic amphiphile to activate superoxide
generation and phosphorylation of p47phox in a cell-free system from human
neutrophils.

Burns, D.J. and Bell, R.M. (1992) in ‘PKC current concepts and future perspectives’
(Lester, D.S. and Epand, R.M. Eds.) Ellis Horwood, New York 1992, ch2. Lipid
regulation of PKC.

Caldwell, S.E. McCall, C.E. Hendricks, C.L. Leone, P.A. Bass, D.A. McPhail, L.C.
and phosphorylation of a 48 kD protein(s) by a cytosol factor defective in
autosomal recessive chronic granulomatous disease.

Chem. 265; 20241-20246. Interferon-gamma induces in human neutrophils and
macrophages expression of the mRNA for the high affinity receptor for
monomeric IgG(Fc-γ-R-1) or CD64.

Chem. 257; 7847-7851. Direct activation of calcium activated phospholipid-
dependent protein kinase by tumour promoting phorbol esters.

Biochem. 194; 799-804. Effector dependent conformational changes in PKCγ
through epitope mapping with inhibitory monoclonal antibodies.

254;10262-10267. Calmodulin and development and application of a sensitive
radioimmunoassay.

Chanock, S.J. El Benna, J. Smith, R.M. Babior, B.M. (1994) J.Biol. Chem. 269;24519-
24522. The respiratory burst oxidase.

Superoxide production by B-lymphocytes lacking respiratory burst oxidase
subunit p47-phox after transfection with an expression vector containing a p47-
phox cDNA.

Two cytosolic components of the human neutrophil respiratory burst oxidase
complex translocate to the plasma membrane during cell activation.

toxin and neomycin on G-protein-regulated phosphoinositide phosphodiesterase - a
comparison between HL60 membranes and permeabilised HL60 cells.

guanine nucleotide binding proteins in the activation of polyphosphoinositide
phosphodiesterase.

generation by digitonin stimulated guinea pig granulocytes.

2898. Stimulation of human neutrophil respiratory burst by formyl peptides is
primed by a protein kinase inhibitor, staurosporine.

Cooper, D.R. Watson, J.E. Acevedo-Duncan, M. Pollet, R.J. Standaert, M.L. Farese, R.V.
PKC isoenzymes following chronic phorbol ester treatment in BC3H-1 myocytes.


Curnutte, J.T. Erickson, R.W. Ding, J. Badwey, J.A. (1994) 269;10813-10819. Reciprocal interactions between PKC and components of NADPH oxidase complex may regulate superoxide production by PMN stimulated with phorbol ester.


Dekker, L.V. Parker, P.J. (1994) TIBS 19; 73-74. PKC a question of specificity.


CSF and other cytokines prime human neutrophils for enhanced arachidonic acid and leukotriene B4 synthesis.


Evans, A.T. Sharma, P. Ryves, W.J. Evans, F.J. (1990) FEBS letts. 267; 253-256. TPA and Rx-mediated activation of NADPH oxidase a possible role for Rx-kinase augmentation of PKC.


Gschwendt, M. Kittstein, W. Marks, F. (1991) TIBS 16; 167-169. Protein kinase C activation by phorbol esters: do cysteine-rich regions and pseudosubstrate motifs play a role?


binding and activation of PKC on Triton X-100 mixed micelles containing phosphatidylserine.

Hannun, Y. A. Loomis, C. R. Bell, R. M. (1985) J. Biol. Chem. 260; 10039-10043. Activation of PKC by Triton X-100 micelles containing DAG and PS.


Hata, A. Akita, Y. Komo, Y. Suzuki, K. Ohno, S. (1989) FEBS lets. 252; 144-146. Direct evidence that the kinase activity of PKC is involved in transcriptional activation through a TPA responsive element.

Hecker, E. (1988) Cancer Res. 28; 2338-2349. Cocarcinogenic principles from the seed oil of Croton tiglium and other Euphorbiaceae


Heyworth, P. G. and Badwey, J. A. (1990b) Biochim. Biophys. Acta. 1052; 292-305. Continuous phosphorylation of both the 47 and 49 kDa proteins occurs during superoxide production by neutrophils.

Heyworth, P. G. and Segal, A. W. (1986) Bioch. 239;723-731. Further evidence for the involvement of a phosphoprotein in the respiratory burst oxidase of human neutrophils.


Maly, F.E. Schuerer-Maly, C.C. Quilliam, L. Cochrane, C.G. Newburger, P. Curnutte,
Reconstitution of superoxide generation in autosomal cytochrome - negative
chronic granulomatous disease (A22(0)CGD)-derived b-lymphocyte cell lines by
transfection.

Studies on the priming sequence requirements for PKC-α, -β, and -γ peptide
substrates.

phorbol ester/diacylglycerol-binding protein encoded by the Unc-13 gene of C.
elegans.

Masmoudi, A. Labourdette, G. Mersel, M. Huang, F.L. Huang, K.-P. Vincendon, G.
Malviya, A.N. (1989) J. Biol. Chem. 264; 1172-1179. PKC located in rat liver
nucli.

and the extinction coefficient of cytochrome C.

of intracellular calcium mobilization in the regulation of PKC mediated
membrane processes.

for superoxide production by neutrophils and other phagocytic cells.

3053. Priming of the human neutrophil respiratory burst by GM-CSF and TNFα
involves regulation at a post-cell surface receptor level - enhancement of the
effect of agents which directly activate G-proteins.

NADPH oxidase of human polymorphonuclear cells.

activate PMN NADPH oxidase.

Melloni, E. Pontemoli, S. Michetti, M. Sacco, O. Sparatore, B. Salamino, F. Horecker,
calpain in the activation of PKC in neutrophils stimulated by TPA.

Biochem. J. 289; 919-926. Involvement of calcium modulation of neutrophil
function by phorbol esters that activate PKC isotypes and related enzymes.

Mikawa, K. Maekawa, N. Goto, R. Yaku, H. Obara, H. Kishimoto, A. Kusunoki, M.
(1991) Italian J. Biochem. 40; 133-142. Limited proteolysis of PKC subspecies by
calpain: stimulation by basic polypeptides.

Biochim. Biophys. Acta. 971; 46-54. Charge dependent regulation of NADPH
oxidase activities in intact and subcellular systems of polymorphonuclear
leukocytes.

14868. Intracellular receptors for activated PKC.

Regul. 1; 693-706. A PKC isozyme is translocated to cytoskeletal elements on
activation.

Collagen activates superoxide anion production by human polymorphonuclear neutrophils.


The cytosolic components of the respiratory burst oxidase exist as a M(r) approximately 240,000 complex that acquires a membrane-binding site during activation of the oxidase in a cell-free system.


Parkos, C. Dinauer, M. Jesaitis, A. et al. (1989) Blood 73; 1416-1420. Absence of both the 91 kDa and 22 kDa subunits of human neutrophil cytochrome b in two genetic forms of chronic granulomatous disease.


human neutrophils.


target for genetic correction of p47-phox-deficient chronic granulomatous disease.
Stabel, S. and Parker, P.J. (1991) Pharmac. Ther. 51; 71-95. Protein Kinase C
calcium stores during phagocytosis in human neutrophils.


Vries (De), D.J. and Blumberg, P.M. (1989) Life Sci. 44; 711-715. Thermoregulatory effects of Rx in the mouse, comparison with capsaicin.


Young, S. Parker, P.J. Ullrich, A. Stabel, S. (1987) Biochem. J. 244; 775-252. Down-regulation of PKC is due to an increased rate of degradation.

