STIMULATION OF PHAGOCYTIC CELLS BY PHORBOL ESTERS

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Submitted for the Degree of Doctor of Philosophy

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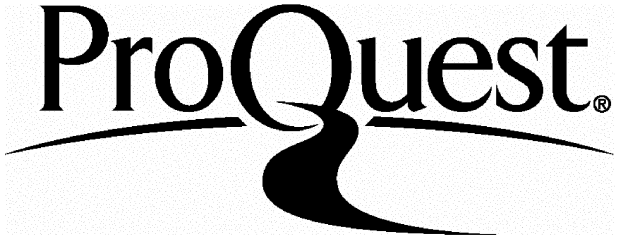

..... Sunny for all her support and understanding and Jenade for making everyday a joy

Abstract

Initial studies using phorbol ester stimulation of superoxide anion (O_2^-) production from mouse peritoneal macrophages revealed a degree of selectivity. The action of a daphnane diterpene, Resiniferatoxin (Rx) was synergised by the presence of particulate zymosan and this effect only occurred from starch-elicited derived cell populations but not in resident macrophages. Previously the phorbol ester receptor has been found to be a family of protein kinases (PKC's). More recently a novel kinase activity which was stimulatable by Rx only in the absence of added calcium was discovered (termed Rx-kinase). A similar activity was also isolated in greater quantity from starch-elicited mouse peritoneal macrophages under identical conditions.

To further understand the nature of this enzyme extensive studies of co-factor requirements, phorbol dibutyrate binding and, tissue distribution were investigated on Rx-kinase derived from hydroxyapatite FPLC from various sources. Interestingly Rx-kinase activity was discovered to be enhanced by priming of cells such as macrophages prior to purification. Proteins from elution fractions were purified on SDS-PAGE and when immunoblotted with specific antisera for PKC revealed a novel enzyme unrelated to previously known isotypes of PKC.

A comparison of macrophage Rx-kinase and pooled rat brain PKC stimulation of the reconstituted membrane NADPH-oxidase system to generate superoxide anion *in-vitro* was investigated in a cell free assay system. Rx-kinase was found to

potently activate the physiological substrate *in-vitro* in the presence of Rx and absence of calcium (i.e. conditions corresponding to its histone kinase assay requirements *in-vitro*.)

To further evaluate the signalling pathways leading to the activation of oxidase, involvement of calcium ions in modulation of phagocytic cells and pure PKC isotypes stimulation of the oxidase itself was studied. Phorbol esters inhibited fMLP stimulated divalent cation influx but synergised with intracellular calcium to produce superoxide anion in neutrophils. Unstimulated PKC activity showed that calcium independent PKC's were more potent than the calcium dependent isotypes and corresponding to the action of Rx-kinase.

Additionally the ability of daphnane derivative Thymeleatoxin A (TxA) and Rx to stimulate superoxide anion from neutrophils and monocytes were investigated. TxA was more potent in stimulating superoxide from neutrophils compared to monocytes. The relationship between biological response and biochemical mechanism involved in this system was investigated by studying PKC isotype profiles of neutrophils via hydroxyapatite chromatography.

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Abbreviations.

A23187 = Calcium ionophore
AA = Arachidonic acid
ADP = Adenosine diphosphate
ATP = Adenosine triphosphate
cAMP = Cyclic adenosine monophosphate
β-cell = β-lymphocytes
β-MSH = β-mercaptoethanol
bFGF = basic Fibroblast growth factor
BSA = Bovine serum albumin
C_1-C_4 = Conserved region of PKC
C-1, C-2 etc.. = Carbon-1, Carbon-2 etc..
Ca^{2+} = Calcium
$[Ca^{2+}]_i$ = Calcium intracellular
CAPS = Capsaicin
CB = Cytochalasin B
CGD = Chronic granulomatous disease
Con A = Concanavalin A
CNS = Central nervous system
CTL-L = IL-2 dependent clone
Cyt C = Cytochrome C
DAG = Diacylglycerol
Daudi = Transformed β-cell line
DEAE = Diethylaminoethyl cellulose
$DiC_{18:1}$ = 1,2-sn-Dioctanoylglycerol
DMBA = Dimethylbenz [a] anthrance
DMSO = Dimethylsulphoxide
DNA = Deoxyribonucleic acid
cDNA = Complementary DNA
DTT = Dithiothreitol
EBV = Epstein Barr virus
EDTA = Ethylenediaminetetracetic acid
EGF = Epidermal growth factor
EGFR = EGF receptor
EGTA = Ethylene glycol bis(β-aminoethyl ether)-N,N,N^1,N^1-tetracetic acid
FAD = Flavin adenine dinucleotide
FCS = Foetal calf serum
FDGF = Fibroblast derived growth factor
fMLP = N-formylmethinoylleucylphenyalanine
FPLC = Fast protein liquid chromatography
GABA = γ-amino butyric acid
G-CSF = Granulocyte colony stimulating factor
GDP = Guanosine diphosphate

Abbreviations (cont..)

Gi = Inhibitory G-protein
rho-GDI = GDP dissociation inhibitor factor
Gs = Stimulatory G-protein
GEP = GTP/GDP exchange proteins
GH-4 = Pituitary cell lines
G-protein = GTP binding protein
GTP = Guanosine triphosphate
cGMP = Cyclic guanosine monophosphate
gp91-phox = β-subunit glycoprotein 91KDa of Cyt b_{-245}
GM-CSF = Granulocyte macrophage colony stimulating factor
5-HT = 5 Hydroxytryptamine
H^+ = Hydrogen ions
HCl = Hydrochloric acid
H-7 = 1-(5-isoquinoline sulphonyl)-2-methyl piperazine (dihydrochloride)
HEPES = 4(2-hydroxyethyl)-1-piperazine ethane sulphonic acid
HL-60 = Human leukaemic cell line
HMBA = Hexamethyl bisacetamide
HMNC = Human mononuclear cells
HPLC = High pressure liquid chromatography
IgG = Immunoglobulin G
IL-2 = Interleukin 2
IL-2R = Interleukin 2 receptor
IONO = Ionomycin
IP_3 = Inositol 1,4,5 trisphosphate
IP_4 = Inositol 1,3,4,5 tetrakisphosphate
IP_5 = Inositol 1,3,4,5,6 pentakisphosphate
IP_6 = Inositol hexaphosphate
K^+ = Potassium ion
KDa = Kilodaltons
K_a = Association constant and activation constant
K_d = Dissociation constant
K_m = Michelis-Menton constant
LTD_4 = Leukotriene D_4
M = Molar
MAP-K = Mitogenic activated kinase
MAP-2 = Microtubule associated protein-2
Mg^{2+} = Magnesium ion
Mn^{2+} = Manganese ion
MLCK = Myosin light chain kinase
Mw = Apparent molecular weight
mRNA = Messenger RNA
Na^+ = Sodium ion

Abbreviations (cont..)

NaCl = Sodium chloride
NADPH = Nicotinamide dinucleotide phosphate reduced form
NADP = NADP oxidised form
^{32}P = Radiolabelled phosphate group
p22-phox = α-protein 22KDa of Cyt b_{-245}
p47-phox = Cytosolic protein 47KDa *ph*agocyte *ox*idase
p67-phox = Cytosolic protein 67KDa *ph*agocyte *ox*idase
p21-rac1 = *ras* related small G-protein
p21-rac2 = Human *ras* related small G-protein
PA = Phosphatidic acid
PAF = Platelet activating factor
PAH = Polyaromatic hydrocarbons
PBS = Phosphate buffering saline
PC = Phosphatidylcholine
PDGF = Platelet derived growth factor
PGE_2 = Prostaglandin E_2
PHA = Phytohaemagglutanin
Pi = Inorganic phosphorous
PI = Phosphatidylinositol
PIP = Phosphatidylinositol-4-phosphate
PIP_2 = Phosphatidyl-4,5-bisphosphate
PIPES = Piperazine-N,N^1-bis[2-ethanesulphonic acid]
PKA = cAMP-dependent protein kinase
PKC = Protein kinase C
PKCal = Calmodulin dependent protein kinase
PKG = cGMP-dependent protein kinase
PLA_2 = Phospholipase A_2
PLC = Phospholipase C
PLM = Plumbagin
PMSF = Phenyl methyl sulphonyl fluoride
pp68 = 68KDa product of the *ros*-oncogene in virally transformed cells (v-ros), or normal cells (c-ros)
pp60 = 60KDa product of *src*-oncogene
PS = Phosphatidylserine
PTK = Protein tyrosine kinase
RNA = Ribonucleic acid
mRNA = Messenger RNA
RPA = 12-O-retinoylphorbol-13-acetate
RT = Room temparature
SEM = Standard error of the mean
SDS/PAGE = Sodium dodecyl sulphate-polyacrlyamide gel electrophoresis
SEPM = Starch elicited mouse macrophages

Abbreviations (Cont...)

SOD = Superoxide dismutase
TAS = Transferrable aggregatory material
T-cell = T-lymphocytes
TCA = Trichloroacetic acid
TEMED = N,N,N^{1},N^{1}-tetramethylethylenediamine
TFP = Trifluoroperazine
TMB-8 = 3,4,5-trimethyoxybenzoic acid 8-[diethylamino]-octyl
TNF α,γ = Tumour necrosis factor α and γ
Tris = Trsi (hydroxymethyl) aminoethane
TRE = TPA responsive element
TSH = Thyroid stimulating hormone
V_1-V_5 = Variable regions of PKC-isotypes
V_{max} = Maximum velocity of enzymatic reaction
W-7 = N-[6-aminohexyl] 5-chloro-1-naphthalene sulphonamide

Phorbol esters

4-me-TPA = 4-O-methyl-TPA
DOPP =12-deoxyphorbol-13-phenylacetate
DOPPA = 12-deoxyphorbol-13-phenylacetate-20-acetate
PDBu = Phorbol-2,13-dibutyrate
Rx = Resiniferatoxin, 2=9,13,14-Orthophenylacetyl resiferonol-20-(3-methoxy,4-hydroxy)-phenylacetate
Sap-A = Sapintoxin A, 12-O-(2-methylaminobenzoyl)-4-deoxyphorbol-13-acetate
TxA = Thymeleatoxin A, 9,13,14-Orthobenzoyl-6,7-epoxy,5-hydroxy resiniferonol-12-cinnamate
TPA = 12-O-Tetradecanoyl phorbol-13-acetate

CHAPTER 1 Introduction

Introduction.

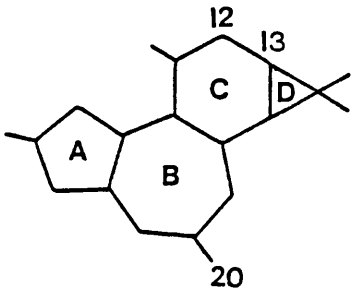
1.1 The Phorbol Esters.

The plant families Euphorbiaceae and Thymeleaceae have provided diterpene esters which, due to their potent and toxic effects, have been critical in furthering our understanding of the disease states of cancer and inflammation. Esters based upon the tigliane, ingenane and dahnane diterpene nuclei have been derived from these plant families (See Fig1.1.1; Hecker and Schmidt, 1971; Evans and Taylor, 1983). Tigliane derivatives were shown to be responsible for the tumour promoting effects of seed oil of *croton tiglium. L.* (Hecker, 1986). The subsequent use of these diterpenes to study the mechanism of disease states has resulted in some confusion of nomenclature; these three distinct classes of diterpenes are now often collectively termed "phorbol esters", although the term phorbol strictly refers only to tigliane derivatives.

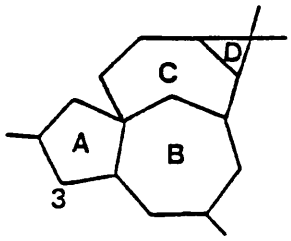
1.1.1 Tigliane Esters

The tiglianes consist of a group of tetracyclic compounds which occur in plants as an acylated polyhydroxy diterpene (Evans and Soper, 1987). They are toxic, tumour - promoting and pro-inflammatory agents of many species of Euphorbiaceae (Evans and Taylor, 1983). Over recent years these substances have been increasingly utilised as pharmacological and biochemical tools for investigating the mechanism of tumour promotion in both *in-vivo* and *in-vitro* models. 12-O-tetradecanoyl-phorbol-13 acetate (TPA) has been shown to be the most potent tigliane ester and is certainly the most widely used in biological systems (Evans and Soper, 1983; Blumberg, 1980).

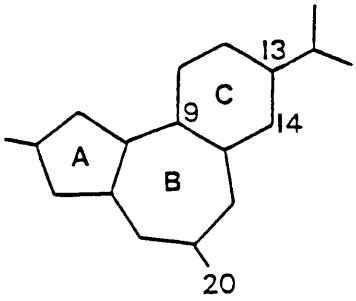
Tigliane diterpenes are tetracyclic, consisting of a five-membered A ring, a seven



Tigliane

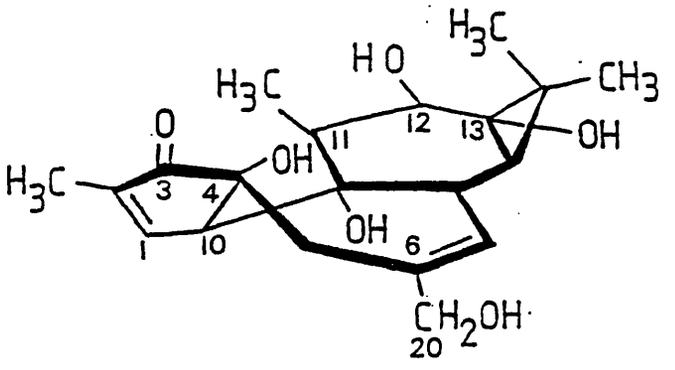


Ingenane

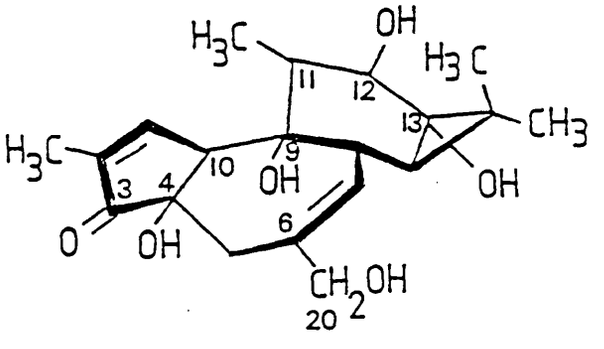


Daphnane

Figure 1.1.1 Tigliane, Ingenane and Daphnane hydrocarbon skeletons



4 β - phorbol
(biologically active)



4 α -phorbol
(biologically inactive)

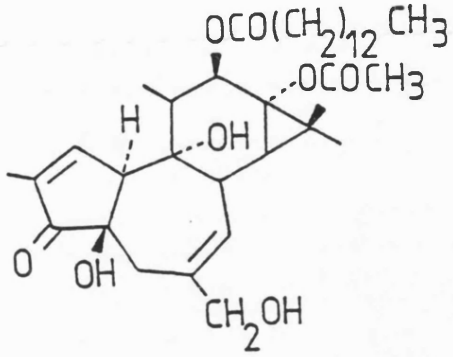
Figure 1.1.2 4β and 4α - phorbol

membered B ring, a six membered C ring and a cyclopropane D ring (see Fig 1.1.1). The parent alcohol, phorbol, exhibits an α,β– unsaturated ketone at C-3, tertiary hydroxyl groups at C-4, C-9 and C-13, a secondary hydroxyl group at C-12 and primary hydroxyl at C-20 (See Fig 1.1.2). The A/B and B/C ring junctions are both in the *trans* configuration whereas the C/D rings are *cis* fused. The isomer of phorbol is characterised by *cis* fusion of the A/B rings and the C-4 hydroxyl group is α- oriented (termed 4 α- phorbol). This alteration of A/B ring stereochemistry has been proposed as the reason for esters of 4 α- phorbol possessing no biological activity (Hecker and Schmidt, 1971; Hecker, 1968).

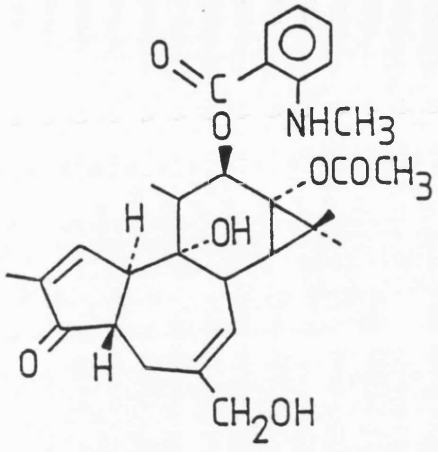
Phorbol esters occur naturally as 12-13 diesters. A typical phorbol diester, which was the original tumour promoting ester isolated from croton oil (Hecker, 1968) is TPA (also known as PMA, phorbol-12-myristate-13-acetate; see Fig 1.1.3). TPA is generally a toxic substance but possess a wide spectrum of biological activity and as a result is usually the positive control phorbol ester used in tests. The phorbol nucleus also exists naturally in different states of oxygenation. Some compounds based upon these nuclei which have been of biological interest are shown in Fig 1.1.3.

1.2 Ingenane Diterpenes.

In 1968, Hecker's group isolated an irritant principle of *Euphorbia lathyris* (Hecker, 1968), later identified by X-ray crystallography as being ingenol triacetate (Zechmeister *et al*, 1970). Ingenane diterpenes, like the tiglianes are tetracyclic. The parent alcohol, ingenol (Fig 1.1.4), exhibits similar features to phorbol except the C ring is seven membered and is *cis* fused to cyclopropane D ring. Also present are C1-2 and C6-7 double bonds, the C-4 β-tertiary hydroxyl group and the C-20 primary hydroxyl group. Ingenol exhibits secondary hydroxyl groups at C-3 and C-5, and a β,γ- unsaturated ketone at C-9. Naturally occurring aliphatic or aromatic

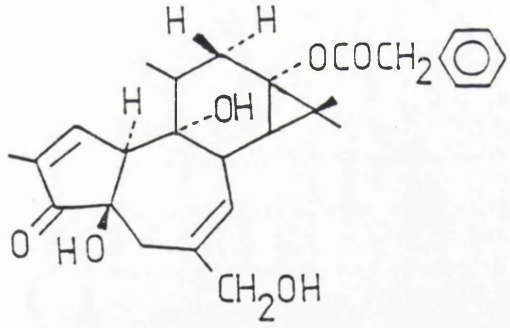


12-0-tetradecanoyl
phorbol-13-acetate
(TPA)

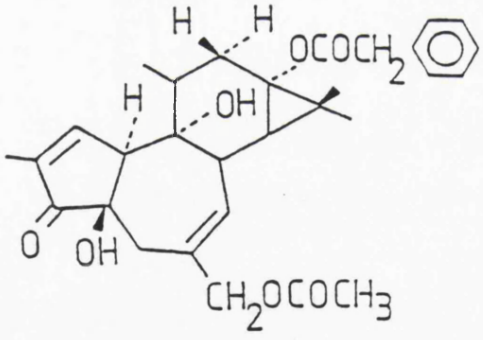


12-0-[2-methylaminobenzoyl]
-4-deoxyphorbol-13-acetate
(Sapintoxin A)

4-deoxyphorbol ester



12-deoxyphorbol-13-
phenylacetate
(DOPP)



12-deoxyphorbol-13-
phenylacetate-20-acetate
(DOPPA)

Figure 1.1.3 Some biologically important esters with different phorbol nuclei.

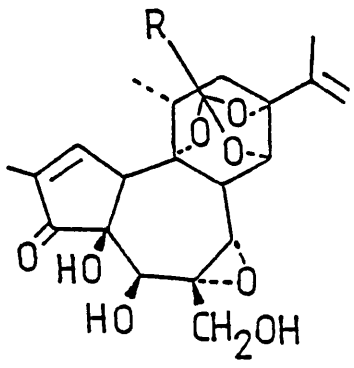
esters of ingenol are linked to the nucleus via the C-3 and C-5 hydroxyls (as opposed to C-12 and C-13 for phorbol) as well as via C-20 hydroxyl.

In common with many tigliane derivatives, some ingenanes have been reported to be tumour promoting (Hergenhahn *et al*, 1974), although ingenol-3,20-dibenzoate has been reported to be anti-leukaemic (Kupchan *et al*, 1976), and ingenol and 20-deoxyingenol esters from *E.paralias* latex are cytotoxic *in-vitro* (Sayed *et al*, 1980). Apart from these activities, the biological effects of the ingenanes do not seem to have been extensively studied and they are in general considered to be of similar activity to the tigliane. Molecular modelling of ingenanes (Wender *et al*, 1986) and recent studies on the binding of ingenol to PKC (Hasler *et al*, 1992) support this supposition that ingenanes are biologically similar to tiglianes.

1.1.3 Daphnane diterpenes.

The daphnane diterpenes are found in both the plant families *Euphorbia* and *Thymeleaceae* but their distribution is limited. Esters based upon the 5-deoxydaphnetoxin and 1α-alkyldaphnane nuclei are present only in the Thymeleaceae; resiniferonol esters are only present in the Euphorbiaceae, whilst daphnetoxin and 12-hydroxydaphnetoxin esters are present in both plant families (Schmidt, 1986). The daphnane ring structure is identical to that of the tigliane except that the cyclopropane D ring has opened to form an isopropanyl side chain (Fig 1.1.1). The daphnanes also exhibit an orthoester function bridging the α-oriented hydroxyl groups at C-9, C-13, and C-14 of the parent alcohol, resiniferonol. Only one compound, pro-resiniferatoxin, is an exception to this and it is thought to be the immediate precursor to resiniferatoxin (Schmidt, 1978; Fig 1.1.5).

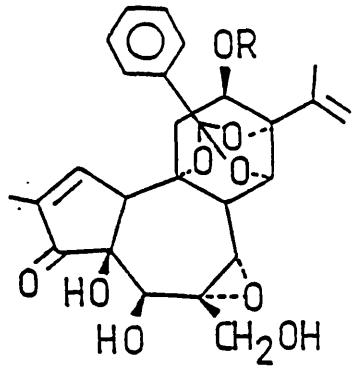
12-hydroxydaphnetoxin esters have been useful in understanding the process of



Daphnetoxin R=C_6H_5

Simplexin R=$(CH_2)_8CH_3$

Daphnetoxin type



R

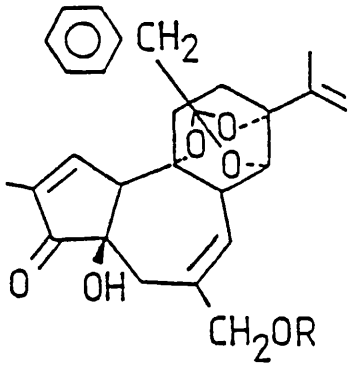
Gnidicin (also termed Thymeleatoxin A) $CO.CH{=}CHC_6H_5$

Gniditrin $CO(CH{=}CH)_3(CH_2)_2CH_3$

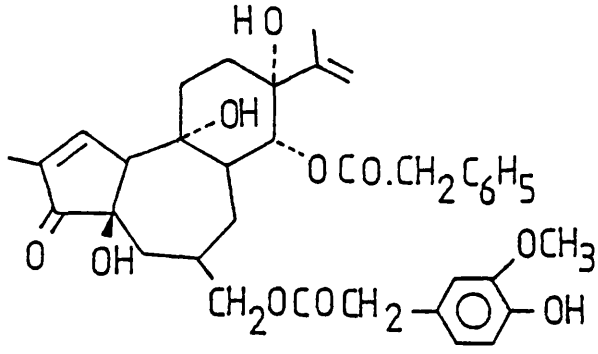
Gnididin $CO(CH{=}CH)_2(CH_2)_4CH_3$

Mezerein $CO(CH{=}CH)_2C_6H_5$

12-Hydroxydaphnetoxin type

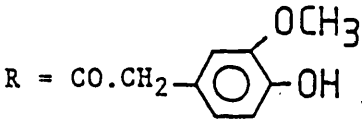


Resiniferonol 9,13, 14 orthophenylacetate R = H



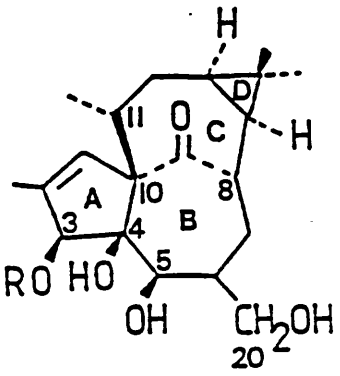
Proresiniferatoxin

Resiniferatoxin



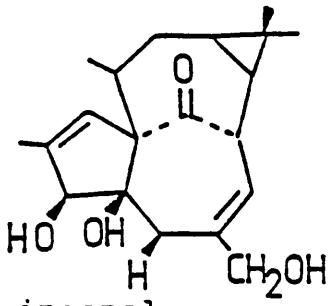
Resiniferonol type

Figure 1.1.5 Daphnane diterpene esters

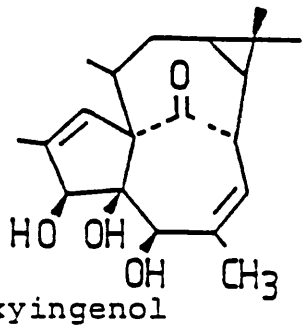


Ingenol R=H
3-0-tetradecanoylingenol (3TI) R=CO(CH_2)$_{12}CH_3$

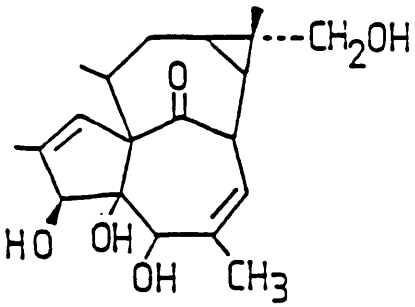
Ingenol derivatives (includes configuration)



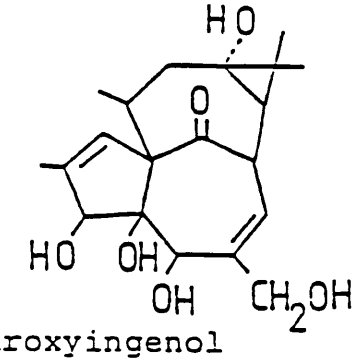
5-deoxyingenol



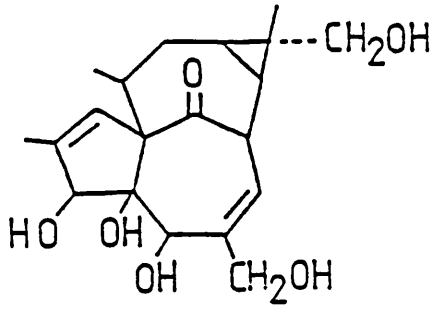
20-deoxyingenol



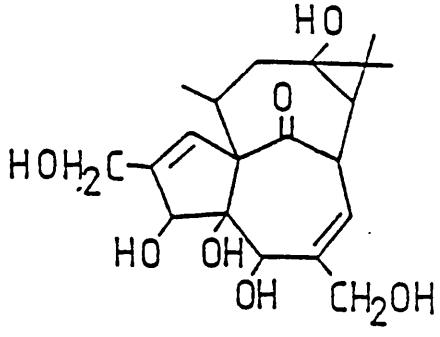
16-hydroxy-20-deoxyingenol



13-hydroxyingenol



16-hydroxyingenol



13,19-dihydroxyingenol

Figure 1.1.4 Ingenol and derivatives.

tumour promotion (see section 1.2.2).Mezerein (Fig 1.1.5), the compound of this type, was isolated from *Daphne mezereum* (Ronlan and Wickber, 1970), and shown to posses anti-leukaemic properties against P388 leukaemia in mice (Kupchan and Baxter, 1974). However it is now recognised as a second stage tumour promoter (Slaga *et al*, 1980). Other anti-leukaemic compounds, Gnididin, Gniditrin and Gnidicin, have been isolated from *Gnidia lamprantha* (Kupchan *et al*, 1975). More recently gnidicin has been isolated from *Thymeleae linsuta* (Rizk *et al*, 1984) along with a series of other compounds, termed Thymeleatoxins. Thymeleatoxin A (Gnidicin) has been shown to be like mezerein, a second stage tumour promoter (Brooks *et al*, 1989) and this further suggests the anti-cancer and co-carcinogenic actions of these diterpenes may be difficult to distinguish.

The resiniferonol daphnane esters (Fig 1.1.5) most closely resemble those of phorbol in that they retain the C-6, C-7 double bond. The first compound to be characterised was resiniferatoxin (Rx), which was isolated from *Euphorbia resinifera* (Hergenhahn *et al*, 1975). This compound has a phenolic side chain, which was concluded to be (4-hydroxy-3-methoxy)-phenylacetic acid on the basis of ^{1}H -NMR spectra (Adolf *et al* 1982) (See Fig 1.1.5). Resiniferatoxin is the most potent irritant detected as measured by erythema of a mouse ear (Schmidt and Evans, 1979) and the biochemical mechanism(s) by which it exerts its effects has recently been of great interest (see Chapter 3 and 4 and 7).

1.2 Biological Effects of Phorbol Esters

1.2.1 Structural consideration.

The biological actions of phorbol esters are both numerous and wide ranging. Ultimately, phorbol esters can induce gross morphological changes in mammals

Structure activity relationship
(1) An AB ring in the *trans* is required for activity (AB *cis* epimers are inactive
(2) A tertiary hydroxyl group at the C-4 position is necessary for complete tumour promoting action. O-methylation of the C-4 hydroxyl results in the loss of complete promoting activity although stage 1 promoting activity is retained. 4-deoxyphorbol esters are inactive as complete and stage 2 tumour promoters and as hyperplastic agents
(3) An ester moiety at C-12 is not required for activity, although a secondary hydroxyl group at this position reduces activity dramatically
(4) A free primary hydroxyl group at C-20 is required for activity.
(5) Increasing unsaturation in an ester moiety results in decreased complete promoting activity but increased stage 2 promoting activity
(6) A secondary hydroxyl group at C-16 does not affect activity

Table 1.2.1 Structure activity requirements for tumour promoting and hyperplastic activity of phorbol esters. Compiled from Gordge (PhD thesis, 1992); Ryves (PhD thesis, 1991); Brooks (PhD thesis, 1989).

which represent discrete changes at cellular biochemical level. Derivatives of diterpenoids possessing selective actions may provide critical breakthroughs in understanding particular systems studied.

Comparative studies conducted on a range of phorbol derivatives have elucidated many structural requirements for biological activity. If the primary hydroxyl group is substituted (eg esterified), the bioactivity is decreased in proportion to the type of substitution (Hecker, 1985; Evans and Edwards, 1987). The spatial position of this C-20 seems important since slight adjustment (eg. caused by double bond shift from C-6/C-7/C-8) results in activity loss. Similarly switching the hydroxy group on the C-4 position to the α- conformation or derivatisation results in lessening of activity. The importance of such hydrophilic groups appears to involve orienting the phorbol ester to facilitate interaction with the phospholipid head groups and/or PKC protein groups in the membrane. The phorbol esters also require lipophillic moieties in the molecule (typically at C-12 and C-13) which allow anchorage and spatial organisation in the lipid bilayer according to acyl chain length and acyl type. These appear to be important in the biological activity since compounds with short chain acyl groups can bind to PKC but are inactive themselves and inhibitory to biological action. The *trans* configuration of A - ring to B - ring is also considered to be essential for activity although *cis* - α-sapintoxin has been found to possess activity at high concentration (Edwards *et al*, 1985; see also Table 1.2.1)

Computer modelling techniques have been employed to compare the structural similarities between energetically favourable conformation between phorbol esters and diacylgylcerols (Leli *et al*, 1990). In this study the C-1 ester in diacylglycerol and the C-3 free hydroxyl group corresponded to C-13 ester and C-9 hydroxy group (rather than the C-20 (Wender *et al*, 1986)) of the phorbol derivatives in terms of spatial position. Furthermore it was suggested that the C-4 hydroxyl group (and possibly other parts of the molecule such as C-20) which had no correlate in the

diacylglycerol molecule, but which are involved in the biological potencies of the phorbol esters, may play functional roles on separate receptors or different PKC isoforms. In support of this, groups have been found to have correlates with groups in non-phorbol ester molecule (eg Teleocidin) which share some biological activity with phorbol esters *in-vivo* and can activate PKC *in-vitro* (Wender *et al*, 1986; Blumberg *et al*, 1984; Thalstrup *et al*, 1990) without having any resemblance to diacylglycerols.

1.2.2 Tumour Promotion.

As early as 1941, Berenblum first devised experiments to study the mechanism of carcinogenesis. Polyaromatic hydrocarbons (PAHs), for example dimethylbenz [a] anthracene (DMBA) and benz [a] pyrene, were recognised carcinogens. That is, tumours developed after appropriate single dose was applied to the dorsal skin of an animal. If a subthreshold dose was applied, no tumours developed. Application of a subthreshold dose of carcinogen, followed by weekly applications of Croton oil (the seed oil of *Croton tiglium*) resulted in the development of tumours (Berenblum, 1941).

Berenblum later showed (Berenblum and Shubik, 1947) that application of the croton oil only produced tumours if it was applied after the carcinogen; application of croton oil followed by carcinogen produced no tumours. This and other work (Freidewald and Rous, 1944) resulted in the concept of a two stage mechanism for carcinogenesis: an "initiation" stage, and a "promotion" stage (see Fig 1.2.1). Initiators and promoters have distinct biological properties but, essentially, the initiator must be carcinogenic, must be applied before the promoter and its action is irreversible. However, urethane and some of its derivatives are initiators but are not themselves carcinogenic (Salaman and Roe, 1953) and this has led to them being termed "incomplete carcinogens". These properties imply that the initiator acts via a

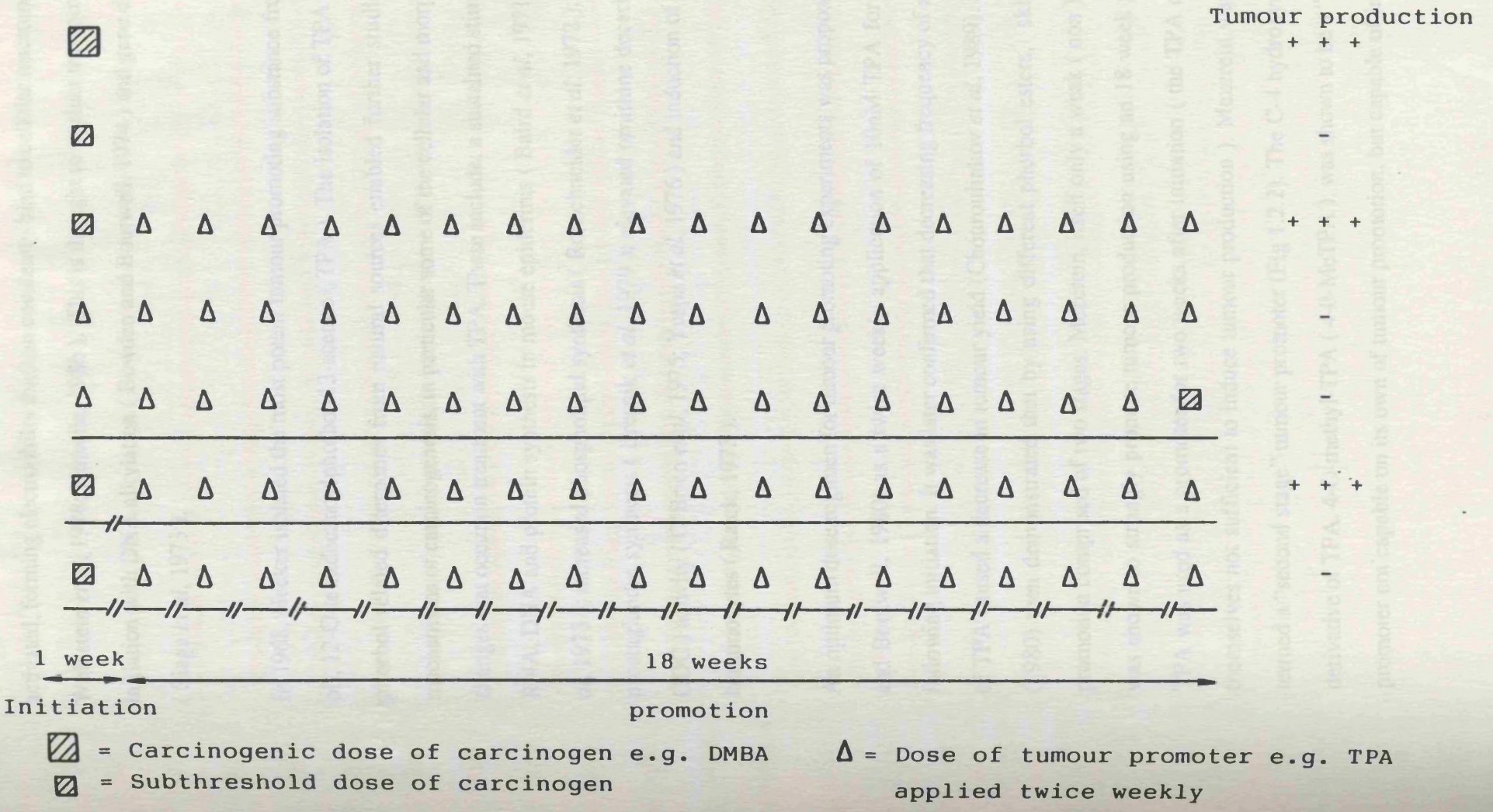


Figure 1.2.1 The concept of tumour promotion

different mechanism to the promoter; initiators are now thought to be metabolically activated, forming electrophiles that can covalently bind to cellular macromolecules (Weinstein *et al*, 1978; Kinsella, 1986). This is thought to explain the correlation of initiation with DNA alkylation (Bowden and Boutwell, 1974) and gene depression (Slaga *et al*, 1973).

In 1968, Hecker isolated the most potent tumour promoting substance from croton oil, 12-O-tetradecanoylphorbol-13-acetate (TPA). The isolation of TPA and other phorbol related diterpenes from natural sources enabled further studies on the mechanisms of carcinogenesis, in particular some of the cellular and morphological changes that occur on treatment with TPA. These include: a sustained stimulation of RNA, DNA and protein synthesis in mouse epidermis (Baird *et al*, 1971; Raick *et al*, 1973); increased phospholipid synthesis (Rohschneider *et al*, 1972); increased prostaglandin synthesis (Bresnik *et al*, 1979); elevated ornithine decarboxylase (ODC) activity (O'Brien *et al*, 1975; Yuspa *et al*, 1976) and induction of dark basal keratinocytes (Raick, 1973).

An optimum dose regimen for tumour promoting experiments was proposed (Verma and Boutwell, 1980) as a twice weekly application of 10nM TPA for 18 weeks following initiation. It was later confirmed that decreasing frequency of application of TPA caused a decrease in tumour yield (Chouroulinkov *et al*, 1989). Slaga *et al* (1980) then demonstrated that by using different phorbol esters, skin tumour promotion comprised of two stages. Mezerein, itself only a weak (non) promoter, was shown to strongly promote tumour production using an 18 week protocol if TPA was used as a promoter for two weeks after initiation (the TPA doses were themselves not sufficient to induce tumour production). Mezerein was therefore termed a "second stage" tumour promoter (Fig 1.2.2). The C-4 hydroxymethylated derivative of TPA, 4-O-methyl-TPA (4-0-MeTPA), was shown to be a "first stage" promoter not capable on its own of tumour promotion, but capable of inducing the

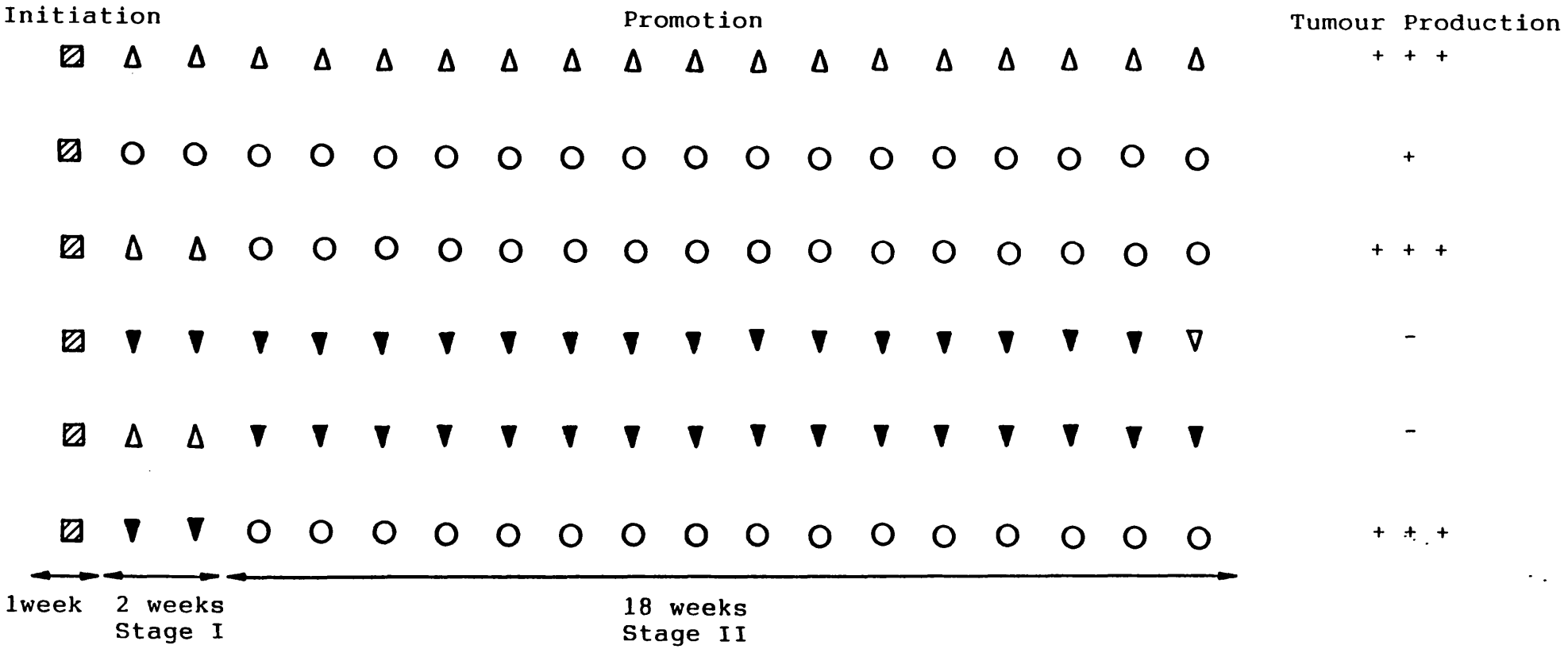


Figure 1.2.2 First and second stage tumour promotion

same effects as TPA to subsequently promote tumour production, if a second stage promoter (or TPA) was then utilised. Furstenberger *et al* (1981) confirmed that phorbol ester tumour promotion was a two stage process, by semi-synthetic manufacture of 12-O-retinoylphorbol-13-acetate (RPA), a compound which was also a potent second stage promoter in NMRI mice. RPA was later shown to be a complete promoter in Sencar mice (Fischer *et al*, 1985). This is of interest as retinoids (Vitamin A analogues) had previously been shown to inhibit TPA induced tumour promotion (Verma *et al*, 1978; Weeks *et al*, 1979).

Mouse strain-specific susceptibility to tumour promotion raises the question of the significance of this biological phenomenon to carcinogenesis in man. In the mouse, the Sencar (Sensitive to carcinogenesis) strain is the most susceptible to carcinogenesis (DiGiovanni *et al*, 1980) and was bred for this purpose. NMRI and CD-1 mice are also likely to develop cancers but B6C3F1 mice are resistant. Phorbol-12,13-diester-12-ester hydrolase was proposed as the enzyme responsible for prevention of phorbol ester induced tumour promotion (Shoyab *et al*, 1982). This enzyme converts 12,13 diesters to 13-monoesters and was shown to be present in rat, hamster, guinea pig and rabbit but not in mice, cat, dog, monkey and humans. They proposed that species possessing this enzyme were resistant to tumour promotion although no *in-vivo* experiments were demonstrated to test this hypothesis, and by implication of species distribution of the enzyme, humans were susceptible. The evidence that phorbol esters could be risk factors of cancer in humans was provided by studies in the Caribbean island of Curacao which has a high incidence of oesophageal cancer (reviewed by Hecker, 1987). The population's drinking water was found to be contaminated with petrol from 1930 - 1964 and this may have caused the initiation dose by PAH carcinogen. Furthermore, the population regularly take tea made from the plant *Croton flavens* (termed 'Welensali tea') and chew the roots of this bush. Hecker *et al* (1983) isolated the active constituents of this plant, which were tumour promoting esters of 16-

hydroxy and 4- deoxy-16-hydroxy - phorbol. The use of this bush in local custom suggested that the promoting stage of the disease could be attributed to the bush (Hecker, 1983; 1984). Interestingly, neighbouring islands did not use the bush in local custom and the incidence of oesophageal cancer was much lower.

The observation that PKC is the major cellular receptor for the phorbol esters (see Section 1.3) has led to investigation of its involvement in tumour promotion. Using normal and TPA-resistant mice strains no differences were apparent in epidermal PKC levels or activities (Mills and Smart, 1989; Hashimoto *et al*, 1989). Not all PKC activators are able to promote tumours, for example Sap-A, a fluorescent phorbol ester was shown to activate PKC but to be neither a complete nor a second stage tumour promoter in its own right (Brooks *et al*, 1987; 1988). However, if co-adminstered with sub-hyperplasiogenic doses of calcium ionophore, A23187, Sap-A was found to be a complete tumour promoter and inducing tumours in a dose dependent manner (Brooks *et al*, 1989). This points to an involvement of calcium in tumour promotion processes and interestingly the tumour promoting sesquiterpene lactone, Thapsigargin, has been shown to act by discharging calcium from stores in the endoplasmic reticulum by specific inhibition of endoplasmic reticulum Ca^{2+}-ATPase (Thalstrup *et al*, 1990).

The involvement of other biochemical systems (eg Ca^{2+} levels) in the tumour promotion process reflects the complex nature of this phenomenon and may help explain why different classes of compounds act as tumour promoters. Tumour promotion by phorbol esters appears to reflect their ability to induce complex interactions at both cellular and biochemical levels.

1.2.3 Pro-inflammatory response.

The acute effects of the exposure to humans of phorbol esters is the production of

inflammation. Phorbol ester induced inflammation is characterised by classical symptoms (Evans, 1978) of redness, swelling, pain and heat. Skin burning by *Euphorbia* species can result in open and weeping pustules which at a later stage may lead to the formation of dry flaking skin, scad formation and eventually necrosis of the tissues (Schmidt and Evans 1980). When ingested a burning sensation develops on the lips, tongue and the mucous membranes of the mouth, followed sometime later by intestinal pain, vomiting and severe purging (Kinghorn & Evans, 1975). Similarly contact with eyes leads to conjunctivitis, swelling of the eye lids and eye closures due to oedema (Evans and Kinghorn, 1979) which may result in blindness.

Observation of mouse ear erythema induced by a single application of irritant phorbol ester detected two types of responses. A transient response (eg as elicited by resiniferatoxin with 100 times the potency of TPA) peaking within 1-2 hours and then declining to normal, and a persistent response (eg as elicited by DOPP) evident in one hour and able to remain for 24 hours (Schmidt and Evans, 1979). TPA was able to achieve either non-persistent (at low doses) or persistent (at high doses) responses (Evans and Schmidt, 1979). Examination of phorbol ester effects at an *in-vitro* level in rabbit skin detected vasoconstriction (Evans and Edwards, 1987), whereas mouse skin vasodilation, oedema and cellular infiltration were evident (DeYoung *et al*, 1989). The response as measured by ear thickness indicated that oedema peaked within 6h for TPA and declined over 24h, whereas for resiniferatoxin (Rx) peaked within 1h and had disappeared within 4h (Szallasi and Blumberg, 1990 b). The possibility of a neurogenic effect for the phorbol esters, by causing polymodal nociceptors to release multiple peptides (such as substance P, a PI cycle agonist) which then potently mediate neurogenic inflammation (Foreman, 1987; Szallasi and Blumberg, 1989) has been suggested. Blumberg has proposed that Rx acts as an 'ultrapotent' analogue of capsaicin (a pungent compound from hot capsicum peppers) which elicits inflammation by neuropeptide release (Szallasi and

Blumberg, 1989b; Szallasi and Blumberg, 1990a) in experiments on an isolated population of dorsal root ganglion neurones. These neurones were voltage clamped, and both Rx at 2nM and capsaicin at 300nM could induce the opening of an unidentified mono/divalent ion channel, without involvement of PKC, causing cell depolarisation (Winter *et al*, 1990), and in the case of Rx, osmotic death followed.

However while pre-treatment of mouse ear with Rx causes desensitisation to Rx and capsaicin - induced oedema (Szallasi and Blumberg, 1989b), capsaicin only pre-treatment inhibited early (transient) erythema induced by Rx and had an enhancing effect on persistent erythema (Gordge *et al*, 1990 ; Evans *et al*, 1992). Higher doses of Rx could reduce the latency time to erythema and caused the erythema to be prolonged (over 12 h) whereas higher doses of other irritant phorbol esters (eg Sap D) only prolonged the duration and had little effect on latency time. Inflammation thus appears to be a complex interaction of endogenous mediators from neurogenic and inflammatory cell sources which synergise in the final response.

1.2.4 Platelet Aggregation.

Zucker *et al* (1974) first demonstrated that a pro-inflammatory and tumour promoting phorbol ester, TPA, would induce a dose dependent aggregation of human platelets. Later studies on the aggregating ability of a range of phorbol esters indicated that daphnane ortho esters were unable to induce aggregation of platelets (Williamson *et al*, 1980) yet 12-deoxyphorbol esters could induce platelet aggregation induced by phorbol esters was rigidly dependent upon its structure (eg *trans* A/B ring; free C-20 hydroxyl group; C-13 ester function). However C-4 tertiary hydroxyl and a C-12 ester function were not essential structural requirements for activity (Westwick *et al*, 1980 ; Edwards *et al*, 1982).

Studies on the mechanism of aggregation induced by 12-deoxyphorbol-13-phenylacetate (DOPP) indicated that it was a true aggregation and not a non-specific agglutination induced by a surfactant mimicking action (Williamson *et al*, 1981). EDTA was able to inhibit phorbol ester induced aggregation. In addition to aggregation, DOPP also stimulated the secretion of a biologically active factor termed a Transferable Aggregating Substance (TAS) into human plasma. This substance was so termed as a result of the following experiment: 100 ul aliquot of plasma whose platelets had been aggregated by 0.86uM DOPP was transferred to fresh recipient platelets, then aggregation was observed (Williamson *et al*, 1981). The concentration of DOPP transferred (0.14uM) was not itself sufficient to induce platelet aggregation. On the basis of trypsin sensitivity and Sephadex G-25 elution, TAS was shown to be a protein (Edwards *et al*, 1987) and pharmacologically distinct from other known platelet aggregators, including phorbol esters. The half-life of TAS in plasma at 37^{o}C is 20 minutes (Edwards *et al*, 1987). However recent use of a Superose 12 column in conjunction with FPLC techniques has provided material that can be stored at -20^{o}C for up to two weeks without loss of activity. This has enabled further characterisation of TAS, confirming it as protein, with bands in 20kDa range when examined by SDS-PAGE electrophoresis (Evans *et al*, 1991 a).

Biochemical studies using phorbol esters possessing abilities to induce platelet aggregation have revealed that this activity may correlate with phosphorylation of a 47 kDa protein by protein kinase C (Brooks, 1989; Brooks *et al*, 1990). TPA and DOPP, the aggregating phorbol esters, were able to stimulate phosphorylation in the presence and absence of Ca^{2+}-ionophore, A23187. Sap A, a weaker aggregatory agent, stimulated phosphorylation of the 47kDa protein, which was enhanced in the presence of Ca^{2+} ionophore. The Ca^{2+}- dependency of this probe seems to correlate with its tumour promoting properties, for which Ca^{2+} is also required (Brooks *et al*, 1989). The non-aggregatory phorbol ester, 12-deoxy- phorbol-13

-phenylacetate-20-acetate (DOPPA), was able to induce phosphorylation only in the presence of ionophore. The Ka for PKC activation by DOPPA decreased from 530nM to 120nM in the presence of Ca^{2+} (Brooks *et al*, 1990) and DOPPA has recently been shown to activate only one PKC isoform (Ryves *et al*, 1991).

These studies further re-enforce the need to use selective phorbol esters to understand a biological response. The role of Ca^{2+} may be critical for phorbol ester induced platelet aggregation, although the role of TAS in the aggregatory response requires further investigations.

1.25 Mitogenic effects and lymphocyte activation.

Among the biological activities of phorbol esters their role in mitogenesis in cells are under intense investigations. Phorbol esters have been found to promote DNA synthesis and induce proliferation in skin (Rose-John *et al*, 1988), glomerular cells (He *et al*, 1992 ; Issandou and Darbon, 1991; Isakov *et al*, 1986; Munoz *et al*, 1991) and fibroblast (Halsey *et al*, 1987). Additionally phorbol esters induce macrophages to produce a variety of cytokines (monokines and lymphokines) which themselves act on lymphocyte populations as soluble mediators and mitogens (Krakauer *et al*, 1982; Haung *et al*, 1987). Without accessory cells (macrophages and monocytes) TPA induced mitogenic activity in lymphocyte cultures are poor, but additions of calcium ionophores (Lin *et al* 1992), plant-derived lectins (Mastro and Mueller, 1974;) or cytokines (eg IL-1 and IL-2; Krakauer *et al* 1982; Mastro, *et al* 1991) can serve to augment the response. It has thus been possible to dissect components of lymphocyte activation and mitogenesis at a biochemical level by study and manipulation of physiological activators (antigens and antibodies), phorbol esters and other PKC activators, calcium levels and plant lectins.

Lectins such as concanavalin A (Con A), phytohaemagglutanin (PHA) act by

coupling surface sugars, attaching to protein as glycoproteins (Grillon *et al*, 1990), and mimicking the activation process of physiological ligands. PHA and Con A have been found to enhance PI turnover and raise intracellular calcium - factors implicated in T-lymphocyte activation (Mire *et al* 1986, Mizushima, 1987). It has been found that mimicking the intracellular calcium rise with calcium ionophores (Ionomycin or A23187) in T-cell cultures does not stimulate mitogenesis in the absence of TPA (Trunch *et al*, 1985). Combined, these agents induce interleukin-2 (IL-2) (Soldaini *et al*, 1991) and appear in the culture after 24 hours which was then responsible for driving mitogenesis. TPA also enhanced IL-2 production induced by Con-A. Concomitant with IL-2 production is the appearance of the surface IL-2 receptor (IL-2R) through which the ligand exerts its mitogenic effect. Experiments on IL-2 dependent clone (CTL-L) demonstrated that Con-A or combined TPA + calcium ionophore could not bypass this requirement for IL-2. (Truneh *et al* 1985)

The effectiveness of phorbol esters on mitogenesis does not correlate with the ability and degree of tumour promoting activity as has been suggested (Mastro and Mueller, 1974; Estensen *et al*, 1978) since both promoting and non-promoting phorbol esters are mitogenic in human lymphocyte cultures (Edwards *et al*, 1982). The non promoting phorbol esters DOPPA and Sap A induced 95% and 70% of the mitogenic effect of TPA. Similarly these effects of DOPPA do not fit in with its poor activation of PKC *in-vitro*. In co-mitogenic experiments on allogeneic mixed lymphocyte cultures, DOPPA was more potent than SapA or TPA in achieving the maximal response (Edwards *et al*, 1989). Inhibition of phorbol ester induced mitogenesis by dexamethasone (a glucocorticosteriod which selectively inhibits IL-2 production (Gillis *et al*, 1979)) was similar for these compounds whereas cyclosporin A was ineffective at inhibiting TPA response (10%) at concentrations that inhibited 50% of the DOPPA response (Edwards *et al*, 1982). Further experiments indicated that cyclosporin A could not negate proliferation induced by

both DOPPA and TPA in IL-2 dependent human and mouse T-lymphocyte clones (Edwards *et al*, 1989; Mizushima *et al*, 1987). It is thought that cyclosporin A acts in a different manner on IL-2 system than dexamethasone by modifying the IL-2R mechanism so that cells fail to acquire responsiveness to IL-2 at concentrations where IL-2 production is unaffected (Larsson, 1980).

The importance of PKC mediated proliferation of T-cells is indicated by the fact that short term treatment of cells by TPA results in IL-2 production and proliferation. However long term TPA-pretreatment of cells, IL-2 and proliferation are depressed (Grove and Mastro, 1992) suggesting down-regulation of PKC lowers IL-2 production and depresses proliferation. In the same study, DOPP and DOPPA were required at 100-fold higher concentration than TPA to suppress IL-2 production and mitogenesis. Similarly in B-cells treatment by PDBu for short period primed the cells for enhanced proliferative response to anti-Ig antibody whereas treatment for a longer period resulted in suppression of proliferation (Mond *et al*, 1991). However studies with chronically depleted PKC levels in T-lymphocytes, IL-2 production was greatly reduced but cells still proliferated when exogenous Il-2 was added (Mastro *et al*, 1991). This was shown due to the fact that these cell still expressed high affinity for IL-2R. Basic Fibroblast Growth factor (bFGF), a potent mitogen of cultured glomerular mesangial cells, induced mitogenesis in both normal and PKC depleted cells. Staurosporine, a protein kinase inhibitor does not prevent bFGF from inducing mitogenesis (Issandou and Darbon, 1991). bFGF clearly stimulates cell proliferation through a PKC independent pathway. All this suggests that although PKC is required in early 'priming' of T-cells, it cannot complete the proliferation process without another pathway(s) (eg. serum, accessory cells or calcium).

1.2.6 Other effects of phorbol esters.

As previously mentioned, the biological effects of phorbol esters are wide ranging. The ability of phorbol esters to promote tumour formation and induce pro-inflammatory response led to the use of these compounds to study the disease state mechanisms of cancer and inflammation.

Phorbol esters can induce both proliferation and differentiation of cell types (Dicker and Rozengurt, 1980). Phorbol esters inhibit proliferation before inducing differentiation in many cell lines (eg Human pro-leukaemic cells (HL-60 ; Rovere *et al*, 1979; Evans *et al*, 1989); human colon cell line (McBain *et al*, 1988); U937 monocytic cell line (Lipsey-Hersh *et al*, 1986); Daudi human B-cell line (Thomas, 1989)). Not all of these cells undergo differentiation (eg Daudi cells appear to be arrested in G_1/ G_0 phase of the cell cycle). Differentiation of HL-60 cells has been linked with the translocation and down regulation of PKC induced by TPA (Solanki *et al*, 1981). Interestingly bryostatin-1 (which actively translocates PKC but does not induce down-regulation) does not induce HL-60 and human colon cell line to differentiation and blocks the effects of TPA (Kraft *et al*, 1986; McBain *et al*, 1988). The effects of phorbol esters on HL-60 cell system are not confined to tumour promoting derivatives (Evans *et al*, 1989). Weak promoters (TxA) and non-promoting agents (Sap-A and DOPP) could equally induce differentiation while DOPPA (a non-promoter) had this effect at high doses. Phorbol ester differentiation potencies correlates with their ability to activate PKC (Evans *et al*, 1989). Resiniferatoxin (of limited biological activity and also a weak activator of PKC) was unable to induce differentiation but however shared the effect of immediate inhibition of cell growth/ divisions with phorbol esters.

TPA has been found to influence arachidonic acid (AA) metabolism and prostaglandin production in many cell types. In mouse keratinocytes TPA stimulates

deacylation of cellular phospholipids through enhanced phospholipase A_2 activity resulting in AA metabolism (Kast *et al*, 1991). In mouse macrophages TPA alone was sufficient to stimulate prostaglandin synthesis and synergises with a calcium ionophore to stimulate thromboxane A_2 synthesis from increased AA pool (Kaever *et al*, 1990). Such evidence points to a central involvement of PKC in eicosanoid metabolism.

TPA has been found to alter gene expression in many oncogenes, including c-*myc*, c-*jun*, and c-*fos* (Weinstein, 1991) by binding to TPA responsive elements (TRE, also referred to as AP-1 region; Angel *et al*, 1987; Tugores *et al*, 1992). Interaction of c-*jun* and c-*fos* is thought to enhance their DNA binding (Allegretto, 1990) and TRE is thought to be a possible convergent point for PKA and PKC mediated signal transduction pathways (Hoeffler *et al*, 1989; Litz-Jackson *et al*, 1992). Interestingly TPA induced activation of PKC is thought to decrease phosphorylation of c-*jun* at sites negatively regulating its DNA binding activity (Boyle *et al*, 1991), whilst the normally recognised effect of PKC activation is an increased phosphorylation, reflecting further complexity of phorbol ester and PKC mediated effects.

Phorbol esters have a variety of effects on cellular secretion . Some of these effects are slow, taking many hours to manifest themselves (eg enhancement of collagenase secretion in U937 monoblast line (Lipsey-Hersh *et al* , 1986), interleukin-1 in macrophages (Krakauer *et al*, 1982)).However some are rapid, for example in cells with specific secretory functions whose actions are primed (eg TAS generation in platelets (Evans and Edwards, 1987)). In phagocytes (neutrophils and macrophages) the production of oxygen radical and secretion of lysosomal enzymes are associated with the activation response elicited by TPA (Pozzan *et al*, 1983). TPA treatment has been linked to phosphorylation of proteins in the NADPH-oxidase complex, generating superoxide anions from phagocytes (Cox *et al*, 1985;

Phorbol ester	Tumour promoter (a)	Irritancy ID50 (b)	Lymphocyte mitogenic action (c)	Platelet aggregation (d)	Co-mitogenic action (e)
Rx	-	0.00021	0	0	-
DOPPA	-	0.075	560	0	0.97
DOPP	-	0.064	45	0.4	1.0
TxA	+/- complete ++ 2nd stage	0.070	-	0.3	-
SAP-A	-	0.042	35	1.8	1.0
TPA	++++ complete	0.016	6	0.3	1.0

Table 1.2.2 Examples of biological effects elicited by selective phorbol esters.
(a) Initiation/promotion standard assay on NMRI mice with DMBA as initiator.
(b) Irritant dose for 50% inflammation of mouse ear after 24h (nmoles).
(c) Dose for 40% maximal increase of human mitogenesis as optimised by TPA (EC_{50}; ng/ml).
(d) Concentration required for 50% maximal aggregation response in human platelets (EC_{50}; μM)
(e) Relative mitogenic effect, compared with TPA, on mixed allogenic human lymphocytes.
Compiled from Ryves (PhD thesis 1991) and Brooks (PhD thesis 1989).

Bronza *et al*, 1988; see section 1.4). TPA treatment induces many different cellular secretions by several cell types; acetylcholine by human blastoma cells (Murphy *et al*, 1992); catecholamines by adrenal chromaffin cells (Burgoyne *et al*, 1988); aldosterone by rat adrenal glomerulosa cells (Hajnoczky *et al*, 1992); dopamine by fetal rat mesencephallic cells (Boksa *et al*, 1992) and fetal brain neurones (Zurgil and Zisapel, 1985); histamine by mast cells (Jacobsen *et al*, 1987); insulin by islets of Langerhans (Jones *et al*, 1992); prolactin by anterior and posterior pituitary cultures (Dymshitz and Ben-Jonathan, 1991) and 5-HT by platelets (Naka *et al*, 1983).

Phorbol esters exert complex and broad spectrum of biological response. However biochemical mechanisms of actions are now possible to begin to comprehend after isolation of a cyclic nucleotide independent kinase and its pro-enzyme from bovine brain (Takai *et al*, 1977) and subsequent identification of this enzyme, termed PKC, as the receptor site for tumour promoting phorbol esters (Castagna *et al*, 1982).

1.3 Protein kinase C - the phorbol ester receptor.

1.3.1 The discovery of protein kinase C

In 1977, Nishizuka reported the isolation of a novel protein kinase, which was termed protein kinase M (PKM; Takai *et al* 1977). This was active, independent of the presence of cyclic nucleotides or Ca^{2+} and capable of phosphorylating histone or protamine. It was suggested that PKM was produced by limited proteolysis of a pro-enzyme. An accompanying paper (Inoue *et al*, 1977) described the purification of the pro-enzyme from rat brain; it had an approximate molecular weight of 77KDa and the protease converting this enzyme to PKM

required Ca^{2+}. The pro-enzyme and protease were present in many tissues including: lung, liver, kidney, cerebellum, heart, skeletal muscle and adipose tissue. Nishizuka subsequently demonstrated (Takai *et al*, 1979) that the pro-enzyme was capable of phosphorylating five histone fractions and muscle phosphorylase kinase. The pro-enzyme required the presence of Ca^{2+} and a membrane associated factor for activity. Other divalent cations were unable to activate this pro-enzyme, but the membrane factor could be replaced by phosphatidylinositol, phosphatidylserine, phosphatidic acid or diphosphatidylglycerol. Other phospholipids (including phosphatidylcholine, phosphatidylethanolamine and sphingomyelin) were less capable of replacing the membrane factor. The pro-enzyme was additionally shown to be non-proteolytically activated in the presence of phospholipid and limited calcium ions, higher calcium ion concentrations were required to convert the pro-enzyme to PKM. Furthermore, activation of the pro-enzyme by Ca^{2+} resulted in its conversions from a soluble form to a membrane - associated form. Nishizuka suggested that kinase activity induced by the non-proteolytic activation of the pro-enzyme, since it was reversible and required only low Ca^{2+} concentrations, may be more physiologically significant than the kinase activity induced by irreversible proteolytic conversions to PKM. He therefore, considered the pro-enzyme a protein kinase in its own right, dependent on the presence of phospholipid and activated by Ca^{2+} and termed it protein kinase C (PKC).

Nishizuka subsequently demonstrated that PKC could be activated *in vitro* by diacylglycerol at concentrations of less than 5% w/w the concentration of phospholipid (Kishimoto *et al*, 1980). Diacylglycerol alone exerted little effect on the enzyme activity, over a wide Ca^{2+} range, indicating that phospholipid presence was required for activity. The enzyme stimulation by diacylglycerol was apparently due to an increased affinity of PKC for phospholipid and concomitant decrease in apparent Ka for Ca^{2+} from 10^{-4} to 10^{-6} M. Since phosphatidylinositol was a potent phospholipid activator of PKC, and phosphatidylinositol turnover yielded

diacylglycerols (Michell, 1979). Nishizuka then proposed that PKC activation by diacylglycerol was due to the release of this substance from phosphatidylinositol metabolism (Kishimoto *et al*, 1980).

1.3.2 The phosphatidylinositol signal transduction cycle.

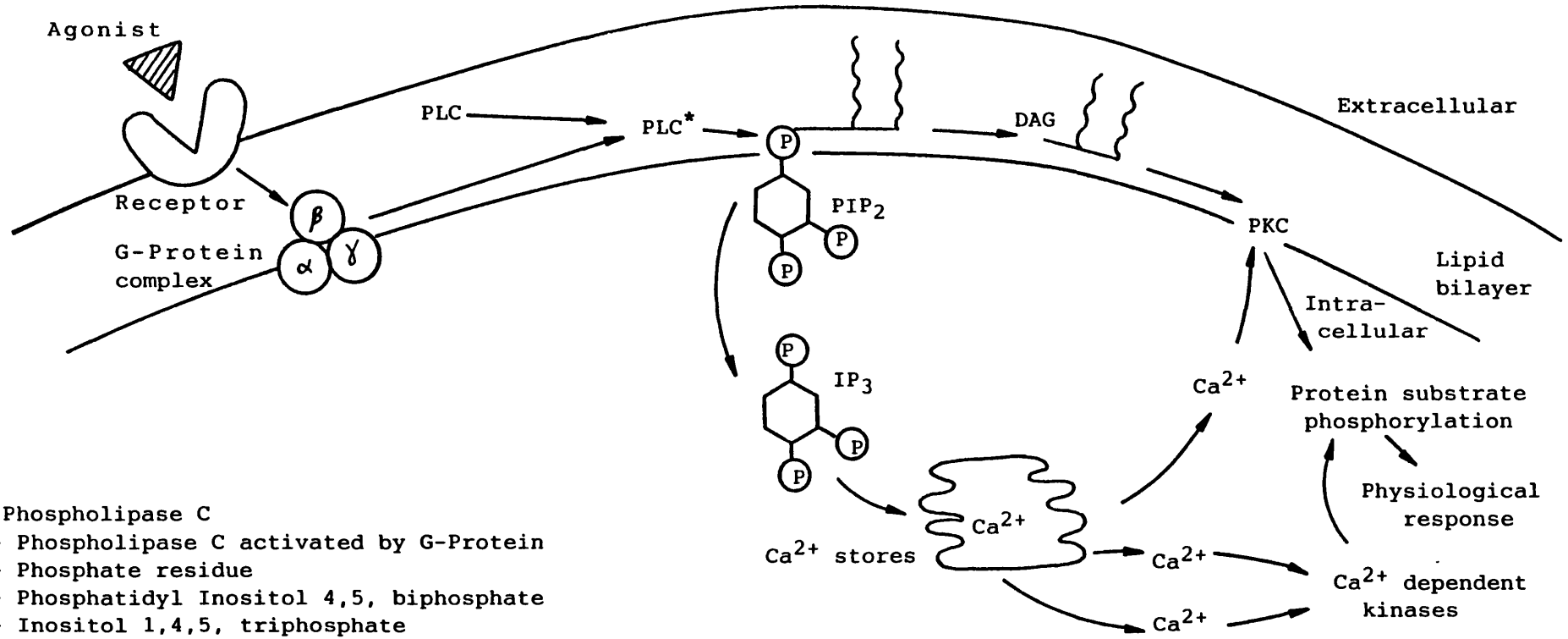
On binding to its cell surface receptor a neurotransmitter, hormone or drug induces a series of events which ultimately results in physiological or pharmacological response. The opening of a ligand-gated ion channel (eg. Acetylcholine binding to the nicotinic receptor, resulting in the influx of Na^+ ions and corresponding change in electrophysiological potential) is an effect induced directly by the binding of the ligand. Alternatively, the agonist may on binding to its cell surface receptor, initiate a sequence of events that result in the primary signal (or "message") being passed through the cell membrane to "secondary messengers". Before the physiological event is observed, secondary messengers cause amplification of the signal by involvement of enzyme cascades. In this case, the ligand receptor is present in the cell membrane and can transiently couple a G- protein(Guanine nucleotide binding protein); these proteins link the receptor with one or more of the following signal transduction pathways:

cAMP / adenylate cyclase system,

phospholipase C / phosphatidylinositol turnover,

ion channel regulation (reviewed by Drummond and Hughes, 1987).

Agonists capable of stimulating phospatidylinositol turnover are numerous (see review Ryves, 1991, Gordge, 1992) and include: neurotransmitters (e.g. adrenaline, GABA, 5HT, histamine); growth factors (e.g. epidermal growth factor, platelet derived growth factor); hormones (e.g. adrenocorticotropic hormone, thyroid stimulating hormone); insulin; inflammatory mediators (e.g. leukotriene D_4, prostaglandin $F_{2\alpha}$, substance P, bradykinin). This is indicative of the importance



PLC - Phospholipase C
PLC* - Phospholipase C activated by G-Protein
Ⓟ - Phosphate residue
PIP_2 - Phosphatidyl Inositol 4,5, biphosphate
IP_3 - Inositol 1,4,5, triphosphate
DAG - Diacylglycerol

Figure 1.3.1 Phophatidyl inositol signal transduction mechanism

Binding of agonist to cell surface receptor induces coupling to G-protein complex which then activates Phospholipase C; PLC hydrolyses Phosphatidyl inositol-4,5,-biphosphate to diacylglycerol and inositol-1,4,5-triphosphate. These second messengers then exert their function; IP_3 causes calcium ion release from intracellular stores and DAG activates PKC

of this signal transduction mechanism in cellular communication, proliferation and differentiation.

Inositol phospholipid turnover has been reviewed (Hirasawa and Nishizuka, 1985; Berridge, 1987; Berridge and Irvine, 1989; Potter,1990); the important inositol in terms of signal transduction is recognised as being phosphatidylinositol -4,5-bisphosphate (PIP_2) although this constitutes only a small percentage of total inositol phospholipids. PIP_2 is formed by sequential phosphorylation from phosphatidylinositol (Berridge and Irvine 1984).

Agonists binding to its cell surface receptor and transient coupling to a G-protein activates a membrane bound phosphodiesterase, phospholipase C (PLC). PLC hydrolyses PIP_2 to the secondary messengers, inositol-1,4,5 -trisphosphate(IP_3) and diacylglycerol (DAG) (See Fig1.3.1), thereby forming a bifurcating signalling pathway. IP_3 is water soluble and diffuses into the cytosol causing the release of calcium ions from intracellular stores, which are probably part of the endoplasmic reticulum (Ross *et al*, 1989). Elevation of intracellular Ca^{2+} levels enables the activation of Ca^{2+}-dependent kinases, including PKC, thereby inducing substrate phosphorylation and a subsequent physiological response. However the role of PIP_2 directly activating PKC remains unclear. Some studies have implicated strong activation of PKC by PIP_2 (Chauhan *et al* 1991; Lee and Bell, 1991), while others have observed weak activation (Huang and Huang, 1991) with respect to DAG. Since these studies were investigated *in vitro*, the significance of such findings *in-vivo* are yet unclear.

DAG produced from PIP_2 hydrolysis remains within the cell membrane lipid bilayer and, on binding to PKC, enables PKC to become membrane associated with a lower Ca^{2+} requirement, and activated. PKC in this membrane associated state phosphorylates its protein substrates to induce a physiological response. Rozengurt

et al (1983) showed that phosphorylation of an 80KDa substrate protein in intact quiescent 3T3 cells was a result of direct (using phorbol esters) or indirect (by addition of PLC or an endogenous agonist, platelet derived growth factor) PKC activation and, therefore, demonstrated a direct relationship between phosphatidylinositol turnover, PKC activation and protein substrate phosphorylation.

Once IP_3 or DAG have fulfilled their second messenger function, metabolic deactivation is necessary for the normal basal state of the cell to be retained. IP_3, which clearly has a critical role in regulation of cellular calcium mobilisation and homeostasis (Mennitt *et al*, 1991), a complex metabolic pathway exists which is mediated by specific kinases (reviewed by Shears, 1989). IP_3 can be metabolised to inositol-1,4-bisphosphate and thence inositol-1 and inositol -4-monophosphates and finally inositol (Shears, 1989) (See Fig 1.3.2). Alternatively, inositol tetrakisphosphates (Batty *et al*, 1985; Shears *et al*, 1987) and thence pentakis - and hexakis - phosphates (Shears *et al*, 1987; Balla *et al*, 1987; Stephens *et al*, 1988) can be formed from IP_3. Inositol tetrakis-, pentakis-, and hexakis-phosphates have been observed in rat brain (Theibert, *et al*, 1992; Smith *et al* 1991), human T-lymphocytes (Guse and Emmrich, 1992) and neuroblastoma cell lines (Sasakawa *et al*, 1992). Physiological functions of these inositol phosphates are poorly understood. However there is increasing evidence that as IP_3 increases in cells, in addition to an intracellular mobilisation there is also an increase in Ca^{2+} entry (Irvine, 1990; Irvine 1992). Until recently there were two principle alternative hypothesis for the way in which IP_3 stimulated Ca^{2+} entry

capacitative Ca^{2+} entry, whereby the intracellular pools control Ca^{2+} entry (IP_3 therefore has an indirect effect) or

entry is controlled by inositol 1,3,4,5 - tetrakisphosphate (IP_4).

Another possibility is that these two hypothesis are in fact different facets of a

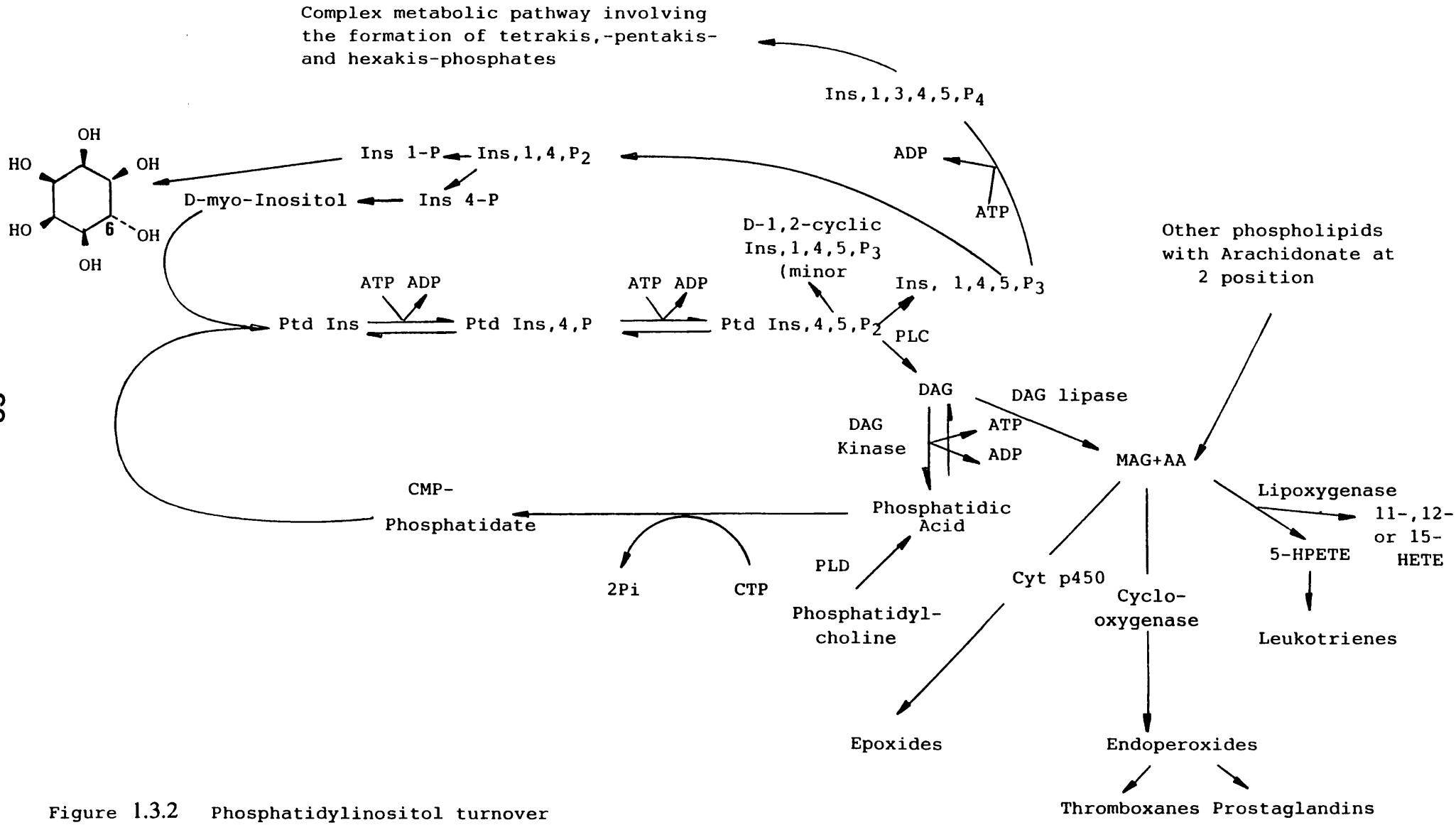


Figure 1.3.2 Phosphatidylinositol turnover

common mechanism. In this hypothesis the receptors for IP_3 and IP_4 interact to control Ca^{2+}- entry, and the IP_3 receptor (located in endoplasmic recticulum), but interacting with IP_4 receptor in plasma membrane, is under modulatory control by luminal Ca^{2+} (ie. Ca^{2+} in the stores; Irvine, 1992; Irvine, 1991). An entirely separate function of phosphoinositides apparently exists in the nucleus. The generation of DAG from nuclear cycle results in translocation of PKC to the nucleus (Divecha *et al*, 1991). However a physiological role for the nuclear phosphoinositide cycle is still to be clarified.

DAG can be metabolised to phosphatidic acid, which after subsequent phosphorylation and addition of cytidine monophosphate, can be coupled with D-*myo*-inositol, thereby forming phosphatidylinositol which re-enters the phosphatidylinositol cycle. DAG can alternatively be hydrolysed to monoacylglycerol, releasing arachidonic acid (AA; reviewed by Smith, 1989). AA can then be further metabolised to produce eicosanoid inflammatory mediators (for review see Gordge, 1992).

The regulation and turnover of the phosphatidylinositol cycle is thus complex; PLC exists as an isoenzymic family (Hirasawa and Nishizuka, 1985) and initial agonist - receptor complex interactions with G-protein subunits may cause preferential activation of one or more isozyme and may partially explain cellular responses to a particular agonist. Interactions with other phospholipid pathways (e.g. phospholipase D metabolism of phosphatidycholine, as an additional source of DAG) may also contribute to the response of the cell. Regulatory and feedback mechanism of phosphatidylinositol turnover are complex (for review see Ryves, 1991), and will not be discussed here, however I will principally focus on one arm of its turnover, the activation of PKC.

1.3.3 PKC - identity with the phorbol ester receptor.

Several laboratories demonstrated that phorbol ester binding activity and PKC enzymatic activity co-purified, ultimately to homogeneity, implicating PKC as the phorbol ester receptor (Castagna *et al*, 1982; Kikkawa *et al*, 1983; Leach*et al*, 1983; Niedel, *et al*, 1983; Ashendel *et al*, 1983; Parker, Stabel and Waterfield, 1984). *In-vitro* work showed that phorbol ester binding to PKC was dependent on phosphatidyl serine and calcium and could be competitively inhibited by DAG (putative endogenous activator of PKC) (Tanaka *et al* 1986; Kikkawa *et al* 1983 ; Sharkey *et al* 1984).

The discovery of PKC as the receptor site for tumour promoting phorbol esters (reviewed by Blumberg, 1988) provided a biochemical mechanism to mediate the biological actions of phorbol esters, and indicated signal transduction systems, particularly the phosphatidylinositol cycle, as critical pathways in cellular communication, proliferation and differentiation.

1.3.4 PKC - isozymes.

The importance of PKC in signal transduction and the complex co-factor requirements led to closer investigations into the structure of the protein itself. Studies on purified bovine brain PKC digested with trypsin enabled the determination of the primary amino acid sequence (Parker *et al*, 1986). Using predicted sequence oligonucleotide probes, clones containing cDNA library from calf brain were screened and hybrids isolated. These were found to contain not one but several closely related but distinct cDNA strands which yielded three sequences upon transcription; PKC-α, PKC-β and PKC-γ (Coussens *et al*, 1986; Kikkawa *et al*, 1987a; 1987b; Housey *et al*, 1987). β-PKC was shown to exist as two isoforms, β_i- and β_{ii}-PKC, arising from alternate splicing of a single gene and

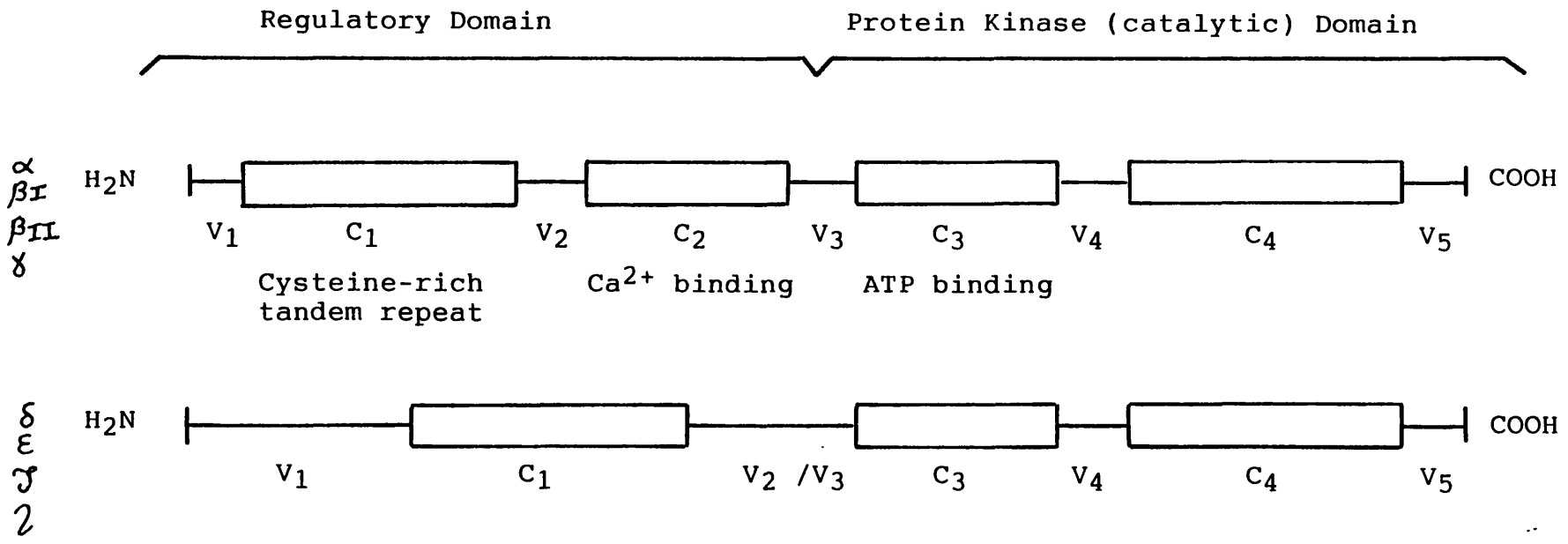


Figure 1.3.3 Schematic structure of PKC isotypes

α , βI , βII PKC possess four coserved (C_1-C_4) and five variable (V_1-V_5) regions. δ, ε, ϑ, η PKC are lacking C_2 region (Ca^{2+} binding region). C_1 contains a tandem-repeat of a cysteine rich sequence (ϑ has only one set of cysteine rich sequence). C_3 has an ATP binding sequence. (Modified from Nishizuka 1988)

differing by only 2 amino acid residues (Ono *et al*, 1987a). Subsequently, low stringency probing of cDNA libraries with oligonucleotide prepared from α–, β– and γ-PKC has revealed more members of the PKC family termed nPKC; δ-, ε–, ζ– (Ono *et al*, 1987b). Recently two more isotypes belonging to nPKC family have been characterised, η- (Osada *et al*, 1990; Bacher *et al*, 1991) and θ-PKC isoforms (Osada *et al*, 1992). The heterogeneity of PKC (reviewed by Nishizuka, 1988; Parker *et al*, 1992; Ono and Kikkawa, 1987) and subtle enzymological properties of isotypes poses the question of individual isotype's role(s) in cellular function. Although PKC function may be poorly understood, several properties of the isotypes may contribute to particular roles:

Distinct patterns of tissue distribution and intracellular localisation (see Table 1.3.4).

Specific substrates for a specific isotype.

Selective activation (or non-activation) of an isotype by different diacylglycerol agonists (This could be the result of extracellular agonist stimulation of phosphatidylinositol turnover, or generation of diacylglycerols from metabolism of other phospholipids).

PKC is a monomeric protein consisting of a protein kinase (or "catalytic") and a regulatory domain (see Fig 1.3). α-, βi–, βii- and γ-isotypes possess four conserved (C_1 - C_4) and five variable (V_1 - V_5) regions (see Fig 1.3.). δ–, ε–, ζ– and η– isotypes lack the C_2 conserved region, which presumably contains the Ca^{2+} binding regions since these isotypes are Ca^{2+} -independent (Schaap *et al*, 1989; Ono *et al*, 1988; 1989). The conserved C1 region contains a tandem repeat of a cysteine rich sequence, Cys - X_2 - Cys - X_{10-14} - Cys - X_7 - Cys - X_7 - Cys, where X represents any amino acid sequence (Osada *et al*, 1992). ζ-PKC has only one set of this cysteine rich sequence (Ono *et al*, 1989a). This is similar to the consensus sequence of a cysteine - zinc - DNA - binding finger found in metallo proteins and DNA-binding proteins (Nishizuka, 1988) and recently direct evidence

Table 1.3.1 Examples of protein kinase C isozyme distribution.

Tissues	Isotypes present	Refs.
Adrenocortical tissues.	α	Pelosin *et al*, 1987.
Brain	α, β, γ, δ, ε, ζ, η	Bacher *et al*, 1991; Brandt *et al*,1987; Huang *et al*,1986;1987; Kosaka *et al*, 1988; Osada *et al*, 1990; Schaap *et al*, 1989; Wetsel *et al*, 1992.
Epidermis	δ, η	Bacher *et al*, 1991; Osada *et al* 1990.
Erytholeukemia (MELC)	α, β, ε	Melloni *et al*,1989;Powell *et al* 1992.
Fibroblasts	α, β, δ, ζ	Borner *et al*,1992; Kosaka *et al* 1988; Rose-john *et al*, 1988; Guadagno *et al*, 1992.
Glomerular Mesengial Cells	α, ε	Huwiler *et al*, 1991.
Heart	α, β, ε, η	Kosaka *et al*, 1988; Osada *et al* 1990; Schaap *et al*, 1989.
Kidney	α, β, ε	Kosaka *et al*, 1988; Schaap *et al*, 1989
Liver (incl. hepatocytes)	α, β, ε	Kosaka *et al*, 1988; Robles-Flores *et al*, 1991; Schaap, 1989.
Lung	α, β, ε, η	Bacher *et al*, 1991; Kosaka *et al*, 1988; Osada *et al*, 1990.
Neutrophils	α, β, ζ	Pontremoli *et al*, 1990; Stasia *et al*, 1990
Pancreatic Acinar cells	α	Wooten and Wrenn, 1988.
Pituitary cells (GH-4)	α, β, ε	Jaken *et al*, 1990.
Platelets	"a" and "b" α, β, δ, ζ	Brooks *et al*, 1990; Crabos *et al*, 1992; Baldassare *et al*, 1992.

Skeletal muscle	α, β, θ	Nakano *et al*, 1992; Osada *et al*, 1992.
Sperm	α, β	Chaudhry and Casillas, 1992.
Spleen	α, β, η	Brandt *et al*, 1987; Kosaka *et al*, 1988; Osada *et al*, 1990.
Testes	α, β, η	Kosaka *et al*, 1988; Osada *et al*, 1990.
Thymocytes	α-mRNA, β-mRNA, ε-mRNA, ζ-mRNA	Freire-Moar *et al*, 1991.
T-Lymphocytes	α, β	Berry *et al*, 1989; Shearmean *et al*, 1988.

Table 1.3.1 cont...

has been obtained that βi-PKC over expressed in insect cells tightly binds four zinc ion per molecule (Hubb *et al*, 1991). The conserved C_3 regions has an ATP binding sequence Gly - X - Gly - X - X - Gly.....Lys. C_4 contains a similar sequence Gly - X - Gly - X - X - Gly.....(X), although its significance is unknown (Nishizuka, 1988). Phorbol ester/ diacylglycerol and phospholipid binding sites are thought to be contained in C_1 region (Ono *et al*, 1989b).

Initially studies on activation of PKC isozymic mixtures from rat brain by phorbol esters revealed that phorbol ester activators could be classified into four groups (Ellis *et al*, 1987; Evans 1989):

i) Compounds, which like TPA could activate PKC at low concentrations (100nM) eg SAP-A, SAP-D.

ii) Compounds which only maximally stimulate PKC to approximately 30% of the activity induced by TPA eg DOPPA, Rx.

iii) Compounds which fully activate PKC but at high concentrations (1uM - 10uM) eg α-SAP-A.

Compounds which fail to activate PKC even at concentrations of 10uM eg α-sapine.

The ability of phorbol esters TPA, SAP-A, Rx, DOPP, DOPPA and TxA to activate purified α–, βi–, γ–, δ–, ε-PKC isoforms confirmed that α–, β- and γ-PKC were dependent on Ca^{2+} for activation whereas δ- and ε- isoforms were Ca^{2+}-independent. Additionally the range of phorbol esters used to study the isotypes gave differential responses in terms of activating ability (Evans *et al*, 1991; Ryves *et al*, 1991). DOPPA was shown to be specific βi-isotype activator and Sap-A was a specific δ-isotype non-activator.

These data support the idea that individual PKC-isotype may play distinct roles in cellular function. Additionally since isotypes can be selectively activated or non-

activated by particular phorbols, then these probes could be utilised to selectively study PKC-isotype functions in cells.

1.35 Translocation and Down Regulation

Binding of phorbol esters to PKC had other effects concomitant with activation. PKC was observed to be translocated from cytosol to the cell membrane (Kraft, 1982; Kraft and Anderson, 1983). When the translocating action of 1,2-dioctanoylglycerol (DiC_8, a membrane permeable diacylglycerol analogue) was compared to phorbol ester action in T - lymphocytes, it was found that both agents elicited similar rapid translocation of cytosolic PKC to the particulate fraction (~50%) but the phorbol ester response was prolonged (4 hours +) compared to DiC_8 (transient rise peaking at 20 minutes) (Berry *et al*, 1990). This stabilisation of membrane PKC possibly reflects the differences between slow metabolism of phorbol esters compared to the rapid breakdown of diacylglycerols (De Gaffroy de Courcelles *et al*, 1989 ; Mayorek and Bartan, 1985; Evans and Soper, 1987), although in some systems this is not the case (Kreutter *et al*, 1985). Translocation has been demonstrated with physiological agonists of PI turnover, with maximum translocation varying (eg. 10 mins with IL-3 treated T-cell line (Farrar *et al*, 1985) and 40 minutes in TSH - treated thyroid cells (Ginsberg *et al*, 1988). When phorbol esters are compared with PI agonists in the same cell type (eg. anti-IG in B-lymphocytes (Nel *et al*, 1986) and IL-2 in T- lymphocytes (Giri , 1988)) the translocation induced by phorbol esters significantly exceeded the agonist in quantity as well as duration. A recent study has shown that selective translocation of PKC isotypes can occur with a PI agonist (Baldassare *et al*, 1992). Treatment of human platelets with thrombin translocated PKC–α, β– and ζ– while PKC–δ remained unaltered. The significance of selective translocation is yet to be clarified.

In many cell types chronic incubation with phorbol esters results in the translocation

followed by the decrease in total PKC levels from the cells due to proteolytic degradation or 'down - regulation' (Rodriguez-Pena and Rozzengurt 1984; Ballester and Rosen, 1985; Adams and Gullick, 1989). Down- regulation can be mediated by the action of trypsin (Huang *et al*, 1989) or calcium dependent neutral protease (Calpain I or II (Kishimoto *et al*, 1989)). The activated form of PKC is thought to be required for down-regulation , since the rate of down-regulation is enhanced by the presence of PKC co- factors (Huang *et al*, 1988). A variable sequence region (V_3) has been predicted as the major site for proteolytic degradation of these enzymes (Parker *et al*, 1986; Huang and Huang, 1986; Lee and Bell, 1986). Possibility that PKC - isotypes in cells may be subjected to differential down-regulation because of the differences in their susceptibilities to proteolysis has been suggested. Investigations into the rate of down regulation of α–, β– and γ– isozymes of PKC by trypsin and calpains I and II have revealed that these isozymes are differentially down regulated (Huang *et al*, 1989; Kishimoto *et al*, 1989). γ–PKC was the most rapidly down-regulated by both trypsin and calpain, followed by β–PKC, whilst α–PKC was the most resistant to proteolysis. Prolonged TPA treatment of intact rat basophillic leukaemia RBL-2H3 cells (which contain β– and α– PKC) confirmed the *in vitro* results. TPA induced faster down- regulation of β– than α– PKC (Huang *et al*, 1989). Differential down - regulation of PKC isotypes is now regarded as a common phenomenon in many cell lines, and it may be possible, by comparing rates of degradation of each isotype with physiological responses, to associate the role of each isotype with a given physiological function.

1.3.6 Substrates of PKC

Activation of PKC leads to a change in cellular physiological function and the first step in this chain of events is substrate phosphorylation by PKC. However, whilst numerous proteins have been shown to be phosphorylated by PKC, the effects and relevance of this phosphorylation remain, in most cases, poorly characterised. Indeed, whether or not some of these proteins are physiological PKC substrates is contentious. Both *in vitro* and *in vivo* techniques have been used to examine the characteristics of potential PKC substrates. These normally utilise ^{32}P radiolabelled phosphate of ATP to assess incorporation into the acceptor or substrate protein as a measure of phosphorylation. *In vivo* experiments on intact cells involve loading the culture media with ^{32}P-ortho-phosphate until the intracellular phosphate pool has been labelled to equilibrium. Cells can then be treated with activator (phorbol ester or diacylglycerol) and the ^{32}P-phosphoproteins can be analysed by autoradiography following one or two dimensional electrophoresis on SDS-PAGE gels (Laemmeli, 1970). The ultimate aim of this analysis is to determine the amino acids sequence of the substrate protein, enabling assessment of the serine/threonine residues phosphorylated by PKC, in an attempt to clarify the physiological significance of substrate phosphorylation. There appears to be no absolute consensus sequence required for PKC-induced substrate phosphorylation, although PKC recognition shows independence from other serine/threonine kinases (Gallis *et al*, 1986). Experiments with synthetic substrates (O'Brian *et al*, 1984; Ferrari *et al*, 1985; Schaap *et al*, 1989) seem to suggest that basic amino acids (arginine or lysine) are required on both the carboxyl- and amino-sides of the target residue for PKC phosphorylation.

For protein to be considered a potential physiological target of PKC mediated phosphorylation, the following minimum criteria should be fulfilled:

i) Correlation of *in vitro* and *in vivo* phosphorylation sites.

ii) Functional difference of phosphorylated/dephosphorylated forms of the substrate.

iii) Stoichiometric *in vitro* phosphorylation of substrate is dependent on the presence of PKC co-factors under conditions that will induce activation of PKC.

iv) *In vivo* accessability of the substrate to PKC (i.e. co-localisation).

Table 1.3.2 shows the numerous substrates that are phosphorylated by PKC; these include: receptor/signalling proteins; enzyme proteins; structural/contractile proteins; nuclear proteins; and some undesignated proteins. Many of these substrates can be phosphorylated by other kinases, either at the same serine/threonine residue e.g. C-protein (Lim, Sutherland and Walsh, 1985), at different residues e.g. GABA-modulin (Wise, Guidotti and Costa, 1983), or at a mixture of these e.g. MAP-2 (Akiyama *et al*, 1986). Therefore, the physiological significance of PKC induced phosphorylation is often unclear. Identification of PKC substrate proteins can be further hampered by several factors: i) Substrate species variation; the 87kDa protein from bovine sources is an 80kDa protein in rat (Albert *et al*, 1986); ii) Changes in isoelectric properties on phosphorylation (McFarlane, 1986); iii) Tissue-specific forms of a substrate exhibiting differences in phosphorylation (Wu *et al*, 1982; Rider and Hue, 1986); iv) Changes in substrate with maturation (Turner *et al*, 1984); v) Generation of PKC phosphoprotein fragments following autophosphorylation (Mochley-Rosen and Koshland, 1987; Huang

et al, 1986); vi) Phosphatase action (Cantrell *et al*, 1989; Hannun, Loomis and Bell, 1985).

A further factor involved in the study of PKC substrate proteins is the heterogeneity of PKC isozymes. Different isotypes may be triggered by particular agonists (or phorbol esters) and may possess their own set of protein substrates, or may phosphorylate substrates at different rates e.g. *in vitro* EGF-receptor phosphorylation has slight variation $\alpha > \beta > \gamma$ (Ido *et al*, 1987). These possibilities have implications in terms of co-localisation and tissue/cell-specific distribution of both PKC isotypes and the protein substrates.

Table 1.3.2 Protein substrates for protein kinase C.

Protein.	Tissue.	Effect of phosphorylation.	Reference.
1. Receptor/signalling proteins			
Nicotinic-acetylcholine receptor*: α-chain 40k δ-chain 65k	Torpedo electric organ	Increased rate of desensitisation	Hemmings *et al*, 1989
Adenylate cyclase catalytic unit 130k	Bovine brain; frog erythrocytes	Enhances hormone-sensitive activity	Yoshimasa *et al*, 1987
α_1-adrenergic receptor * 55k subunit	Hamster DD1 MF2 cells; vas deferens; smooth muscle	Decreased binding affinity for adrenaline; uncoupling from PtdIns metabolism	Leeb-lundberg *et al*, 1985
β-adrenergic receptor* 52k	Duck/turkey erythrocytes	Desensitisation to agonist, uncoupling from adenylate cyclase activation	Kelleher *et al*, 1984; Sibley *et al*, 1984
Dihydropyridine receptor* (L-type calcium channel) 165k, 55k	Rabbit skeletal muscle	Calcium channel regulation	Nasainczyk *et al*, 1987
Epidermal growth factor receptor (EGFR)* 170k	Fibroblasts; A431 cells	Decreased affinity of EGF for EGFR; decreased tyrosine kinase activity and autophosphorylation	Cochet *et al*, 1984; Davis and Czech, 1984; Davis *et al*, 1985a, b; Downward, Waterfield and Parker, 1985; Ferrari *et al*, 1985; Ido *et al*, 1987

GABA-modulin receptor* 16.5k	Rat Brain	?	Wise, Guidotti and Costa, 1983
Glucose transporter 50-60k	Human fibroblasts and erythrocytes	?	Witters, Vater and Lienhard, 1985
G-protein free α subunit* 41k $G_{1\alpha}$ $G_{2\alpha}$	Rabbit liver; human platelets; S49 lymphoma line	Decreased ability to interact with adenylate cyclase; regulation of signal transduction	Carlson, Brass and Manning, 1989, Mendoza *et al*, 1986
Guanylate cyclase 150k	Rat brain	Increased activity	Zwiller, Revel and Malviya, 1985
HLA class I antigens* h chains 47k	Human platelets, lymphocytes; HL-60 cells	?	Feuerstein, Monos and Cooper, 1985
Insulin receptor β subunit* 95k	Human placenta; B-cell line 1M-9; rat liver; FAO hepatoma cells	Decrease in receptor tyrosine kinase activity	Bollag *et al* 1986; Takayama *et al*, 1984
Interleukin-2 receptor* (T-cell activation antigen) 50-55k	Human T-cell line HUT102B2; transfected mouse C127 line	?	Gallis *et al*, 1986; Leonard *et al*, 1985; Shackleford and Trowbridge, 1984
Mouse T-cell antigen receptor δ and ε chain	Mouse T-cell hybridoma 2B4 line	Down regulation of receptor complex	Minami *et al*, 1987
Ovotransferrin 78k	Chick oviduct	?	Hom, Gschwendt and Marks, 1985
Somatomedin C receptor* 92-95k	Human B-cell line 1M-9	?	Jacobs *et al*, 1983.
T_3/T-cell antigen receptor* (Ti) T_3 γ chain 26k CD_3 δ chain 21k	Human lymphoblasts and T-cell line HPB-ALL	Down regulation of T_3/T-cell receptor complex	Alexander *et al*, 1990; Cantrell *et al*, 1989; Chetila and Geha, 1988.

Transducin (G_t, a G-protein)* 41k free α subunit in GDP-bound form	Bovine retina	?	Zick *et al*, 1986.
Transferrin receptor 180k	HL-60 cell line	Phosphorylation mediates receptor internalisation	May, Jacobs and Cuatrecasas, 1984; May *et al*, 1985
Voltage sensitive sodium channel* α subunit 260k	Rat brain	Regulation of sodium channel	Costa and Cantrell, 1985
2. Enzyme proteins			
Acetyl Co-A carboxylase * 230-250k	Rat lactating mammary gland	Inhibition of activity	Hardie *et al*, 1986
ATP-citrate lyase	Rat lactating mammary gland	No effect (?)	Hardie *et al*, 1986
Cytochrome p450 scc* 50k	Bovine adrenal cortex	Increases stereogenesis activity	Vilgrain, Defaye and Chambaz, 1984.
Enolase 41k	Rabbit muscle	Modification of bidirectional enzyme activity	Nettleblad and Engstrom, 1987.
Glycogen synthase* 85k subunit	Rabbit liver, skeletal muscle; rat hepatocytes	Inactivation (?)	Ahmad *et al*, 1984; Arino and Guinovart, 1986; Inazu *et al*, 1984.
Harvey-*ras* oncogene product* 21k	Bacterial expression vector	?	Jeng *et al*, 1987.
3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) 100k (53k)	Rat liver	Inactivation	Beg, Stonik and Brewer, 1984.

Inositol trisphosphate 5' phosphomonoesterase 40k protein, p47 (?)	Rabbit/human platelets	Increase of phosphatase activity	Connolly, Lawing and Majerus, 1986; leyasu *et al*, 1982; Lepetina *et al*, 1985; Touqui *et al*, 1986.
NADPH-Oxidase complex	phagocytes	Activation of O_2^- production	Majmunder *et al*, 1993; Ding *et al*, 1993 (see Section 1.4.3.1)
Neutral protease 74k subunit	Bovine heart	?	Hincke and Toinai, 1986.
Phosphofructokinase* 58k (82k)	Bovine heart; rabbit/rat skeletal muscle; rat liver	No effect (?)	Hoffer, Schlatter and Graefe, 1985; Nettleblad *et al*, 1986.
pp60 *v-src** 60k	RSV transformed vole, BALB/C and chicken embryo fibroblast cells	?	Gentry *et al*, 1986; Gould *et al*, 1985.
pp60 *c-src** 60k	Mink lung, chicken embryo fibroblasts; human platelets	?	Gentry *et al*, 1986; Gould *et al*, 1986.
Tyrosine hydroxylase* 62k (66k)	Rat brain; PC12 cells	?	Albert *et al*, 1984a.
3. Structural/contractile proteins.			
Caldesmon* 130k	Chicken smooth muscle	Decreased activation of myosin light chain kinase	Umekawa and Hidaka, 1985
Calmodulin-binding protein* α 130k; β 97k	Human erythrocytes	Modification of membrane skeleton	Ling, Gardner and Bennett, 1986.
C-protein* 145k	Bovine myocardium	?	Lim, Sutherland and Walsh 1985.

Cytoskeletal proteins* Band: 4.1 78-80k 4.9 45-49k	Rabbit/human erythrocytes	Modification of membrane skeleton	Horne, Leto and Marchesi, 1985; Ling and Sapirstein, 1984; Ling, Gardner and Bennett, 1986; Palfrey and Waseem, 1985.
Fibrinogen* α chain 60-70k	Human plasma	?	Humble *et al*, 1984.
Filamin 68k	Chicken smooth muscle	?	Kawamoto and Hidaka, 1984.
Microtubule associated protein* (MAP-2) 270k	Rat/pig brain	Decreased interaction with actin	Akiyame *et al*, 1986; Tsuyama *et al*, 1986.
Myelin basic protein* 14, 18k	Mammalian brain	?	Kishimoto *et al*, 1985; Su *et al*, 1985, 1986; Turner *et al*, 1984.
Myosin light chain kinase* (MLCK) 130k	Turkey smooth muscle	Decreased kinase activity and k_a for calmodulin	Ikebe *et al*, 1985; Inagaki, Kawamoto and Hidaka, 1984; Nishikawa, Shirakana and Adelstein, 1985.
Myosin light chain* 20k	Human/rabbit platelets; turkey smooth muscle	Modification of activated Mg/ATPase activity	Endo, Naka and Hidaka, 1982; Inagaki, Kawamoto and Hidaka, 1984; Nishikawa, Hidaka and Adelstein, 1983.
Talin 215k	Chicken gizzard	?	Litchfield and Ball, 1986.
Troponin I* 27k T* 36k	Bovine heart; rabbit skeletal muscle	Modulation of contractility	Katoh, Wise and Kuo, 1983; Mazzei and Kuo, 1984.
Vincullin* 130k	Chicken embryo fibroblasts, smooth muscle; mouse 3T3 cells	?	Kawamoto and Hidaka, 1984; Werth, Niedel and Pastan, 1983; Werth and Pastan, 1984.

4. Nuclear proteins DNA-methyltransferase 126k	Human placenta	Stimulation	DePaoli-Roach *et al*, 1986.
Eukaryotic initiation factors* 2B 52k; 3 120k; 4B 80k; 4F 220k	Rabbit reticulocytes	Regulation of mRNA translocation	Morley and Traugh, 1989, 1990; Schatzman *et al*, 1983; Tuazon, Merrick and Traugh, 1989; Tuazon *et al*, 1990.
Histone proteins* H-1 33k; H-2B 52k; H-4 33k	Calf thymus; rat H-35 hepatoma cells	?	Ahmad *et al*, 1984; Albert *et al*, 1984b; Butlar, Byus and Slaga, 1986; Iwasa *et al*, 1980.
P1* 48-53k	Rat liver	?	Walaas, Ostvold and Laland, 1989.
Ribosomal S6 protein* 40S subunit	Rat H-35 hepatoma cells; mouse C127 cells; chicken embryo fibroblasts	?	Blenis, Spivak and Lerikson, 1984; Burkhard and Traugh, 1983; LePeuch, Ballester and Rosen, 1983; Tuazon, Merrick and Traugh, 1989.
RNA polymerase II 150k, 180k and 220k subunits	Chicken myeloblastosis leukaemia cells	Modification of RNA synthesis	Chuang *et al*, 1987.
Topoisomerase II* 320k	Drosophilia embryos	Increased activity	Sander, Nolan and Hsieh, 1984.
5. Undesignated proteins Chromaffin granule binding protein 9, 37k	Rat adrenal medulla	Regulation of exocytosis	Summers and Creutz, 1985.

Lipocortin* 1, 35k; 2, 36k	Bovine lung, MDBK kidney line; human AG1523 fibroblast line	Decreases the inhibition of lipocortin on lipase activity	Gould *et al*, 1986; Ieyasu *et al*, 1982; Khanna, Tokuda and Waisman, 1986; Lapetina *et al*, 1985; Touqui *et al*, 1986.
Middle T-antigen* 58k (Polyoma viral antigen)	Polyoma infected mouse 3T3 cells	Stimulates tyrosine kinase activity	Hirata *et al*, 1984.
Neuromodulin* (GAP-43; F1; pp46; p57; B50) 47k	Bovine/rat brain, hippocampus	Inhibition of calmodulin binding activity; modulation of synaptic plasticity	Akers and Routtenberg, 1985; Aloyo, Zwiers and Gispen, 1983; Dekker *et al*, 1989.
Phospholamban* 27k	Canine heart sarcolemma	Modification of Ca^{2+} uptake	Movesesian, Nishikawa and Adelstein, 1984; Yuan and Sen, 1986.
Retinoid binding proteins 14.6k	Calf thymus, liver	?	Cope *et al*, 1984.
B and C stress proteins* 28k	Rat embryo fibroblasts	?	Welsh, 1985.
Vitamin D binding protein 56k	Rat pancreatic acinar cells	?	Wooten *et al*, 1985.
80-87k (MARCKS protein)	All tissues	?	Albert *et al*, 1984a, 1986; Brooks and Brooks, 1990; Brooks, Evans and Aitken, 1987; Graff, Gordon and Blackshear, 1989; Morris and Rozengurt, 1988; Rozengurt, Rodriguez-Pena and Smith, 1983; Rozengurt *et al*, 1984; Smith and Colburn, 1988; Thelen *et al*, 1991; Wang *et al*, 1989; Witters and Blackshear, 1987; Wu *et al*, 1982.

17k, 37k and 69k	Rat liver mitochondria	?	Backer, Arcoleo and Weinstein, 1986.
40k*	Rat adipocytes	?	Graves and McDonald, 1985.
70k	A431 epidermal carcinoma cells	?	Cochet *et al*, 1984; Ido *et al*, 1987.
47k[1]	Human platelets	?	Brooks *et al*, 1900; Kaibuchi *et al*, 1983; Sano *et al*, 1985.

Key and footnotes:

* Denotes that protein is a known target for other protein kinases.

k Denotes approximate molecular weight in kilodaltons.

[1] It has been proposed that this protein would be inositol trisphosphate 5' phosphomonoesterase (Connolly, Lawing and Majerus, 1986), lipocortin (Khanna, Tokuda and Waisman, 1986; Touqui *et al*, 1986), or a class I histocompatability antigen (Feuerstein, Monos and Cooper, 1985).

1.3.7 Phorbol ester Interaction with other kinases.

The major kinase systems discovered (PKCal, PKA and PKG) are involved in cellular processes in much the same manner as PKC (receptor - ligand recruitment of second messengers). However there may be vast array of kinases and kinase systems yet to be discovered which may be potential target sites for phorbol esters. In addition to recognised isotypes of PKC (α-, βi and βii-, γ–, δ–, ε–, ζ–, η–, and θ-; see section 1.3.4), the existence of other PKC or PKC-related kinases have been reported. These include two components of Type III (α–) PKC (Buday *et al*, 1989) with different substrate specificities and Ca^{2+} dependencies and, a Ca^{2+} unresponsive, phorbol ester/phospholipid activated kinase (Gschwendt *et al*, 1989). Gschwendt *et al*, (1989) established that their kinase was not a PKC subtype by using a PKC inhibitor K252a which was unable to inhibit the novel 76 kDa kinase. Similarly, the novel Type III component reported by Buday *et al*, (1989) could be a member of nPKC sub-family.

More significant data concerning phorbol ester stimulation of novel kinases has been reported by Evans *et al*, (1991). Using TPA and Thymeleatoxin A (TxA) it has been shown that these probes can stimulate seven kinase peaks from rat brain. Peaks designated "2" - "5" appear to be conventional PKC isotypes confirmed by Western blotting analysis using anti- PKC α–, β–, γ– and ε– antisera. TxA stimulated peak "1" in a Ca^{2+}-independent manner compared to other peaks (Ryves, 1991). These data suggest the existence of phorbol ester stimulated kinases, other than the PKC isotypes at presently defined. The group of Evans have also identified a novel Ca^{2+}- inhibited kinase, termed Rx-kinase, which is stimulated by Resiniferatoxin (Rx) to a greater extent than by TPA. This kinase was originally isolated from human mononuclear cells (Ryves *et al*, 1989), and now from mouse peritoneal macrophages (Evans *et al*, 1990; see Chapter 3) and preliminary tissue distribution studies has been investigated (Evans, A.T. *et al*,

1991; see Chapter 4). The Rx -kinase elutes at a much higher phosphate gradient concentration than PKC's and it is, therefore likely that this kinase is distinct from known isotypes of PKC.

It is therefore, a possibility that some of the biological effects of phorbol esters could be mediated by novel kinases which may be part of PKC family but which, at present, are distinct from known forms of PKC. These novel kinases may synergise or counteract with PKC subtypes to cause a biological response.

1.4 Superoxide and phorbol esters

1.4.1 Superoxide Anion

When exposed to certain soluble agonists or upon phagocytic stimulation, phagocytes (neutrophils, eosinophils, monocytes and macrophages) undergo a cyanide insensitive respiratory burst. Their rate of oxygen uptake increase greatly, sometimes more than 50-fold (Babior, 1984), and they begin to produce large amounts of superoxide anion (O_2^-) and hydrogen peroxide. Simultaneously there is large quantities of glucose uptake by the way of hexose monophosphate shunt. Because of the sharp increase in oxygen uptake, this series of events is often referred to as 'respiratory burst'.

The respiratory burst results from the activation of an enzyme, NADPH-oxidase, dormant in resting cells, that catalyzes the one-electron reduction of oxygen to O_2^- at the expense of NADPH:

$$O_2 + NADPH \quad \text{-----> } O_2^- + NADP^+ + H^+$$

O_2^- reacts rapidly with itself and enzymatically or spontaneously dismutes to H_2O_2. At the same time glucose metabolised through hexose monophosphate shunt generates NADPH that has been consumed by O_2^- generating enzyme.

O_2^- and H_2O_2, the immediate products of the respiratory burst are not used by the phagocytes of microbial killing but are further catalysed to singlet oxygen and

hydroxyl radicals (Babior, 1984; Rossi, 1986) which are potent microbicidal oxidants.

Reactive oxygen intermediates are assumed to play a role in the killing of bacteria, fungi and parasites (Nathan *et al*, 1980; Nathan, 1982; Dallegri, *et al*, 1989) and in the pathogenesis of myocardial ischemia reperfusion injury (Hammond *et al*, 1985; Kloner *et al*, 1989), adult respiratory distress syndrome (Malech and Gallin, 1987), cerebral vascular damage (Weiss, 1989), tumourigenesis (Weitzman *et al*, 1985), and noninfectious acute and chronic inflammatory processes (Lunec *et al*, 1987, Halliwell *et al*, 1988; Blake *et al*, 1987). Thus, as reactive oxygen species potentially possess both beneficial and deleterious effects, the production of the primary radical, $O_2^{\cdot}$, would be expected to be carefully regulated.

1.4.2 NADPH-Oxidase: A superoxide - Forming Enzyme Complex

NADPH-Oxidase catalyses the univalent reduction of O_2 to $O_2^{\cdot}$ according to the reaction described above. However, NADPH-oxidase has also been shown to catalyse the divalent reduction of O_2 to H_2O under certain experimental conditions (Green and Wu, 1986). The apparent K_m value for NADPH of NADPH-oxidase has been calculated to 30-80 µM (Babior *et al*, 1976; Gabig and Babior, 1979, Cohen *et al*, 1980; Tamura *et al*, 1988), and that for NADH to 0.4 to 0.9 mM (Gabig and Babior, 1979). NADPH oxidase shows a pH optimum at about 7.0 and is inhibited by various thiol reagents but more significantly not by inhibitors of the respiratory chain (Babior *et al*, 1976; McPhail *et al*, 1976; Tauber and Goetzl, 1979, Green and Schaefer, 1981).

NADPH-oxidase was extremely unstable due to thermolability and inhibition by salts and thus the purification of this enzyme was hampered (Babior and Peters, 1981; Green and Pratt, 1988; Sakane *et al*, 1987). Dimethyl sulfoxide, ethyleneglycol, glutaraldehyde and glycerol were some of the compounds proposed to stabilise NADPH-oxidase (Tauber and Goetzl, 1979, Sakane *et al*, 1987). The particulate NADPH oxidase from stimulated human neutrophils has been solubilised with various detergents (Gabig and Babior, 1979; Tauber and Goetzl, 1979; Tamura *et al*, 1988). Solubilization with Triton X-100 resulted in K_m for NADPH of NADPH-oxidase of 33μM (Gabig and Babior, 1979) and with deoxycholate plus Tween 20 resulted in 2-fold increase in enzyme activity (Tamura *et al*, 1988). A mild solubilization on treatment was required in order to preserve the enzymatic activity by Bellavite *et al*, (1985), however this led to the isolation of a large proteolipid complex containing several contaminating proteins.

The activity of solubilized NADPH-oxidase has been discovered to be modulated by phospholipids. Phosphatidylethanolamine but not phosphalidylcholine or phosphatidylserine was found to enhance the activity of Triton-X-100 solubilized NADPH-oxidase (Gabig and Babior, 1979). However, Tamura *et al*, (1988) reported that oxidase solubilized with deoxycholate plus tween 20 was substantially augmented by phospholipids, in the order of effectiveness phosphatidylserine > cardiolipin > phosphatidylenthanolamine > phosphatidylinositol, whereas phosphatidylcholine was inactive.

NADPH-oxidase is reported to be regulated by divalent cations. The enzyme activity

isolated from myristic acid stimulated guinea pig neutrophils was enhanced by Mg^{2+} but not by Ca^{2+} (Yamaguchi *et al*, 1983). They reported that Mg^{2+} enhances V_{max} and reduces apparent K_m for NADPH, and the effect of Mg^{2+} is maximal at 40-50μM. However oxidase activity from TPA treated human neutrophils and monocytes was stimulated by both Mg^{2+} and Ca^{2+} but inhibited by EDTA (Green *et al*, 1983; Suzuki *et al*, 1985). In addition to stimulation, Suzuki *et al* (1985) suggested that Ca^{2+} and Mg^{2+} protect the enzyme against thermal inactivation. They also suggested that divalent cations do not interact with NADPH or modulate its binding to NADPH-oxidase, but rather they bind to a structural or regulatory component of the enzyme.

1.4.2.1 Structural components of NADPH-Oxidase

NADPH-oxidase is supposed to be an enzyme complex consisting of multiple components. These components are localised in plasma membranes, specific granules and in the cytosol of phagocytes. A number of components has been suggested to be involved in the redox chain of NADPH-oxidase, including FAD, quinones and cytochrome b_{-245}. In this section I will dicuss the plasma membrane associated components but the cytosolic components will be discussed later (see Section 1.4.4.2). Table 1.4.1 summarises some properties of putative plasma membrane associated components of NADPH-oxidase.

Table 1.4.1 Some properties of putative plasma membrane associated components of NADPH-oxidase

COMPONENT	PROPERTIES	REFS
1. Flavoprotein	Contains FAD. Redox midpoint potential = -280mV; contains neutral semi-quinone intermediates and is reduced by NADPH. Flavoprotein component 51kDa; NADPH reductase component 87kDa. This reductase reduces cyt b_{-245} and is thought to be phosphorylated by PKC with O_2- production. Diphenylene iodonium inhibits reduction of FAD	Tamoto *et al, (1989);* Kakinuma *et al*, 1986 Fujii and Kakinuma (1991); Zenson *et al*, (1992) Doussiere and Vignais (1992); Lutter *et al*, (1984); Green and Pratt (1985); Ellis *et al*, (1989); Bellavite *et al* (1984) Berton *et al*, (1985).
2. Cytochrome b_{-245}	Redox midpoint potential = -245mV; haemprotein with α and β subunits, with absorba maxima at 558; α-subunit 20-23kDa, β subunit 76-92 kDa (glycoslated). Correlation between O_2^- formation and cyt reduction, and reduction by NADPH. Cyt b_{-245} missing in X-chromosomal CGD; Point mutation in cytoplasmic domain of p22 subunit associated with CGD; Both subunits are phosphorylated by protein kinase C (not in parallel with O_2^- formation) Cyt b_{-245} thought to be flavin binding component of oxidase. The β-subunit contains FAD and NADPH binding sites.	Segal *et al*, (1992); Rotrosen *et al*, (1992) Miki *et al*, (1992); Dinauer *et al*, (1991) Doussiere and Vagnais, (1985); Kakinuma *et al*, (1987); Edwards and Lloyd (1988); Garcia and Segal (1988); Parkos *et al* (1988a) Qunin *et al* (1989); Yamaguchi *et al* (1988b); (1989) Segal and Abo (1993).
3. Quinones	Role as component of NADPH-oxidase is controversial.	Lutter *et al*, (1984); Gabig and Lefker (1985); Murakami *et al*, (1986).

COMPONENT	PROPERTIES	REFS
4. 45kDa protein	Work done in neutrophils and macrophages. Binds FAD therefore could be flavoprotein component. Labelled by diphenylene iodonium, and inhibits O_2^- formation.	Yea *et al* (1990); Ellis *et al* (1988, 1989); Hancock and Jones (1987).
5. 31.5kDa protein	Work done in pig neutrophils. Phosphorylated in intact cells and *in vitro* by PKC. Role in redox chain unknown.	Bellavite *et al*, (1984); Papini *et al*, (1985).
6. 14 to 18 kDa protein	Work done in bovine and pig neutrophils. Identified by immunoblotting with antibodies raised against purified NADPH oxidase. Function and identity unknown.	Fukuhara *et al* (1988); Doussiere and Vignais (1988).

Over the last few years a standardised nomenclature was introduced for components of NADPH oxidase which is now widely accepted (Clark, 1990). The α-subunit of cytochrone b_{-245} is referred to as p22-phox (p, protein; 22, apparent molecular mass; phox, *phagocyte oxidase*). The β-subunit of cytochrome b_{-245} is designated gp91-phox (gp, glycoprotein). By analogy, the cytosolic 47kDa and 67kDa proteins are designated p47-phox and p67-phox respectively (see Section 1.4.4.2.1).

NADPH binds to a proximal component of the redox chain, possibly to a flavoprotein. Affinity-labelling techniques have been used to identify the NADPH-binding component of NADPH oxidase. The 2'3'- dialdehyde derivative of NADPH serves both as an electron donor and as a competitive antagonist for NADPH (Umei *et al*, 1986). Inhibition of O_2^- by NADPH analogue is prevented by NADPH and NADPH dialdehyde has been shown to label a protein with apparent molecular mass 66 kDa protein (Umei *et al*, 1986). Pre-treatment of the NADPH oxidase with p-chloromercuribenzoate or with NADPH at excess prevents affinity labelling of the protein. A protein with similar apparent molecular mass was enriched during the purification of NADPH oxidase by Doussiere and Vignais (1985). These data suggest that a 66-kDa protein carries the NADPH-binding sites and acts as NADPH dehydrogenase. However, several lines of evidence from recent studies support the view that NADPH binding sites are contained within the β-subunit of cytochrome b_{-245} (Segal & Abo, 1993; see below).

A flavoprotein has been postulated to be involved in the redox chain of NADPH-oxidase (Babior, 1984; Babior and Kipnes, 1977). This assumption is supported by the findings that FAD and FAD analogues 8-F-FAD, 8-phenyl-FAD, and 8-S-FAD

are co-factor for O_2^- formation, whereas the $2e^-$ donor 5-carba-deaza-FAD was inhibitory (Ligh *et al*, 1981; Parkinson and Gabig, 1988). Membranes of human neutrophils contain FAD and cytochrome b_{-245} at similar concentrations (Gabig & Lefker, 1984; Green & Pratt 1988). The putative flavoprotein of NADPH oxidase possesses characteristics of the dehydrogenase class and recently it was postulated that β-subunit of cytochrome b_{-245} is in fact FAD-containing flavoprotein dehydrogenase (Segal *et al*, 1992; Segal & Abo, 1993, also see below).

Cytochrome b_{-245} is a haemprotein with absorption maxima at 426, 528 and 558nm, and is sometimes also referred to as cytochrome b_{558} (Iizuk *et al*, 1985; Lutter, *et al*, 1985; Yamaguchi *et al*, 1989). Cytochrome b_{-245} is a heterodimer consisting of a 20- to 23- kDa α-subunit (p21 phox) and 76-92 kDa β-subunit (gp91-phox) which is glycosylated (Harper *et al*, 1989; Tyagi *et al*, 1992; Segal & Abo, 1993). The α-subunit of the cytochrome is assumed to carry haem, and the β-subunit is supposed to play a role in the functional assembly of the dimer (Verhoeven *et al*, 1989; Heyworth *et al*, 1989; Nungent *et al*, 1989). The amino acid sequence deduced from the cDNA encoding the α-subunit of cytochrome b_{-245} shows apparent homology to other known cytochromes but it also contains certain structural motifs common to other haem-carrying proteins (Parkos *et al*, 1988). It was also claimed that α-subunit protein could be purified with a haem attached, although the amino acid composition did not correspond very closely with that derived from the nucleotide sequence of the gene (Yamaguchi *et al*, 1989).

The gene for the β-subunit of cytochrome b_{-245} has also been cloned, and its identity confirmed by comparison with amino acid sequence of purified protein and by immunological studies (Royer-Pokora *et al* 1986; Dinauer *et al*, 1987). The gene for the β-subunit is defective in most cases of X-chromosomal chronic granulomatous disease (CGD), and neither the α-nor the β-subunit are expressed in phagocytes of these patients (Royer-Pokora, *et al*, 1986; Teahan *et al*, 1987; Parkos *et al*, 1989).

Most mammalian cytochrome b are composed of a single polypeptide about the size of α-subunit. Thus work has concentrated on the β-subunit. This has revealed that the β-subunit is in fact FAD containing flavo-protein dehydrogenase (Segal *et al*, 1992). A number of lines of evidence support this conclusion. Membranes of neutrophils from X-CGD patients, which selectively lack the two subunits of the cytochrome b, contained grossly reduced levels of both FAD and haem. The purified cytochrome b could be reflavinated, but only after relipidation (Koshkin and Pick, 1993). The most convincing data was the identification of strong homology between amino acid sequence of the β-subunit of cytochrome b and members of the ferredoxin -$NADP^+$ reductase (FNR) family of reductases, particularly in the conserved nucleotide binding regions of these modular proteins (Segal *et al*, 1992). Also *in vitro* studies of reconstituted oxidase from purified proteins has shown that the flavocytochrome b is the only one which there is any evidence for a redox function (Abo *et al*, 1992, Rotrosen *et al*, 1992; 1993).

The cytochrome b_{-245} β-subunit has also been implicated in binding NADPH. Much

of the evidence for this comes from the work done with ^{3}H-azido-NADPH binding studies. These show that azido-NADPH binds to larger β-subunit of cytochrome b_{-245} (Doussiere *et al*, 1993). When enzymatic deglycosylation of photolabelled neutrophil membrane was investigated the masses of both photolabelled band and immunoreactive β-subunit shifted from 80-100 to 55-65 kDa in accordance with glycoprotein nature of β-subunit (Doussiere *et al*, 1993). Thus the electron-transporting apparatus of this NADPH-oxidase is very unique in being entirely contained within one molecule, a membrane bound flavocytochrome.

1.4.3 Activation of NADPH-oxidase

1.4.3.1 PKC, phorbol esters and other PKC agonists

It has been known for several years that neutrophils and mononuclear phagocytes undergo a respiratory burst upon exposure to phorbol esters, and TPA is one of the most potent and effective activators of NADPH-oxidase (De Chatelet *et al*, 1976; Badwey *et al*, 1980). Primed peritoneal macrophages show higher capacities than resident cells to generate O_2^- upon stimulation with TPA (Bryant *et al*, 1982; Badwey *et al*, 1983). Unlike resident peritoneal macrophages, unprimed bone marrow derived murine macrophages generate O_2^- upon exposure to zymosan but not TPA (Phillips and Hamilton, 1989; Phillips *et al*, 1992). The ability of bone marrow derived macrophages to respond to TPA was restored by treatment with cytokines, GM-CSF, TNF-α, TNF-γ and interleukinl α (IL-1α; Phillips & Hamilton 1989). They suggested

that responsiveness of resident peritoneal macrophages to TPA may be the result of *in vivo* exposure to cytokines (Phillips & Hamilton, 1989). However since down-regulation of PKC in unprimed bone-marrow macrophages results in diminished O_2^- production by zymosan, Phillips *et al* (1992) suggested that PKC can mediate both stimulatory and suppressive signals for macrophage O_2^- production.

There seems to be a close correlation between the ability of various phorbol esters to activate PKC and to induce O_2^- production (Robinson *et al*, 1985). In addition, occupancy of phorbol ester binding sites with agonist there was in parallel an increase in rate of O_2^- formation (Tauber *et al*, 1982). TPA induced respiratory burst was characterised by a lag-time, requires temperatures above 17°C, has a pH optimum of 7.0, and was long lasting (Zimmerman *et al*, 1985; Manara and Schneider, 1985; Utsumi *et al*, 1992). Upon stimulation with TPA, PKC is translocated from cytosol to plasma membrane, which precedes O_2^- formation and may explain the observed lag-time (Wolfson *et al*, 1985; Gennaro *et al*, 1986).

TPA induces phosphorylation of numerous proteins in phagocytic cells (Andrews & Babior, 1983; Feuerstein & Cooper, 1984; Ohtsuka *et al*, 1986; Gaut & Carchman, 1987). At that time, most attention focused on a group of protein with apparent molecular mass 44- to 49- kDa (later identified as 47 kDa protein (p47-phox)), which was phosphorylated in neutrophils of healthy volunteers but not in those of patients with autosomal recessive chronic granulomatous disease (CGD). This p47-phox is one of the cytosolic activating factor (see Section 1.4.4.2.1). Badwey and colleagues

showed a close correlation between phosphorylation of p47-phox and O_2^- formation induced by tumour promoters or a cell-permeable diacylglycerol (DAG), and interestingly they reported that the phosphorylated 47kDa protein apparently associates with cytochrome b_{-245} (Badwey *et al*, 1989a; Heyworth *et al*, 1989a).

Phosphorylation of p47-phox by PKC and/or other kinases occurs at multiple sites upon activation in intact cells (Rotrosen & Leto, 1990), and this has also been observed in cell-free systems (Ohtsuka *et al*, 1990b; Uhlinger *et al*, 1991). β-PKC isotype expressed in human neutrophils (see Section 1.3.4) phosphorylates both endogenous and recombinant p47-phox and is suggested to play a role in assembly or maintenance of active NADPH-oxidase (Majmunder *et al*, 1993). Recent studies have indicated that cytosolic activity translocates upon exposure to TPA (Umei *et al*, 1993). Other studies have revealed that translocation is independent of PKC, and is linked to transmembrane signalling involving Ca^{2+} transients and production of lipidic second messengers (Dusi *et al*, 1993). However, there seems to be some controversy as to whether phosphorylation leads to activation of NADPH-oxidases since some investigations have shown that phosphorylation was not obligatory for activation, either in intact cells (Badwey *et al*, 1989b) or in cell-free systems (Uhlinger *et al*, 1991).

Although a large body of evidence points to the central role of the 47 kDa protein as target for PKC, a role for additional PKC dependent and/or independent mechanisms cannot be excluded, for example, TPA has been reported to induce phosphorylation

of α and β subunits of cytochrome b_{-245}, but the time courses of cytochrome b phosphorylation and O_2^- formation do not correlate (Garcia and Segal, 1988). In addition, TPA induces phosphorylation of NADPH cytochrome C reductase of guinea pig phagocytes, and this covalent modification correlates with the activation of O_2^- formation (Tamoto *et al*, 1989).

It is well known that activators of PKC and Ca^{2+} ionophores can interact synergistically to activate various cell functions (Berridge, 1984; Nishizuka, 1984). In phagocytes, the Ca^{2+} ionophore, A23187 potentiates the TPA induced respiratory burst, resulting in reduction of EC_{50} for TPA induced O_2^- formation, reduction of the lag-time and an enhanced rate of O_2^- formation (Dale and Penfield, 1984; Robinson *et al*, 1984). This synergism between phorbol esters and Ca^{2+} ionophores is partly explained by the fact that an increase in cytoplasmic Ca^{2+} enhances the affinity of PKC for phorbol esters without altering the number of binding sites (Dougherty and Niedel, 1986; French *et al*, 1987). Interestingly, synergistic activation of O_2^- formation by activators of PKC and Ca^{2+} mobilizing agents have been reported to be accompanied by increased phosphorylation of 47 kDa protein (Heyworth *et al*, 1989b).

TPA at suboptimal doses 'primes' phagocytes for enhanced O_2^- formation upon further exposures to chemoattractants (McPhail, *et al*, 1984; Tyagi *et al*, 1988; Seifert *et al*, 1989; Smith *et al*, 1988). The mechanism of priming by TPA has been suggested to involve membrane depolarization, enhanced generation of diacylglycerol (DAG), and activation of PKC independent processes (Tyagi *et al*, 1988; Ohsaka *et al*, 1988;

Sha'afi *et al*, 1989). TPA may block agonist-induced activation of phospholipase C in human myeloid cells with parallel potentiation of exocytosis and O_2^- formation, and interestingly priming by phorbol esters does not depend on phosphorylation of the p47-phox (Sha'afi *et al*, 1988; Della Bianca *et al*, 1986; Cockcroft and Stutchfield, 1989). In contrast, Gay and Stitt (1990) suggested that chemotactic peptides and phorbol esters synergistically activate the respiratory burst through translocation of PKC. In addition to TPA, a DAG analogue, 1-oleoyl-2-acetylglycerol (OAG) potentiates fMLP induced O_2^- formation (Bass *et al*, 1989; Smith *et al*, 1988). OAG shortens the lag time, increases the rate of O_2^- formation, and prolongs the respiratory burst (Bass *et al*, 1988). OAG-induced priming apparently does not depend on extracellular Ca^{2+} and may involve activation of phospholipase A_2 (Dewald, *et al*, 1984; Bauldry *et al*, 1992). Since H-7 inhibits priming by OAG, its effects have been suggested to be mediated by protein kinase C (Smith *et al*, 1988). In contrast, Bass *et al*, (1988) did not observe translocation of PKC by OAG, and the PKC inhibitor 1-(5-isoquinolinesulfonyl) piperazine (C-1) did not prevent priming.

Several investigations were carried out with PKC inhibitors in order to clarify its role in activation of NADPH-oxidase. Staurosporine, one of the most potent PKC inhibitors, effectively inhibits protein phosphorylation and O_2^- formation with IC_{50} 15-20nM for TPA induction, and 10-30nM for fMLP and C5a (Smith *et al* 1988; Dewald, *et al*, 1989; Seifert *et al*, 1990). Unexpectedly, at nanomolar concentrations, Combadiere *et al* (1990) found that staurosporine enhanced the chemoattractant induced respiratory burst, suggesting that PKC may also play an inhibitory role in receptor agonist induced O_2^- formation. Unfortunately staurosporine is not specific

since they are also potent inhibitors of other kinases as well (Rüegg and Burgess, 1989). In addition staurosporine paradoxically induces exocytosis of specific granules from neutrophils and shows some functional similarities with TPA in this regard (Dewald *et al*, 1989).

There are numerous PKC agonist and inhibitors which have been studied and are beyond the scope of this review. For example, isoquinolinesulfonamide H-7 is extensively studied. The results concerning the effects of H-7 on O_2^- formation are controversial. H-7 is thought to inhibit at least in part, the TPA and fMLP induced respiratory burst (Fujita *et al*, 1986; Sha'afi *et al*, 1986; Shibanuma *et al*, 1987; Seifert and Schächtele, 1988). In contrast Wright and Hoffman (1986; 1987) reported that H-7 inhibits neither TPA nor fMLP induced O_2^- production. On the other hand, Berkow *et al*, (1987) and Sha'afi *et al* (1988) showed that H-7 inhibits stimulatory effect of TPA but not fMLP on the respiratory burst. Finally some of the vast numbers of PKC agonists and agents which cause a rise in PKC are summarised in Table 1.4.2.

Table 1.4.2 Activation of Respiratory Burst by PKC agonist and agents which lead to increased PKC

Agent	Cell Type	Effect	Mechanism Discussed	Refs.
1. Phorbol Esters (TPA)	Neutrophils and Macrophages (intact & cell free)	Potentiation (priming) and activation of O_2^-	Translocation of PKC and calpain, proteolytic activation of PKC, phosphorylation of several proteins (eg p47-phox), accumulation of DAG and alkylglycerol.	Cox *et al*, (1985); Papini *et al*, (1985); Garcia and Segal (1988); Sha'afi (1989); Badwey *et al* (1989a); Tyagi *et al*, (1988); Umei *et al*, (1993); Kessels *et al* (1993); Rider *et al* (1988), Gennaro *et al*, (1986); Ohtsuk *et al*, (1986).
2. DAG and anlogues (eg. OAG)	Neutrophils	Potentiation and activation O_2^-	Similar to phorbol esters except less potent. Phosphorylations, PKC translocation. PKC-independent mechanisms. Translocation of cytosolic components.	Park & Babior (1992); Tyagi *et al* (1992), Bass *et al* (1989); (1988); Dale and Penfield (1985); Smith *et al* (1988); Wang and Chew (1986).

Agent	Cell Type	Effect	Mechanism Discussed	Refs.
3. Akylacylglycerols	Neutrophils	Controversial : activation and inhibition of O_2^-	Modulation of PKC activity stimulation of arachidonic acid.	Bauldry *et al* 91988). Ford *et al*, (1989); Bass *et al*, (1988).
4. Phosphatidic Acid	Neutrophils (intact & cell free)	Activation of O_2. Potentiation of O_2^- formation	Phosphorylation of p47-phox. Direct activation of NADPH-oxidase Ca^{2+} independent processes. May act at downstram site of PKC.	Ohtsuka *et al* (1989); Bellavite *et al* (1988) Mitsuyama *et al*, (1993b).
5. Bryostatin	Neutrophils	Activation of O_2^-	Activation of PKC	Wender *et al*, (1988); Berkow and Kraft (1985), Kraft *et al* (1986).
6. Mezerein	Neutrophils	Activation of O_2^- formation	Activation of PKC. Phosphorylation of p47 phox and translocation.	Miyake *et al* (1984); Balazovich *et al* (1986); Heyworth *et al* (1989b).
7. Benoxprofen	Neutrophils	Activation of O_2^-	Activation of PKC.	Anderson & Eftychis (1986); Lukey *et al* (1988).
8. R59022	Neutrophils	Potentiation of O_2^- formation	Inhibition of DAG-kinase. Possible activation of phospholipase C&D.	Mahadevappa (1988); De Chaffoy de Courcelles *et al* (1985); Mege *et al* (1988)
9. Indomethacin	Neutrophils	Potentiation of OAG induced O_2^- formation	Inhibition of DAG Kinase and DAG Lipase	Dale and Penfield (1985; 1987)

Agent	Cell Type	Effect	Mechanism Discussed	Refs.
10. Inhalation anaesthetics (eg halothane, chloroform)	Neutrophils	Controversial : inhibition or activation of O_2^- formation	Membrane perturbation activation, of PKC.	Tsuchiya *et al*, (1988); Welch (1984); Rogham *et al* (1987).

1.4.3.2 Calcium Dependent processes

Calcium plays an important role as an intracellular signal molecule mainly in the regulation of calmodulin-dependent enzymes (Tomlinson *et al*, 1984). Following receptor mediated activation of phospholipase C, IP_3 is released from PIP_2 and mobilizes intracellular Ca^{2+} stores (Berridge & Irvine, 1984; see Section 1.3.2). In addition, fMLP induces mobilisation of plasma membrane bound Ca^{2+} influx from extracellular space (Schell-Frederick, 1984; Anderson *et al*, 1986; Nasmith and Grinstein, 1987).

Similar to the role of PKC, the role of Ca^{2+} in the receptor mediated activation of NADPH oxidase is very controversial (see section 1.4.3.1). There are correlations between activation of O_2^- formation and an increase in cytoplasmic Ca^{2+}, and Ca^{2+} ionophores i.e. A23187 and ionomycin, may activate the respiratory burst in phagocytes (McPhail and Snyderman, 1983; Seifert *et al*, 1989; Dahlgren and Follin, 1990; Neilsen *et al*, 1993; Dahlgren *et al*, 1992). In addition, Ca^{2+} ionophores prime phagocytes for an enhanced respiratory burst upon subsequent stimulation with chemotactic peptides, cytokines, cell-permeable DAG, phorbol esters and possibly many other stimuli (McPhail *et al*, 1984; Strand and Wong, 1985, Finkel *et al*, 1987; Dahlgren, 1989; Koenderman *et al*, 1989). With regard to the chemoattractant-induced respiratory burst, an inhibition of cytoplasmic Ca^{2+} procedes O_2^- formation. Inhibition of the increase in cytoplasmic Ca^{2+} and removal of extracellular Ca^{2+} are associated with suppression of fMLP induced O_2^- formation (Seifert *et al*, 1990; Dahlgren 1987; Hruska *et al*, 1988). Not surprisingly, extracellular Ca^{2+} seems to restore the ability of phagocytes to generate O_2^- upon exposure to chemotactic peptides (Stickle *et al*,

1984).

The putative inhibitor of intracellular Ca^{2+} release, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octoyl ester (TMB-8) also inhibits O_2^- formation induced by fMLP and TPA (Korchak *et al* 1984; Elferink and Deierkauf, 1985). CI-922 has also been reported to inhibit A23187 and receptor agonist induced O_2^- formation presumably through interference with calmodulin dependent processes (Wright *et al* 1987a, b). The antipsychotic drugs chlorpromazine and trifluoperazine and the naphthalene sulfonamide, W7 inhibit both the TPA and fMLP induced O_2^- formation and phosphorylation of 47kDa protein (Heyworth and Segal 1986; Shibanuma *et al* 1987; Seifert and Schächtele 1988, Holian *et al*, 1988; Goodman and Tenner 1992). These compounds are primarily inhibitors of Ca^{2+}/calmodulin processes, and hence it has been suggested that the effects of these substances on O_2^- release are due to inhibition of calmodulin-dependent processes (Wright and Hoffman, 1987; Shibanuma *et al*, 1987).

Neutrophils possess a Na^+/Ca^{2+} exchanger which mediates Ca^{2+} influx in the resting state (Simchowitz *et al*, 1990). The order of effectiveness of various cations (eg. La^{2+}, Zn^{2+}, Cd^{2+}) and analogues of amiloride (eg. benzamil, phenamil) at inhibiting Na^{2+}/Ca^{2+} exchange and fMLP induced O_2^- are supposed to be similar. Additionally, the above substances inhibit fMLP induced rise in cytoplasmic Ca^{2+}. Thus these data suggest that Na^{2+}/Ca^{2+} exchange contributes to the chemotactic peptide mediated increase in cytoplasmic Ca^{2+} and is therefore thought to be involved in the activation of NADPH-oxidase.

However, there are a number of studies which indicate that cytoplasmic Ca^{2+} does not play a key role in O_2^- release. In neutrophils and cultured human monocytes, fMLP induced O_2^- formation and an increase in cytoplasmic Ca^{2+} do not seem to correlate (Rebut-Bonneton *et al*, 1989; Bernado *et al*, 1988). 1,25-Dihydroxyvitamin D_3 differentiated U937 cells generate O_2^- upon exposure to opsonized zymosan and TPA but not fMLP, although the latter induces a rise in cytoplasmic Ca^{2+} (Polla *et al*, 1989). fMLP activates oxygen consumption and induces translocation of p47-phox and p67-phox in both normal and Ca^{2+}-depleted neutrophils (Grinstein and Furuya 1988; Dusi *et al*, 1993). Furthermore, the fact that fMLP, C5a, LTB_4 and PAF induce a rapid increase in cytoplasmic Ca^{2+}, but that only fMLP and C5a are effective activators of NADPH-oxidase, provides another example for the dissociation of Ca^{2+} mobilization and O_2^- formation (Hartiali, *et al* 1987).

1.4.3.3 Miscellaneous Mechanism Involved in activation of NADPH-oxidase

There are several putative mechanistic pathways which have been described in activation of NADPH-oxidase, with almost certain cross-talk taking place. Some of the signalling pathways involved are summarised in Table 1.4.3.

1.4.4 Reconstitution of NADPH-Oxidase activity in Cell-free Systems

The development of cell-free systems for the reconstitution of NADPH-oxidase activity with components from resting phagocytes was a break through for the understanding of NADPH-oxidase. Cell free systems were first described by

Table 1.4.3 Pathways Involved in Activation of NADPH Oxidase

Putative Pathway	Cells Studied	Evidences For	Evidence Against	Refs
Phospholipase A_2 and Archidonic Acid (inc. AA metabolites)	Neutorophils and Macrophages (intact and cell free systems). Kuffper cells and perfused liver	AA activates O_2^- in intact and cell free phagocytes. Exogeneous PLA_2 and AA potentiates fMLP response. Direct activation by exogeneous PLA_2 and AA NADPH-oxidase associated H^+ channel opened by AA. AA increases NADPH-oxidase activity by elevating number of active forms and increasing its affinity to substrate. AA induces translocation of p47-phox, p67-phox, p21 rac, particularly in presence of GTP-γ-s. PLA_2 inhibitors p-bronophenyacyl bromide, mepacrine and inhibitors of lipoxygenase, ETYA esculetin inhibit agonist induced respiratory burst. Ibuprofen inhibits O_2^- generation	No correlation between lipoxygenase inhibitors, O_2^- and LTB_4. PLA_2 inhibitors may inhibit O_2^- by non-specific mechanisms. ETYA and esculetin may directly inhibit NADPH-oxidase	Uhlinger *et al.* (1983); Henderson *et al* (1993); Henderson and Chappell (1992); Sawai *et al* (1993); Rubinek and Levy (1993); Bautista *et al* (1992); Bautista and Spitzer (1993); Badwey *et al* (1984); Lackie and Lawerence (1987); Ginsburg *et al* (1989); Sakata *et al* (1987); Maridonneau-Parini *et al* (1986); Kaplan *et al* (1984); Wang and Teng (1992); Watson *et al* (1992). Ozaki *et al* (1986); Schultz *et al* (1985); Seifert and Schultz (1987).

Putative Pathway	Cells Studied	Evidences For	Evidence Against	Refs
Protein Tyrosine phosphorylation and tyrosine phosphatases.	Neutrophils, Macrophages, HL-60	Phagocytes and HL-60 contain substantial amount of Protein Tyrosine Kinase (PTK) and phosphatases. fMLP stimulates PTK activity. TNF, GM- CSF, G-CSF prime neutrophils for O_2^- formation via tyrosine phosphorylation. Tyrosine phosphorylation thought to be an early event in O_2^- formation, possibly a step before DAG formation by PLC. Agonist induced O_2^- results in several tyrosyl phosporylation, 28-130kDa proteins. fMLP induces PTK to phospharylate 40-42 MAP-Kinase isotypes. G-proteins regulates PTK activity. PTK inhibitors ST638, erbstein and genstein inhibit fMLP and zymosan induce O_2^-, Vanadate inhibits tyrosine phosphatase but activates O_2^-, persuambly by inducing phosphotyrosine accumulation.	PDGF induces PTK activity but inhibits fMLP induced O_2^- formation, but not TPA. IgG stimulates PTK, but erbstein, genstein did not inhibit IgG induce O_2^-. Tyrophostin-23, a specific PTK inhibitor, inhitibed zymosan induced tyrosine phosphorylation but not O_2^- production.	Kraft & Berkow (1987); Boutin *et al* (1980); Gomez-Cambronero *et al* (1989) Nasmith *et al* (1989); Grinstein *et al* (1989); Grinstein *et al* (1990); Grinstein and Furuya (1991); Trudel *et al* (1991); Dryden *et al* (1992); Kusunoki *et al* (1992); Wilson *et al* (1987); Mitsuyama *et al* (1993a); Sanguedolce *et al* (1993); Manakami *et al* (1993); Torres *et al* (1993); Yuo *et al* (1993)

Putative Pathway	Cells Studied	Evidences For	Evidence Against	Refs
Cyclic Nucleotides (including cAMP and cGMP dependent protein kinases)	Neutrophils	Phagocytes contain cAMP and cGMP dependent protein kinases. Unlike previous mechanisms, cAMP and cGMP are inhibitory signals. Cell permeant analogues Bt2-cAMP and Bt2-cGMP play inhibitory role in fMLP induced O_2^-. Theophylline, forskolin which elevate cAMP, inhibit O_2^- generation cAMP inhibited O_2^- generation by TPA, GTP γ-s, but this is reversed by PKA inhibitor, H-89. PKA inhibits respiratory burst at down stream site of PKC. PKA and PKC cross talk down regulates O_2^- via induction of 78kDa phosphatase. KT5720, PKA inhibitor, reversed inhibition of O_2^- induced by cAMP. Rolipram, cAMP phosphodiesterase inhbitor, inhibits O_2^- formation induced by zymosan.	fMLP induces transient increase in cAMP. fMLP enhances cAMP levels induced by phosphodiesterase inhibitors cGMP plays a stimulatory role in C5a induced O_2^-	Elliot *et al*, (1986); Cronstein *et al*, (1988); Bjornson *et al*, (1989); Schudt *et al* (1991); Wenzel-Seifert *et al* (1991); Ervens *et al*, (1991); Tyagi *et al* (1991); Cronstein *et al* (1992); Simpson *et al* (1992); Joseph *et al* (1992); Mitsuyama *et al* (1993c); Savitha and Salimath (1993).
Proteases	Neutrophils and Macrophages	Neutrophils contain chymotrypsin-like protease and neutral proteases. Exogeneous cathepsin G, chymotrypsin, trypsin, papain and elastase, all potentiate fMLP O_2^- formation. Anti-chymotrypsin inhibits O_2^- generation induced by TPA.	PMSF & TPCK inhibit fMLP induced O_2^- TPCK binds to 15kDa protein prior to respiratory burst. Protease binding α_2-macroglobulin, a glycoprotein inhibits O_2^- formation	Painter *et al*, (1988); Kusner and King (1989); Speer *et al* (1981); Basci & Shah (1987); Duque *et al* (1983); Conseiller *et al* (1992); Sottrup and Jenson, (1989); Kilpatric *et al* (1992)

Putative Pathway	Cells Studied	Evidences For	Evidence Against	Refs
Cytoskeletons	Neutrophils	Cytochalasin B cause alteration in cell morphology and inhibit glucose transport. CB bind to actin filament and inhibit polymerization. CB potentiate fMLP induced O_2^- formation. Botulinum C-2 toxin inhibit actin polymerization by ADP-ribosylating G-actin. BC2 toxin potentiate fMLP, Con-A and PAF induced O_2^- formation.	CB inhibit respiratory burst induced by digitonin, substance P, and opsonized zymosan	Korn (1982); Flanagan and Lin (1990); Al-Mohanna *et al* (1987); Aktories *et al* (1986, 1987); Norgauer *et al* (1988).

Heyneman and Vercauteren (1984) and Bromberg and Pick (1984). The former reported that oleic or linoleic acid activate O_2^- formation in post-nuclear fractions of horse neutrophils. Bromberg and Pick (1984) obtained similar result but with guinea pig neutrophils. Subsequently analogous reconstitution systems have been established for human neutrophils (Curnutte, 1985; McPhail *et al*, 1985; Seifert *et al*, 1986), human monocytes (Thelen and Baggiolini, 1990), differentiated HL-60 cells (Seifert and Schultz, 1987b), bovine neutrophils (Ligeti *et al*, 1988; Doussiere *et al*, 1988), pig neutrophils (Fujita *et al*, 1987). In general, fatty acids, and/or SDS, membranes, and cytosolic activating factors of phagocytes are all required to reconstitute O_2^- formation, and omission of one of these components abolishes enzyme activity.

1.4.4.1 Activation by Fatty Acids and SDS

Arachidonic acid, other *cis*- unsaturated fatty acids, *trans*-unsaturated fatty acids, and the detergent SDS activate NADPH-oxidase in crude membrane preparations and in purified plasma membranes of neutrophils and macrophages from various sources (Bromberg and Pick, 1984; 1985; Curnutte *et al*, 1987a; Seifert and Schultz 1987a, b; Nozawa *et al*, 1988). In membranes of human neutrophils, saturated fatty acids, esters of unsaturated fatty acids and ETYA do not activate the enzyme (Seifert and Schultz 1987a). In addition other detergents such as Triton X-100, Lubrol PX, sodium deoxycholate do not activate O_2^- formation in various systems (Seifert and Schultz, 1987a). Fatty acids and SDS seem to activate NADPH-oxidase due to their anionic amphiphillic character. This assumption is supported by fact that positively charged alkylamines but not neutral amphiphillic alkylalcohols inhibit fatty-acid induced O_2^-

formation in cell-free systems of guinea pig neutrophils and in intact cells (Miyahara *et al*, 1987; 1988). Clark *et al* (1990) showed that activation of NADPH-oxidase by phorbol esters in intact cells and by arachidonic acid in the cell free system was associated not only with the translocation of the p47-phox to plasma membrane but also translocation of p67-phox. Using immunochemical and phosphorylation methods, several authors have reported translocation of p47-phox in response to anionic amphiphile (Ohtsuka *et al*, 1990; Nauseef *et al*, 1990; Tyagi *et al*, 1992). The mechanisms by which anionic amphiphile activate NADPH-oxidase is under major discussions for which reviews are available (Seifert and Schultz, 1990).

1.4.4.2 Cytosolic Activation Factors

Reconstitution of NADPH-oxidase requires membranes anionic amphiphiles and cytosols. Components of membranes were discussed in section 1.4.2.1. Initial studies suggested that this factor or at least one of these factors may be protein kinase C (McPhail *et al* 1984b; 1985), but subsequent studies have provided convincing evidence that PKC is not one of the cytosolic activating factors.

The cytosolic factor seems to be heat labile and sensitive to proteolytic inactivation (Seifer *et al*, 1989; Fujita *et al*, 1987; Ligeti *et al*, 1988; Bolscher *et al*, 1989). The results of functional studies suggest that more than one cytosolic activation factor is involved in the regulation of NADPH-oxidase. The analysis of kinetics of the NADPH-oxidase in a fully soluble system revealed that the enzyme was activated in a three-stage process (Babior *et al*, 1988; Curnutte *et al*, 1989a). According to this

model, in the first step the membrane component of NADPH-oxidase (M) takes up a cytosolic factor(s) to form complex [M.S]. In the second step, this complex was converted into precatalytic species $[M.S]^*$ possibly through the action of anionic amphiphiles. In the third step, this complex takes up two additional cytosolic components, term C_α and C_β. This process results in low-activity (high K_m) NADPH-oxidase ($[M.S]^*C_\alpha$), and subsequently in the formation of high activity (low K_m) NADPH-oxidase ($[M.S]^*$ C_α C_β).

The concept that multiple cytosolic activation factors are involved in the regulation of NADPH-oxidase was also supported by the results of protein purification studies. These early studies revealed an array of cytosolic activation factors, with each group of authors referring to their own nomenclature, resulting in confusing terminology. I will briefly try to compare the identity of these cytosolic activation factors with the cytosolic components which are known today, such as the p47-phox, p67-phox and p21-rac.

Using anion exchange chromatography, Nunoi *et al* (1988) identified three proteins which were operationally termed neutrophil cytosol factor 1 (NCF-1), NCF-2 and NCF-3. All three were required for reconstitution of NADPH-oxidase activity. Autosomal recessive CGD was associated with the more common defect of NCF-1 or with the less common defect NCF-2 but apparently not with defect of NCF-3 (Nunoi *et al*, 1988, Clark *et al*, 1989). By isoelectric focusing, Curnutte *et al*, (1989a) identified four cytosolic component referred to as C1 and C4. Autosomal recessive CDG was associated with a defect in C2 or C4 (Curnutte *et al*, 1989a). Using

carboxymethyl Sepharose, Bolscher *et al*, (1989) isolated two cytosolic components. The component which did not bind to this matrix was referred to as soluble oxidase component (SOCI), and the component binding to the column was termed SOCII. Additionally, this group also isolated SOCIII which seems to participate in GTP [γS] - dependent activation of NADPH-oxidase (Bolscher *et al*, 1990; Ambruso *et al*, 1990). Sha'ag and Pick (1988) and Pick *et al* (1989) discovered two cytosolic activation factors, referred to as sigma-1 and sigma-2. Both were required for reconstitution of O_2^- formation. Sigma-1 was a low molecular mass and sigma-2 was of higher molecular mass. Two components, C_1 and C_2 were isolated by Fujiti *et al* (1987) and Ishida (1989) using gel filtration chromatography. C_1 consisted of at least two sub-components. C_1 alone was not very effective in reconstituting O_2^- formation, but was greatly potentiated by GTP analogue or C_2.

Apparently, the p47-phox as cloned by Volpp *et al* (1989) and Leto *et al* (1991) corresponds to NCF-1 (Nunoi *et al*, 1988), C4 (Curnutte *et al*, 1989a), SOC-II (Bolscher *et al*, 1989) and one of the two C_1 components described by Ishida *et al* (1989). Thus one of the cytosolic activation factors for NADPH-oxidase represents the p47-phox which is defective in most cases of autosomal recessive CGD. The p67-phox (Volpp *et al*, 1988; Leto *et al*, 1991) is apparently identical to NCF-2 (Nunoi *et al*, 1988), C2 (Curnutte *et al*, 1989a) and one of the components of C_1 (Ishida *et al*, 1989). The second cytosolic activation factor represents the p67-phox. Small GTP-binding protein of the *ras* super family p21-rac (Abo *et al*, 1991; Didsbury *et al* 1989) is apparently identical to SOCI, NCF-3, sigma-1, C-1 and C_2. Thus the third cytosolic activation factor represents the small molecular mass GTP binding proteins.

1.4.4.2.1 p47-phox and p67-phox

The first indications that factors other than cytochrome b_{-245} are required for activation was the discovery that the majority of patients with autosomal recessive CGD lack a 47 kDa and 67-kDa cytosolic phosphoproteins, p47-phox and p67-phox as they are now referred to respectively (Segal *et al*, 1985; Erickson *et al*, 1992, Nunoi and Matsuda *et al*, 1992). Patients inherit autosomal recessive CGD due to mutations in genes encoding p47-phox on chromosome 7, and p67-phox on chromosome 1 (Curnutte, 1993).

The phosphorylations by PKC and/or other kinases of p47-phox and p67-phox coincides with respiratory burst (Segal, *et al* 1985; Rotrosen and Leto, 1990; Nath *et al*, 1992; see also Section 1.4.3.1). Phosphorylations occurs at multiple sites in intact cells and cell-free systems (Rotrosen and Leto, 1990; Uhlinger *et al* 1991; Ding and Badwey, 1993; Ding *et al*, 1993). Phosphorylations leads to translocation of both p47-phox and p67-phox of endogenous proteins and recombinant protein in cell-free system (Ohtsuka *et al*, 1990; Uhlinger *et al*, 1992; Tyagi *et al*, 1992). In intact cells, the presence of p47-phox is required for p67-phox translocation, since CGD cells lacking p47-phox the membrane association of p67-phox does not occur (Heyworth *et al*, 1991). Using [^{35}-S]-methionine-labelled p47-phox and p67-phox, Uhlinger *et al* (1993) investigated the association of these components with both normal and CGD derived plasma membranes. They reported that p47-phox translocation occurred independently of p67-phox, but p67-phox translocation required the presence of p47-phox. It seems therefore that p47-phox forms the initial complex with the cytochrome

and is required for assembly of other components.

The cloning and sequence of the genes encoding both p47-phox and p67-phox have been described (Volpp *et al*, 1989; Leto *et al*, 1991; Lomax *et al*, 1989). The cDNA for p47-phox codes for a 41.4 to 41.9 kDa protein with a calculated pI of 10.4. The protein possesses an arginine- and serine-rich COOH-terminal domain with putative phosphorylation sites for protein kinases and an N-terminal glycine. The cDNA for p67-phox encodes a protein with 526 amino acid sequence and possesses acidic middle and COOH-terminal regions (Leto *et al*, 1990). Recombinant fusion proteins encoded by cDNAs have been used to demonstrate restoration of superoxide generation activity in phagocyte cytosols of CGD patients (Volpp *et al*, 1989; Lomax *et al*, 1989; Leto *et al*, 1990). Both of these proteins contained two SH3 domains (SH, *src* homology region), which are present in several signalling proteins and are likely to be involved in interaction of proteins, possibly small G-proteins, although the precise functional role of these domains are yet to be established.

Epstein Barr Virus (EBV) transforms lymphocyts to generate O_2^- in response to agonists, similar to phagocytes (Volpp and Lin, 1993). p47-phox cDNA clone inserted into EBV-expression vector and transfected to β-lymphocytes from CGD patients deficient in p47-phox, generated normal levels of O_2^- *in-vitro*, and had detectable cytosolic p47-phox (Cobbs *et al*, 1992; Volpp and Lin, 1993; Ruedi *et al*, 1992). These studies are the initial experimental approaches to genetic reconstitution in CGD.

1.4.4.2.2 Small GTP-binding Proteins

Initial development of cell-free assays revealed involvement of small GTP-binding proteins (Bromberg and Pick, 1984). However it was not until some years later that fractionation of cytosols indicated factor(s) other than p47-phox and p67-phox were required to activate respiratory burst. Upon purification, these additional factors resolved into a heterodimeric complex of proteins with molecular weights of 21kDa and 26kDa (Abo and Pick, 1991). These were subsequently identified as the *ras*-related small G-protein, p21 rac1 and *rho* GDI (GDP-dissociation inhibition factor), respectively (Abo *et al*, 1991; Didsbury *et al*, 1989; Fukumoto *et al*, 1990). p21 rac1 was initially purified from guinea pig macrophages and, similar results were obtained from human neutrophils, in which the GTP-binding protein was identified as p21 rac2. (Knaus *et al*, 1991; 1992). These two proteins, which share 92% amino acid sequence homology, probably play similar roles in these different cells (Dorseuil *et al*, 1992).

Small G-proteins have interconvertible GDP-bound inactive and GTP-bound active forms (for review see Bokoch and Der, 1993; Bokoch, 1993; Takai *et al*, 1991) which is regulated by GDP/GTP exchange proteins (GEP). Other GEP's namely the *smg* GDS (GDP dissociation stimulator) have been reported to stimulate NADPH-oxidase (Mizuno *et al*, 1992) and interesting *rho*-GDI was shown to inhibit NADPH-oxidase (Mizuno *et al*, 1992). However, Kwong *et al* (1993) found *rho*-GDI to be non-essential for cell-free oxidase reconstitution. Pure recombinant p21 rac's augment superoxide productions in cell-free system (Knaus *et al*, 1992; Kwong *et al*, 1993; Abo *et al*, 1992), but only after exchange of the nucleotide into GTP form. p21 rac's have a unique C-terminal amino acid sequence of Cys- A-A-Leu (A=aliphatic amino acid) (Ando *et al*, 1992) which can undergo post-translation processing such as

prenylation, proteolysis and carboxyl methylation. Post-translation processing may be important for activity of NADPH-oxidase (Ando *et al*, 1992) possibly by aiding nucleotide exchange (Heyworth *et al*, 1993).

Activation of the oxidase, both in intact cell and the cell-free assay system, is associated with translocation of Rac, unaccompanied by GDI, to the membranes (Sawai *et al*, 1993). The stimulus induces dissociation of these two molecules, possibly by phosphorylation of the components or separation by lipids such as arachidonic acid, might be the switch that initiates activation of oxidase. The current theory favours the formation of an activation complex involving Rac, p47-phox and p67-phox. This complex docks with the flavocytochrome inducing a conformational change favourable to electron transfer (for review see Segal and Abo, 1993). However, the mechanisms responsible for the formation of this complex and the way in which it induces electron transport remain to be established.

CHAPTER 2 Materials and Methods

2.1 Materials.

Throughout this project the following materials were used, from the supplier listed.

2.1.1 From Sigma.

β-mercaptoethanol
sn-1,2 Dioleolglycerol ($DiC_{18:1}$, DAG)
8-methyl-N-vanillyl-6-noneamide (Capsaicin)
12-O-tetradecanoylphorbol-13-acetate (TPA)
Adenosine Triphosphate (ATP)
Ammonium persulphate
Benzamidine
Bovine serum albumin (BSA)
Brillant blue G (Coomassie blue)
Bromophenol blue
Calcium ionophore (A23187)
Cytochrome C
Deoxycholic acid
Dipotassium hydrogen orthophosphate (K_2HPO_4)
Disodium hydrogen orthophosphate (Na_2HPO_4)
Dithiothretiol (DTT)
Ethylene diamine tetra-acetic acid (EDTA)
Ethyleneglycol bis (β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA)
Glycerol
Glucose
Histone IIIs
Histopaque 1077/1119
Leupeptin hemisulphate
Lubrol Type PX
N-Formyl-Met-Leu-Phe (fMLP)
Molecular weight markers (Mr 29, 45, 66, 97.4, 116, 205 KDa)
Nicotinamide adenine dinucleotide phosphate reduced form (NADPH)
Pepstatin A
Percoll
Platelet actvating factor (PAF)
Phenylmethylsulphonylfluoride (PMSF)
Piperazine-N,N'-bis[2-ethanesulphonic acid] (PIPES)
Phosphate buffered saline (PBS)
Plumbagin
Potassium dihydrogen orthophosphate (KH_2PO_4)
Silver protein kit
Sodium dihydrogen orthophosphate (NaH_2PO_4)
Starch
Substance P
Superoxide dismutase (SOD)
Total protein kit

Trichloroacetic acid (TCA)
Trisma base (Tris)
Trypan blue
Zymosan

2.1.2 From BDH.

Acetic acid
Acetone
Butanol
Calcium chloride ($CaCl_2$)
Ethanol
Glycine
Hydrochloric acid (HCl)
Isopropanol
Magnesium chloride ($MgCl_2$)
N-2-hydroxyethylpiperazine-N-ethanesulphonic acid (HEPES)
Orthophosphoric acid
Potassium hydroxide (KOH)
Scintillant cocktail T
Sodium azide (NaN_3)
Sodium hydroxide (NaOH)
Sucrose
Sulphuric acid (H_2SO_4)
Trisodium citrate
Triton X-100
Tween-20 (Tween)
Water, AnalaR molecular biological grade.

2.1.3 From Whatman.

0.2µm filters
Diethylamine cellulose (DEAE, DE52)
Glass microfibre filters (2.5cm GF/C)
Ion exchange chromatography paper.

2.1.4 From LKB Pharmacia

Acrylamide
Ammonium persulphate
Low molecular weight markers (Mr 14.4, 20, 30, 43, 66, 94 KDa)
N-N-methylene bis acrylamide
N,N,N',N'-tetramethylenediamine (TEMED)
Riboflabin-5'-phosphate
Sephacryl S-300

Sodium dodecyl sulphate
Superose 12 prepacked column.

2.1.5 From Bio-Rad.

Hydroxyapatite (including DNA grade)
N,N,N',N'-tetramethylenediamine (TEMED).

2.1.6 From Gibco.

Foetal calf serum (FCS)
Gentamycin
L-Glutamine
RPMI 1640 medium.

2.1.7 From Boehringer-Mannheim.

Acrylamide solutions
(contains 30% acrylamide and 0.8% N,N methylene bisacrylamide)

2.1.8 From Amersham.

Adenosine 5-[$^{\gamma}P^{32}$]-triphosphate (^{32}P-γ-ATP at 5000 Ci/mmol)
Anti-mouse IgG peroxidaes conjugated antibodies
Hybond C-extra nitrocellulose (0.45μm diameter)
Peroxideas conjugated swine IgG to rabbit IgG.

2.1.9 From ICN Biomedicals.

Adenosine-5-[γ-P^{32}]-Triphosphate triethlammonium salt (^{32}P-γ-ATP at 4500 Ci/mmol)
Tissue culture flasks.

2.1.10 From Lipid Products.

L-α-phosphatidyl serine (PS).

2.1.11 From NEN Research Products.

[^{3}H]- phorbol-12,13-dibutyrate [20-^{3}H(N), PDBu at 18Ci/mmol)
[^{3}H]-Sodium borohydride ($NaBH_4$ at 0.6Ci/mmol).

2.1.12 Phorbol Esters.

The following phorbol esters were isolated from natural sources in the laboratory of Professor F.J. Evans, Department of Pharmacognosy, The School of Pharmacy.

9, 13, 14-orthobenzoyl-6, 7,-epoxy, 5-hydroxy-resiniferonol-12 cinnamate
(Thymeleatoxin A, TxA)

9,13,14-orthophenylacetyl-resiniferonol-20-[3-methoxy-4 hydroxy]-phenylacetate
(Resiniferratoxin, Rx)

12-deoxyphorbol-13-phenylacetate (DOPP)

12-deoxyphorbol-13-phenylacetate-20-acetate (DOPPA)

12-O-[2-methylaminobenzoyl]-4-deoxyphorbol-13-acetate (Sapintoxin A, SAP A).

^{3}H-Rx was chemically synthesized using [^{3}H]-$NaBH_4$. All phorbol esters were stored at -20°C under nitrogen; stock solutions and dilutions were made up in acetone and similarly stored.

2.2 Apparatus.

2.2.1 Chromatographic.

Protein chromatography was conducted on a Pharmacia Fast Protein Liquid Chromatography (FPLC) LCC 500plus System, with a Frac-100 fraction

collector.

2.2.2 Centrifuges.

The following centrifuges were used:

i) small	-	MSE bench top microfuge.
ii) Chilled spin	-	MSE bench top chillspin 2 centrifuge.
iii) High speed	-	MSE High speed 18 centrifuge.
		Beckman L8-55 Ultracentrifuge.

2.2.3 Cell Work.

Sterile cabinet - Slee,London.
CO_2 cabinet - Leec MKII

A haemocytometer (Weber Scientific International Ltd.) was used for cell counting.

2.2.4 PKC Assay Harvesting.

Two methods were employed:

i) The Millipore manifold with glass fibre filters were used.

ii) Titertek semi-automatic cell harvester (ICN Flow) was used by conducting the assay in microtitre wells.

2.2.5 Scintillation Counting.

^{32}P samples were counted on a Beckman LS 6000TA liquid scintillation counter by Cherenkov counting. ^{3}H were also counted on same apparatus using scintillant cocktail T.

2.2.6 Spectrophotometer.

Spectrophotometric assay were investigated using a Pye Unicam SP8-100 Ultraviolet Dual Beam Spectrophotometer.

2.2.7 Electrophoresis.

Two electrophoretic equipment were used:

i) SDS-PAGE on LKB-Bromma 2197

ii) SDS-PAGE on Bio-rad Protein II mini protean.

For western transfer a Alto electrophoresis western transfer apparatus was used (Genetic Research Instrumentation Ltd.).

2.2.8 Miscellaneous.

pH meter	-	Pye Unicam PW9418
Ice maker	-	Scotsman AF10
Vortex	-	Rotamixer
Bath sonicator	-	Type 6442AE Ultrasonics Ltd.
Bag sealer	-	Calor
Bench shaker	-	Rotatest R100 Shaker.

2.3 Methods and Buffers.

The reagents were used to make the following buffers and solutions in deionised water (Milli-Q water system, Millipore) or " analaR/hypersolv water " (BDH).

2.3.1 Cell Culture.

RPMI 1640 medium was supplemented with 10% v/v foetal calf serum (FCS), L-glutamine 2mM, and gentamycin 50µg/ml. FCS was heat inactivated (56°C for 30 mins) before additions.

Whole blood was collected in (1:9) citrate supplement of 3.6% trisodium citrate. Phosphate buffered saline (PBS) used for assay of phagocyte superoxide production was supplemented with 4mM glucose, 10mg/ml bovine serum albumin (BSA).

Superoxide production was monitored spectrophotometrically by measuring the superoxide dismutase (SOD) inhibitable reduction of cytochrome C. The assay contained the following:

100µl Cytochrome C (80µM, final conc.)

100µl SOD (300 U) or PBS

10µl Phorbol/agonist

40µl PBS or Zymosan (1mg/ml, final conc.)

The reaction mixtures were added to monolayer of phagocytes in microtitre wells, and after incubation (2hrs at 37°C) supernatants were recovered, centrifuged and determined for absorbance at 550nm.

2.3.2 Partial Purification of NADPH-oxidase.

a) Cells were collected and lysed by sonication (3 x 10s) in buffer-1.

Buffer-1: 40mM Tris-HCL pH 7.4

10mM EDTA

1mM $MgCl_2$

2mM Na-azide

2mM PMSF, 2mM leupeptin

60mM β-mercaptoethanol

10% sucrose w/v.

b) The homogenate was initially centrifuged at 1100 x g x 10mins to remove nuclei and unbroken cells. Post-nuclear supernatants were layered onto a discontinuous sucrose gradients:

2mls of 10% sucrose in buffer-1 over

4mls of 40% sucrose in buffer-1.

c) This was centrifuged at 100000 x g x hr. Band between 10%/40% sucrose gradients (membrane fraction) was stored at -70°C. The cytosol recovered from 10% sucrose was re-centrifuged at 100000 x g x hr and supernatant stored at -70°C (cytosolic factor).

d) Membranes isolated of phorbol ester stimulated cells were further used solubilized with 0.4% Lubrol PX (v/v) and 0.4% Na deoxycholate (w/v) and both in buffer-2.

Buffer-2: 50Mm Na Phosphate buffer pH 8.0

20% glycerol v/v

5mM EGTA, 1mM $MgCl_2$

2mM Na-azide

2mM PMSF, 2mM leupeptin

1mM DTT and 0.1M NaCl.

2.3.3 Assay of NADPH-oxidase.

2.3.3.1: Simple detection of oxidase from phorbol ester stimulated phagocytes were investigated using the following assay:

100µl enzyme

50µl ± SOD (150 U, dissolved in buffer-2) or buffer-2

150µl Buffer-2

100µl Cytochrome C (80µM) in buffer-2.

All reagents were dissolved in buffer-2. The reaction was started by the additions 100µl NADPH co-factor (100µM, final conc.) and continuously monitored on spectrophometer with change in absorbance measured at 550nm. For studying the effects of phorbol ester (PE) on the oxidase complex, 20µl of PE dissolved in 50% ethanol was added and final volumes were balanced by adjusting the volume of buffer-2.

2.3.3.2: The following method was used for studying the effect of kinases on oxidase.

ATP mix (final conc.) in buffer-2:	+ Calcium ions	- Calcium ions
	100µM ATP	100µM ATP

10mM $MgCl_2$ 10mM $MgCl_2$

5.1mM $CaCl_2$ 5.1mM EGTA

Phorbol ester/micelle mix: PS (final conc. 0.25mg/ml) and PE were dried down under nitrogen and mixed with 0.02% Triton-X 100 in buffer-2 by bath sonication and vortexing.

The assay was prepared by mixing :

50µl membrane fraction, 50µl cytosolic factor

100µl ATP mix buffer

100µl Cytochrome C (80µM final conc.)

50µl ± SOD (300 U)/or buffer

50µl or 100µl Kinase prep.

125µl or 75µl buffer-2

25µl phorbol ester/ micelle mix.

The mixture was incubated at RT for five mins. The reactions are started by the additions of 100µl NADPH co-factor (100µM final conc.) in buffer-2, and continuously monitored on a spectrophotometer with change in absorbance measured at 550nm. All results concerning production of superoxide anion were calculated by using molar extinction coefficient of cytochrome C at $21000M^{-1}cm^{-1}$.

2.3.4 Electrophoresis.

a) Double strength Laemmli denaturing buffer was boiled with sample in 1:1 ratio

for five mins prior to electrophoresis.

Laemmli buffer (double strength): 125 mM Tris-HCL pH 6.8

4% SDS, 2% DTT, 20% Glycerol

10% β-mercaptoethanol

0.0025% Bromophenol blue.

b) 40mls of resolving gels (7.5%) were prepared by mixing the following:

1.5M Tris-HCL (pH 8.8)	-	10mls
Water	-	19.4mls
10% SDS (stock)	-	400µl
30% Acrylamide soln.	-	10mls

Gels were polymerised by adding 20µl TEMED and 200µl 0.1 g/ml ammonium persulphate.

20mls of stacking gels (4%) were prepared by the following method:

0.5M Tris-HCL (pH 6.8)	-	5mls
Water	-	12.2mls
105 SDS (stock)	-	200µl
30% Acrylamide soln.	-	2.6mls

Gels were polymerised by addition of 20µl TEMED and 100µl 0.1 g/ml ammonium persulphate.

The running buffer used was:

25mM Tris-HCL pH 8.3

192mM Glycine

0.1% SDS

2.3.5 Western blotting.

Once gels were casted and samples separated were subjected to immunological analysis against specific PKC isozyme antisera antibodies. Samples were transferred and electroblotted onto nitrocellulose Hybond-C using Western Transfer buffer (WTB):

25mM Tris-HCl

192mM Glycine

20% Methanol.

Non-specific binding sites were bound by Blotto buffer:

10% Marvel (non-fat dried milk) in

PBS, 0.05% Tween-20

0.1% azide

0.05% Triton X-100.

Excess milk solution and other washes were with Washing buffer (WB) :

PBS plus

0.05% Tween-20

0.1% azide

0.05% Triton X-100.

Gel stainings were carried out with coomassie blue stain:

0.3% Brilliant blue G stain

50% Methanol, 1% Acetic acid

and destained with : 10% Acetic Acid, 10% Isopropanol.

2.3.6 Protein kinase separation.

a) Cells and tissues were homogenised in buffer:

Homogenisation buffer: 20mM Tris-HCL pH 7.5

0.25M Sucrose

10mM EGTA, 2mM EDTA

1mM DTT, 60mM β-mercaptoethanol (MSH)

2mM PMSF, 100µg/ml Leupeptin.

DTT, MSH, PMSF and leupeptin were added fresh. The homogenates were centrifuged at 25000 x g x 15min at 4°C.

b) Supernatants were applied to a hydroxyapatite column equilibrated with chromatography buffer-A and connected to FPLC machine. All procedures were carried out at 4°C. All chromatography buffers were filtered through 0.2µm filters and degassed by vacumn.

Chromatography buffer-A: 20mM mono- and dipotassium salts

(19:81 ratio)

10% v/v glycerol

10mM EGTA, 2mM EDTA

60mM β-MSH (added fresh)

Chromatography sodium buffer A was as buffer-A but replacing mono- and divalent potassium salts with sodium salts.

Non-binding and broken protein were eluted with pre-wash in buffer-A. This was monitored using a UV chart recorder connected to FPLC and wash was complete once trace returned to background levels.

c) Protein samples were eluted using various phosphate gradients [20mM (buffer-A) to 500mM (buffer-B)].

Buffer-B : As buffer-A but 500mM phosphate

Sodium buffer B : As sodium buffer B but 500mM phosphate.

d) Eluted fractions were stored in 25% storage buffer;

Storage buffer: Glycerol / 2% Triton - 16.36:1 ratio respectively.

After vortexing samples were snap frozen in liquid nitrogen and stored at -70°C.

2.3.7 Protein kinase assays.

2.3.7.1: Assay for crude preparations using essentially the micellar assay of Hannun and colleagues (Hannun et al, 1985).

Assay buffer: 25mM Tris-HCL pH 7.5

5mM EGTA

Micelle buffer: 2.6% Triton X-100

25mM Tris-HCL pH 7.5

5mM EGTA

Micelle mix : PS (6mg/ml) and phorbol esters are dried down under nitrogen and mixed with micelle buffer by bath sonication and vortexing.

Histone mix : 4mg/ml Histone-IIIs in assay buffer.

ATP mix : 200μM ATP

20mM $MgCl_2$

± 10.4mM $CaCl_2$ or 10mM EGTA

~ 300 000 cpm/assay ^{32}P-γ-ATP

The ^{32}P-γ-ATP quantities vary with the source material and are calculated from specific activity and time elapsed from 100% activity.

The enzyme fraction are typically stored (-70°C):

0.02% Triton X-100

16% Glycerol.

The assay was prepared by mixing:

25μl Micelle mix

50μl Histone mix

25μl Enzyme

The assay was started by adding 100μl ATP mix and after appropriate time was terminated by addition of 1ml 25% TCA to precipitate protein and 100μl 2 mg/ml BSA as a protein carrier.

The precipitate was filtered and counted by Cherenkov method. This micelle assay was conducted with PS from Nutfield Laboratories Ltd., UK, because TLC analysis showed this preparation to be of significantly greater purity than PS from

elsewhere (eg Sigma). Histone preparations also varied significantly in their ability to accept phosphate from batch to batch, so where possible assays were conducted with histone from identical batches per experiment.

2.3.7.2: Assay for pure PKC-isotypes.

Substrate mix	:	100mM HEPES pH 7.5
		25mM $MgCl_2$, ± 1.5mM $CaCl_2$
		2.5 mg/ml Histone III-s or 1mg/ml pseudosubstrate.
Micelle Buffer	:	2mM Tris-HCL pH 7.5
		1% v/v Triton X-100
Micelle mix	:	5 mg/ml PS, (1-1000 ng/ml) Phorbol esters.
ATP mix	:	1mM ATP, ~ 500 000 cpm / assay ^{32}P-γ-ATP

The enzyme are typically stored at -20°C in:

20mM Tris-HCL pH 7.5

2mM EDTA, 50% Glycerol

0.02% Triton X-100, 1mM DTT.

They are diluted with similar buffer except 10% glycerol to give an activity in the range of 1-4 units (nmol/min/ml) of kinase activity, prior to use.

The assay was started by adding 10µl ATP mix, terminated by spotting 25µl

aliquot onto ion exchange paper and washing three times in 30% acetic acid for 10 mins.

The washed papers were counted.

Further techniques used are described in later chapters.

2.3.8 Total Protein Determination.

Total protein was determined by Lowry procedure using a Sigma Diagnostics protein assay kit (No P5656). Briefly, a calibration curve was prepared using BSA (0-400 ug/ml). 1ml of Lowry Reagent solution was added to blank, standards and samples and incubated for 20min at RT. Then 0.5ml Folin & Ciocalteu's Phenol Reagent was added to each tube and, incubated for a further 30mins. Absorbance at 750nM was measured of the standards and samples against the blank.

CHAPTER 3 Stimulation of superoxide anion from Murine Macrophages by Phorbol ester

3.1 Introduction

Appropriate soluble or particulate stimuli induce a number of responses in phagocytes (eg macrophages) which includes the respiratory burst. The immediate product is superoxide anion (O^-_2) whereas subsequent oxygen derivatives are hydrogen peroxide, hydroxyl radical and singlet oxygen (Babior, 1984). These molecules constitute key components in the antimicrobial mechanisms of phagocytes (Babior, 1978).

There is accumulating evidence that suggests a role for active states of oxygen and free radicals in the mechanism of tumour promotion in mouse skin. (Goldstein *et al*, 1983; Cerutti, 1985; Lewis and Adams, 1988). This hypothesis, partially based on studies which demonstrated an inhibition of promotion by antioxidants (Slaga *et al*, 1983), was supported by work which showed a correlation between promoting activity of tumour promoters and their ability to stimulate O^-_2 production by polymorphonuclear leukocytes *in-vitro* (Goldstein *et al*, 1981). Other supporting evidence includes the findings that free radical generating compounds like benzoyl peroxide are tumour promoters (Slaga *et al*, 1981) and that tumour promotion can be inhibited by superoxide dismutase (SOD) mimetic compounds (Kensler, *et al* 1983).

In studies of the production of oxygen radicals by inflammatory cells, i.p. injection of certain tumour promoters into mice stimulated the generation of O^-_2 by peritoneal

exudate cells (Czernieki *et al*, 1986; Witz and Czernieki 1989). The production of $O^{\cdot}_2$ closely correlates with tumour promoting experiments in mouse skin (Czernieki *et al*, 1986).

The tumour promoting phorbol esters such as TPA have been shown to stimulate the production of oxygen radicals by neutrophils (Goldstein *et al*, 1981) and by macrophages following *in-vitro* treatment (Bryant *et al*,1982; Weinberg and Misunkonis, 1983).

In order to study the production of oxygen radicals by macrophages, the present work assessed the ability of known tumour promoters and non-promoters to stimulate the production of $O^{\cdot}_2$. Non-promoting but irritant phorbol esters used were SAP-A and Rx. Irritancy is a common toxic feature of *trans* A-B ring diterpene esters (Evans and Edwards, 1987) but does not correlate directly with *in-vitro* activation of protein kinase C (PKC; Ellis *et al*, 1987). Rx was demonstrated to be more potent than TPA in activating a Ca^{2+}- inhibited kinase activity (Rx-kinase) (Ryves *et al*, 1989). The studies were conducted on elicited macrophages (by i.p. injection of starch 3 days prior to harvesting) and resident macrophages.

3.2 Methods

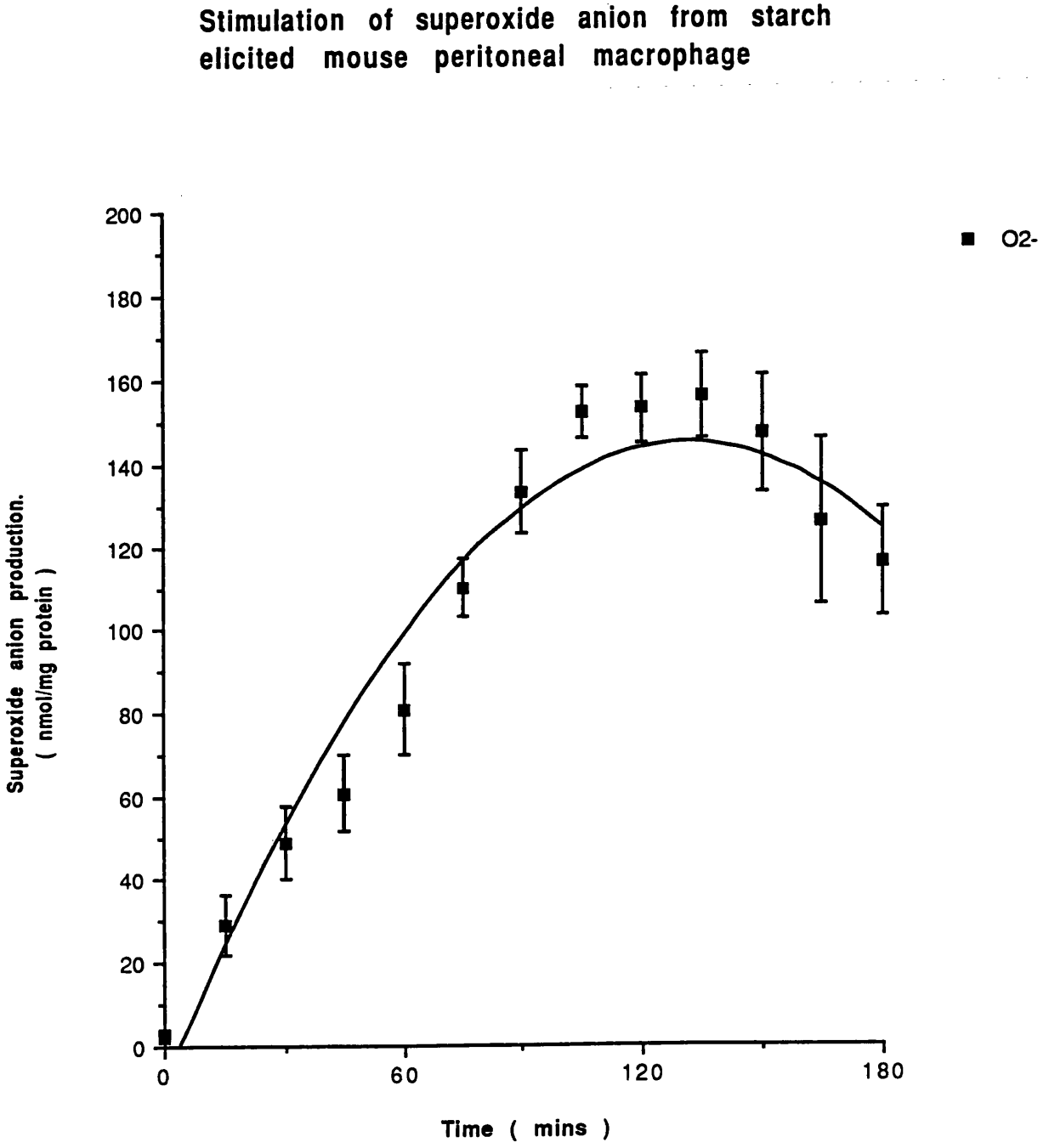


Fig 3.1 Stimulation of superoxide anion from starch-elicited macrophages. O^{-}_{2} production was monitored spectrophotometrically by measuring the SOD (150U) inhibitable reduction of cytochrome C (80μM). TPA (50ngml^{-1}) was used as the standard stimulator. Results are expressed as SEM of triplicates of a representative experiment.

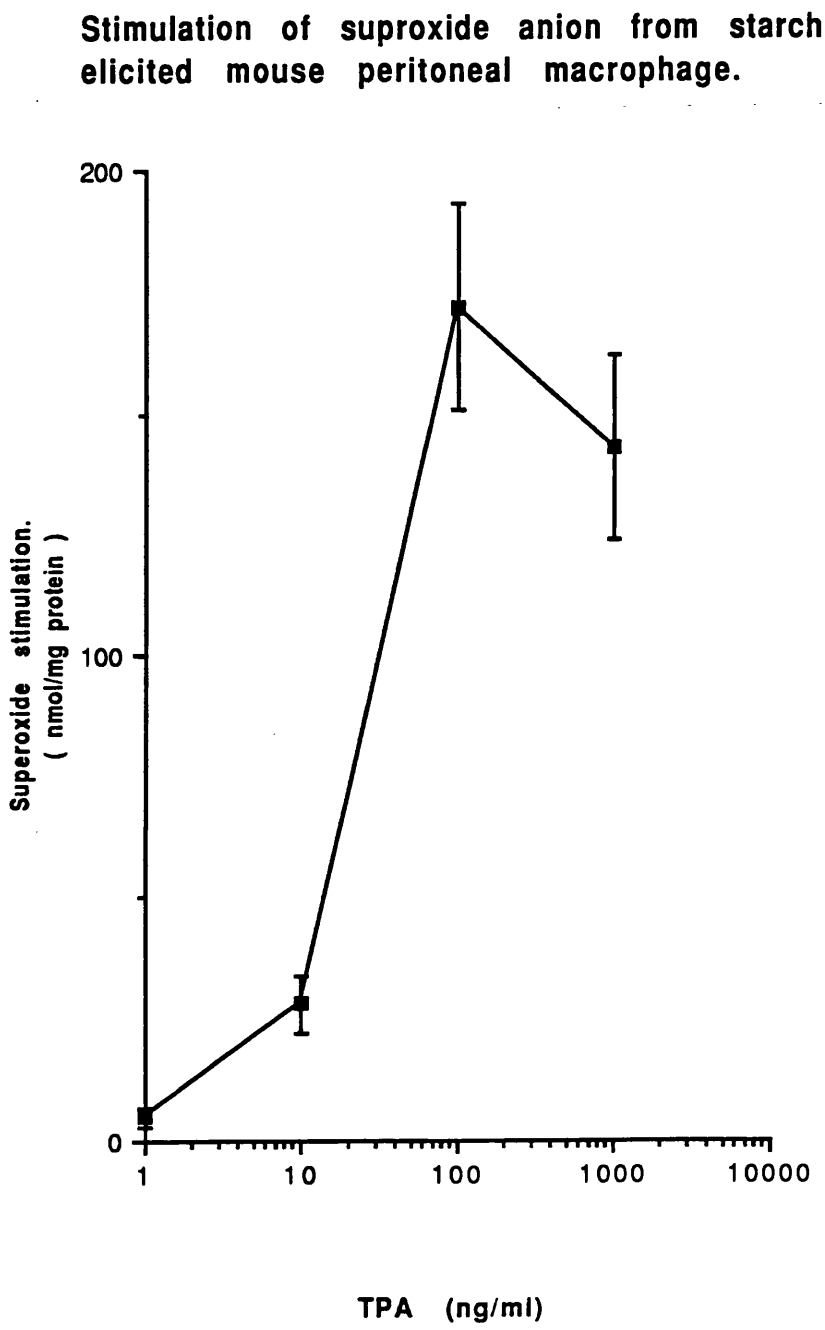


Fig 3.2 Stimulation of superoxide anion from starch-elicited macrophages. O^{-}_{2} production was monitored spectrophotometrically by measuring the SOD (150U) inhibitable reduction of cytochrome C (80μM). TPA was disolved in 50% EtOH and protein contents of cultures were determined using a sigma protein assay kit. Results are expressed as SEM of triplicates of a representative experiment.

3.2.1 Macrophage cultures.

Macrophages were obtained from male CD-1 mice (20g body weight). Peritoneal cells were obtained by aseptic lavage of the peritoneal cavity with 10mls of culture medium (RPMI 1640 supplemented as indicated in Materials and Methods (see Section 2.3.1)). The mice were either untreated or had been given 2mls of 2% starch solution i.p. three days prior to harvesting cells. Alveolar macrophages were isolated from the lungs of CD-1 mice. The lungs were dissected into thin strips and placed in 10mls of oxygen saturated culture medium and agitated for 30mins. Cells were recovered by filtration.

Both sets of cells were washed by centrifugation (400 x g, x 2) and resuspended in culture medium at a density of 1 x 10^6 viable cells/ml, and aliquots (250µl) dispensed into microtitre wells. After 24hr incubation at 37°C (in humidified 5% CO_2 : 95% air) non adherent cells were aspirated off. The resulting monolayer was 95% macrophages as judged by morphology of the cells.

3.2.2 Assay of superoxide anion production.

Superoxide production was monitored spectrophotometrically by measuring the superoxide dismutase (SOD) inhibitable reduction of cytochrome C (see Chapter 2, section 2.3.1). Phorbol esters added were diluted in 50% ethanol and opsonised zymosan was dissolved in PBS. The protein content of representative cultures were determined using a Sigma Protein Assay Kit. For experiments utilising

Resiniferatoxin-sensitivity of cell cultures, several doses of each agonist were initially examined (data not shown). From this a single point was chosen to give an optimal response from cells.

3.2.3 Hydroxyapatite chromatography.

Starch-elicited peritoneal macrophages and alveolar macrophages were enriched by incubation on a sephadex G25 (30min at RT). Following elution of non-adherent cells with PBS, cell were recovered by washing with ice-cold 5mM EGTA in PBS. Approximately 10^8 cells were suspended in homogenisation buffer (see section 2.3.6 a) and lysed by sonication on ice. After centrifugation at 25000 x g, x 15min, the supernatant was incubated with pre-equilibrisied hydroxyapatite (HPT) with protein kinase separation buffer A (section 2.3.6 b). After gentle swirling HPT was allowed to settle and supernatant aspirated off. Initially 3 washes in buffer A was followed by 3 washes in 50% A/B separation buffers (section 2.3.6 c). Finally 100% buffer B was applied and the supernatant stored at -70°C in 16% :0.02% glycerol:Triton X-100.

3.2.4 Assay of Kinase activity.

Protein kinase activity was determined by measuring the transfer of ^{32}P from [γ-^{32}P]-ATP to histone IIIs as described in section 2.3.7.1 (Chapter 2).

3.3 Results.

3.3.1 Stimulation of O_2^- by phorbol esters from starch elicited macrophages.

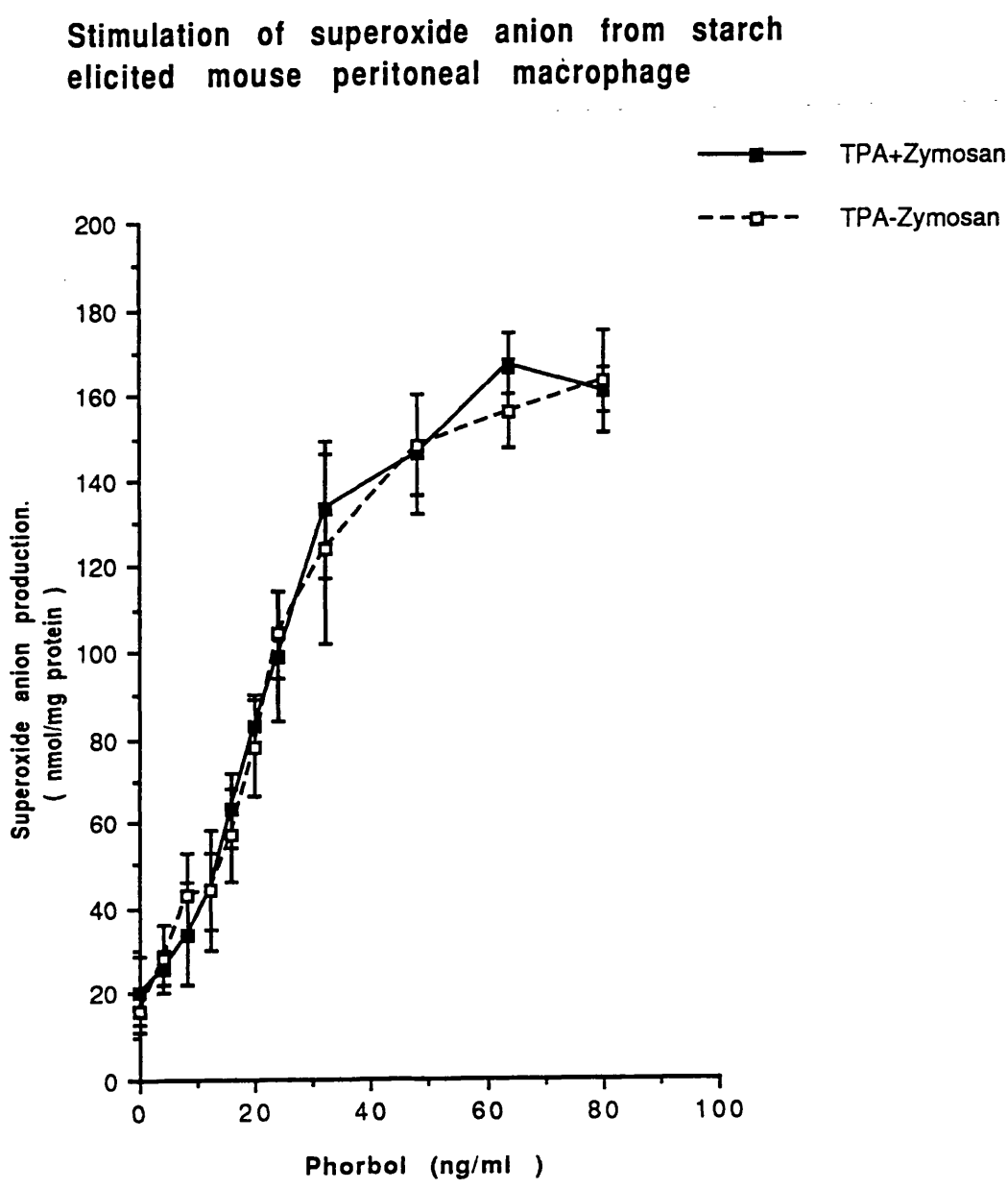


Fig 3.3 TPA stimulation of superoxide anion from starch-elicited macrophages. O^{-}_{2} production was monitored spectrophotometrically by measuring the SOD (150U) inhibitable reduction of cytochrome C (80μM) in the presence and absence of zymosan ($1mgml^{-1}$) . TPA was disolved in 50% EtOH and protein contents of cultures were determined using a sigma protein assay kit. Results are expressed as SEM of triplicates of a representative experiment.

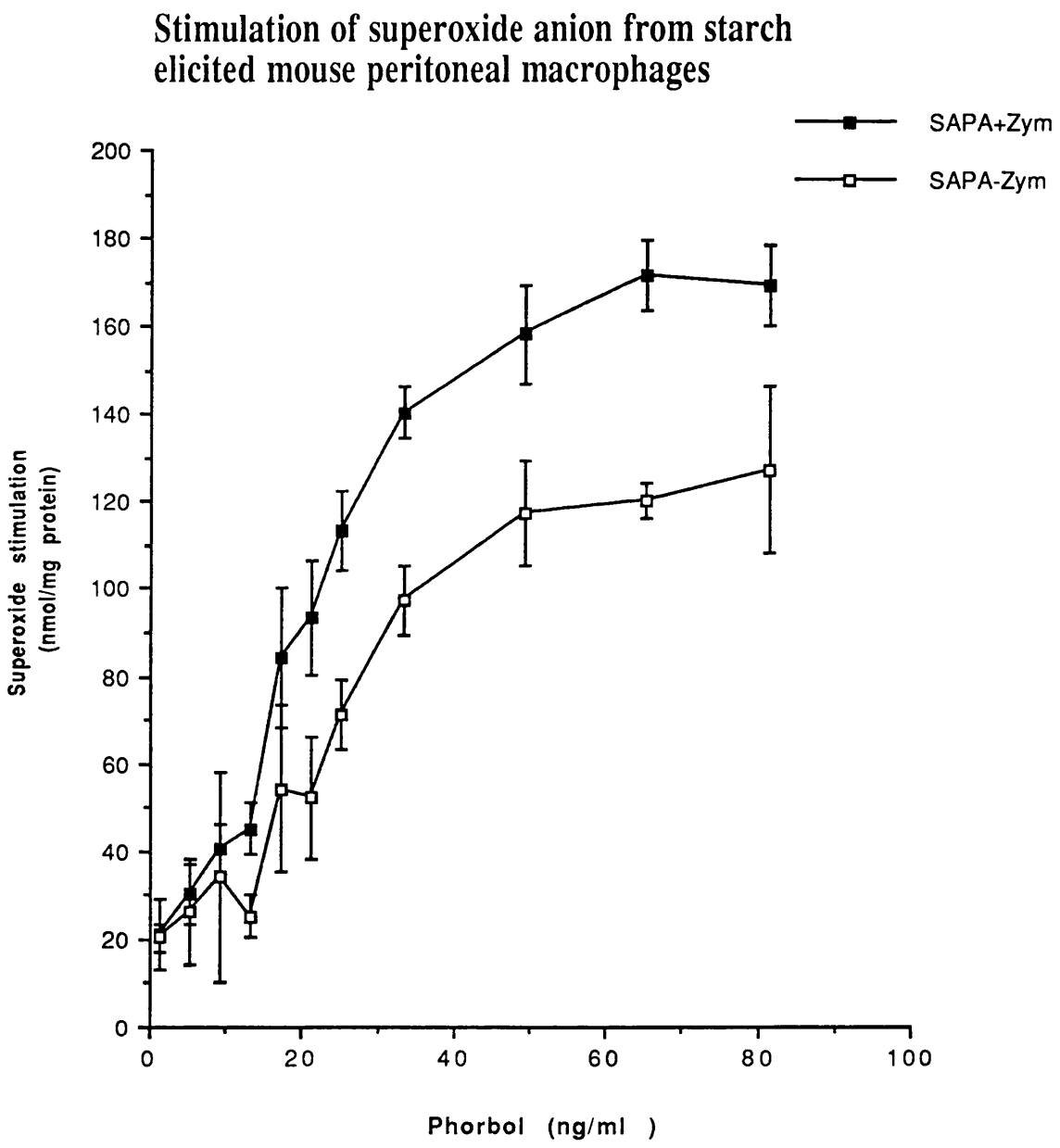


Fig 3.4 SAP-A stimulation of superoxide anion. Results are expressed as SEM of triplicates of a representative experiment.

Stimulation of superoxide anion from starch elicited mouse peritoneal macrophage

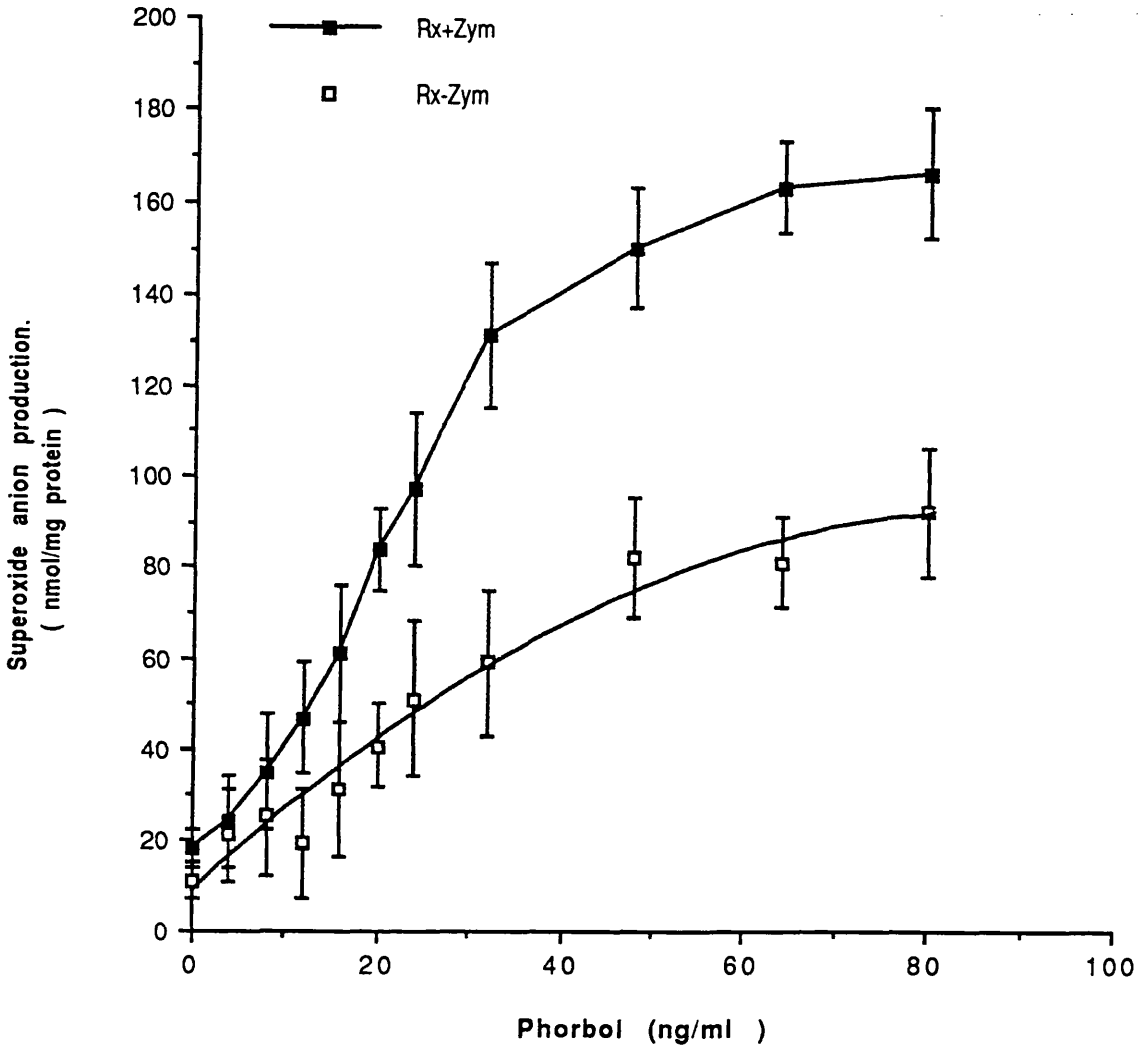


Fig 3.5 Rx stimulation of superoxide anion from starch-elicited macrophages. O^{-}_{2} production was monitored spectrophotometrically by measuring the SOD (150U) inhibitable reduction of cytochrome C (80μM) in the presence and absence of zymosan (1mgml^{-1}) . Rx was disolved in 50% EtOH and protein contents of cultures were determined using a sigma protein assay kit. Results are expressed as SEM of triplicates of a representative experiment.

Time course studies conducted on starch-elicited peritoneal macrophages (SEPM) using TPA showed that O^{-}_2 production plateaued between 1.75 and 2.5 hr (Fig 3.1). TPA was further used under optimal conditions and dose response studies on SEPM revealed maximal stimulation of O^{-}_2 occurred by 100ng/ml concentration (Fig 3.2). As a result of these two studies subsequent experiments utilised an incubation period of 2hr and doses of diterpene esters chosen were in the range of 0-80 ng/ml.

The oxidative burst of SEPM was strongly stimulated by TPA treatment; maximal rate of O^{-}_2 production was 1.3 nmol/min/mg of total protein. Presence and absence of opsonised zymosan minimally affected TPA stimulation of O^{-}_2 (Fig 3.3). However this may be due to assay limits rather than absolute results. SAP-A treatment produced 80% of the magnitude of TPA response (Fig 3.4). However with zymosan co-treatment SAP-A response was synergistically enhanced to TPA levels. Similarly Rx produced approximately 50% of the TPA response and was again synergistically enhanced strongly to TPA levels with zymosan co-treatment (Fig 3.5). Other phorbol esters tested produced weak responses (TxA (Fig 3.6); 4-me TPA (Fig 3.7); Capsaicin (Fig 3.8); DOPPA (Fig 3.9)).

Maximal stimulation of superoxide anion by phorbol esters of SEPM and their EC_{50} values in the presence and absence of zymosan are summarised in Table 3.1. Comparison of EC_{50} shows similar values for TPA and SAP-A with or without zymosan co-treatment. Interestingly there was an approximately 1.5-2 fold increase

in efficacy and potency by Rx with zymosan co-treatment, with EC_{50} lowering from 41 ng/ml to 23 ng/ml. Zymosan alone or with co-treatment produced negligible results suggesting synergistic actions of phorbol esters were real and additive. TxA, DOPPA and 4-me TPA produced weak responses but it could be possible that this may be linked to their relative potencies rather than being inactive at generating O^{-}_{2}. Recent evidence have indicated that DOPPA fails to activate O^{-}_{2} (Merrit *et al*, 1993 ; see Chapter 6).

3.3.2 Stimulation of superoxide anion by phorbol esters from resident macrophages.

Resident peritoneal macrophages (RPM) stimulation of O^{-}_{2} by all phorbol esters were generally lower than SEPM (Table 3.2). TPA produced a maximal response 60% of the magnitude of its response in SEPM. However the presence of zymosan on TPA treatment of RPM increased the total response by 40%. Interestingly efficacy was increased by TPA in the presence zymosan but potency remained approximately similar. SAP-A evoked a weak response in RPM even up to concentration of 80 ng/ml. Higher doses were not investigated and this may have induced a response and therefore requires further investigations. Zymosan co-treatment substantially increased the total response (up to 4-fold higher) produced by SAP-A. Rx also produced a weak response at concentration upto 80 ng/ml; approximately 20% of the magnitude of TPA. Both Rx and SAP-A may have lower efficacies and potencies on resting macrophages but with particulate co-treatment this may be increased. However Rx response was not enhanced by treatment with zymosan.

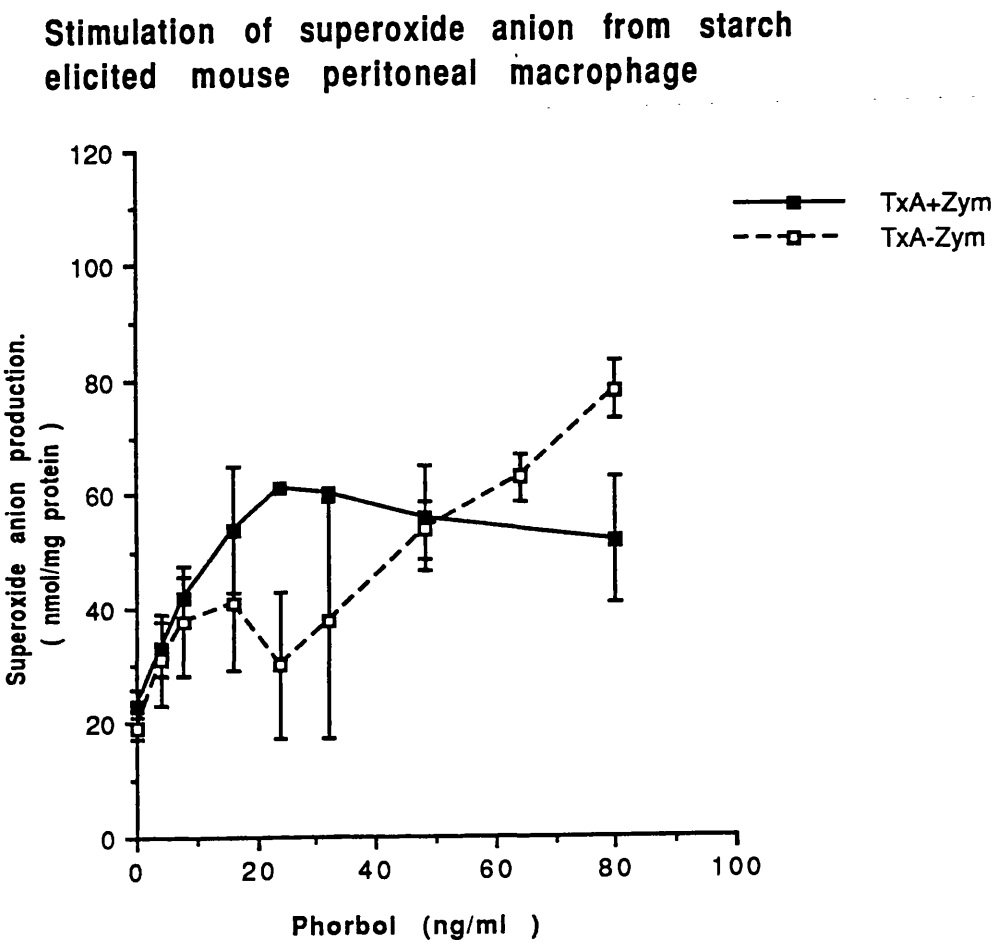


Fig 3.6 TxA stimulation of superoxide anion from starch-elicited macrophages. O^{-}_{2} production was monitored spectrophotometrically by measuring the SOD (150U) inhibitable reduction of cytochrome C (80μM) in the presence and absence of zymosan ($1mgml^{-1}$) . TxA was disolved in 50% EtOH and protein contents of cultures were determined using a sigma protein assay kit. Results are expressed as SEM of triplicates of a representative experiment.

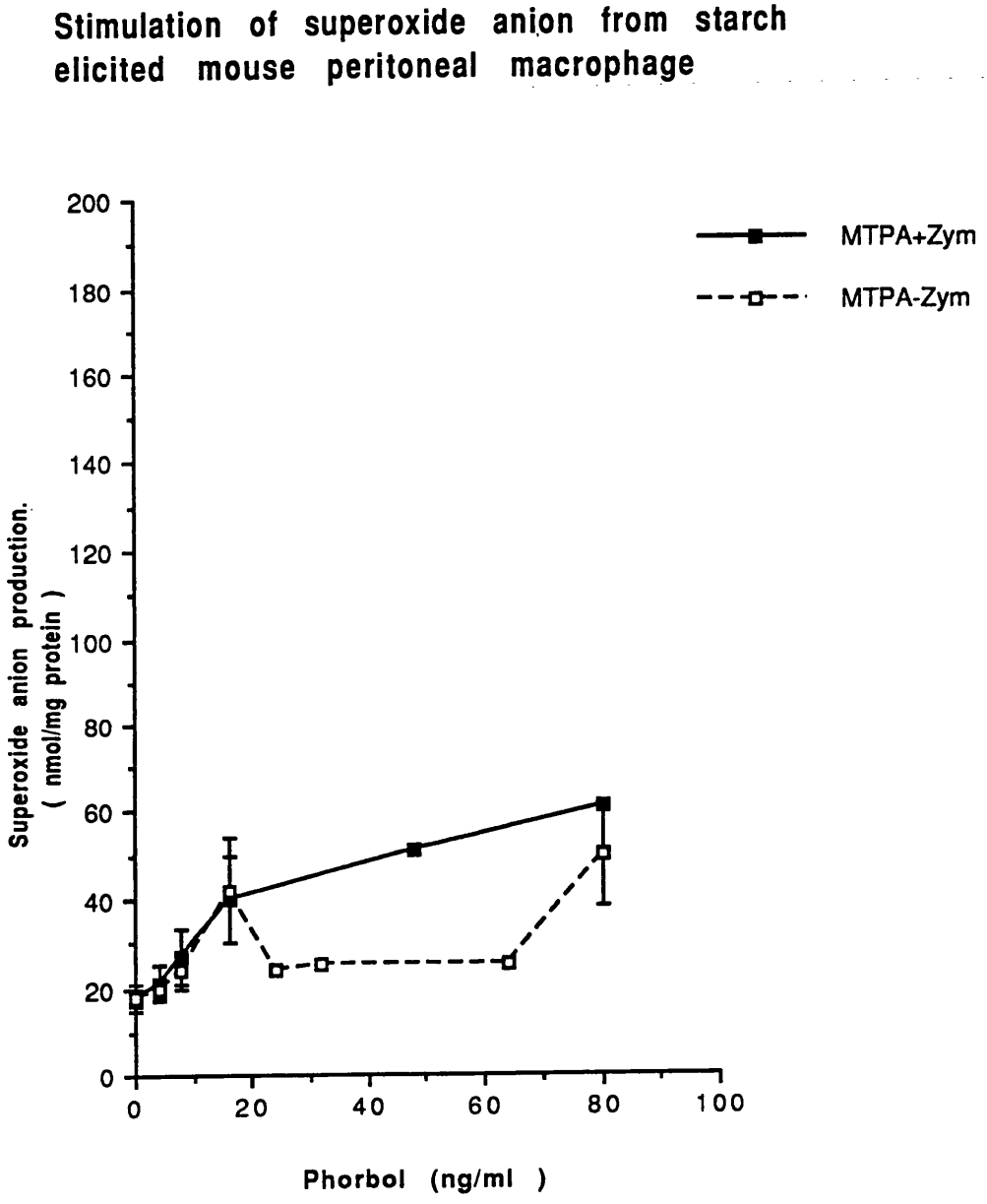


Fig 3.7 4-me TPA stimulation of superoxide anion from starch-elicited macrophages. O^{-}_{2} production was monitored spectrophotometrically by measuring the SOD (150U) inhibitable reduction of cytochrome C (80μM) in the presence and absence of zymosan ($1mgml^{-1}$) . 4-me TPA was disolved in 50% EtOH and protein contents of cultures were determined using a sigma protein assay kit. Results are expressed as SEM of triplicates of a representative experiment.

Stimulation of superoxide anion from starch elicited mouse peritoneal macrophage

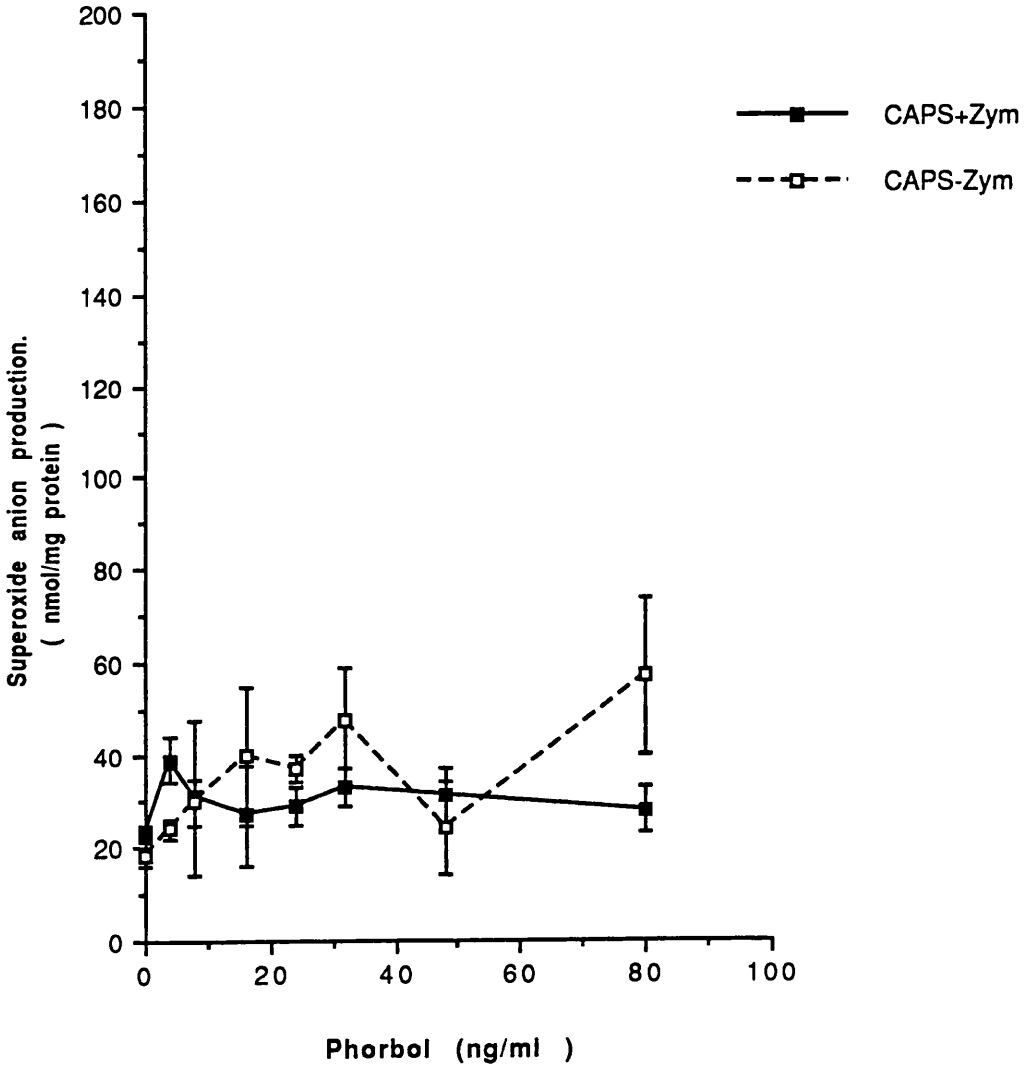


Fig 3.8 CAPS stimulation of superoxide anion from starch-elicited macrophages. O^{-}_{2} production was monitored spectrophotometrically by measuring the SOD (150U) inhibitable reduction of cytochrome C (80μM) in the presence and absence of zymosan ($1mgml^{-1}$) . CAPS was disolved in 50% EtOH and protein contents of cultures were determined using a sigma protein assay kit. Results are expressed as SEM of triplicates of a representative experiment.

Doppa stimulation of superoxide anion from starch elicited mouse peritoneal macrophages

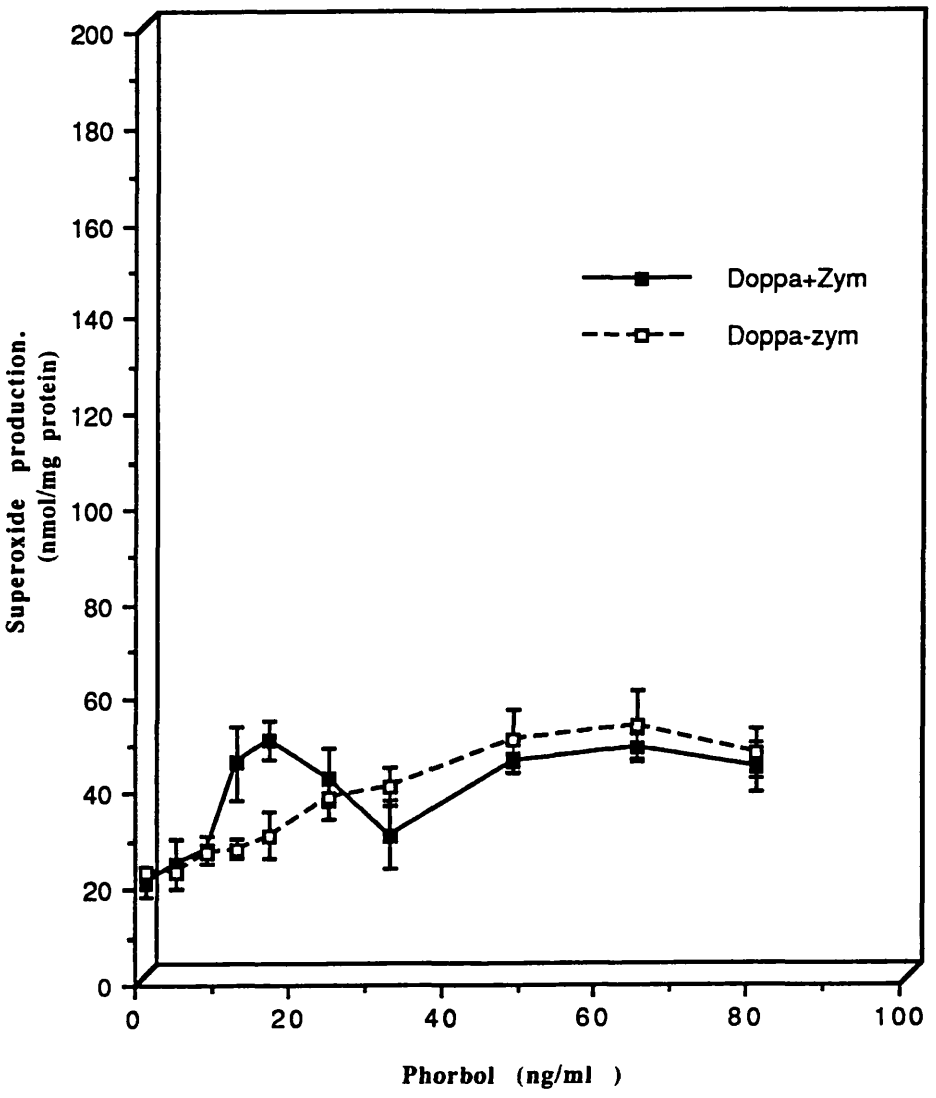


Fig 3.9 DOPPA stimulation of superoxide anion from starch-elicited macrophages. $O^{\cdot}_2$ production was monitored spectrophotometrically by measuring the SOD (150U) inhibitable reduction of cytochrome C (80μM) in the presence and absence of zymosan ($1mgml^{-1}$) . DOPPA was disolved in 50% EtOH and protein contents of cultures were determined using a sigma protein assay kit. Results are expressed as SEM of triplicates of a representative experiment.

3.3.3 Induction of Rx-kinase from murine macrophages.

The lack of response by Rx on resident peritoneal macrophages suggested that the response of SEPM may be the result of induction or priming of Rx-kinase. Therefore kinase isozyme profile from the supernatants of SEPM (resident macrophages were not obtained in large numbers for kinase isolation) homogenates was determined following HPT chromatography. Rx stimulated a kinase activity (maximal 73.5±0.75 pmol/min/assay) in the absence of added calcium which was significantly ($p<0.001$) higher than presence of added calcium (Table 3.3).

Rx sensitivity of cells to various putative 'priming' agents was tested on resident cells. RPM and resident alveolar macrophages were cultured with a variety of putative stimuli at pre-determined doses in culture medium. PAF (10ug/ml), CAPS (10ug/ml), 4-me TPA (10ug/ml), A23187 (10ug/ml) and fMLP (1ug/ml) were all found to be incapable of inducing Rx sensitivity of cells (Table 3.4) with regards to superoxide stimulation. However, substance P (1ug/ml), Rx (0.1ug/ml) and plumbagin (PLM, 0.1ug/ml) all induced a significant increase ($p<0.05$) in superoxide release (Table 3.4). When the alveolar cells were examined for Rx-kinase activity these three agents all yielded a larger kinase activity than control or fMLP ($p<0.05$) (Table 3.5).

3.4 Discussion.

Numerous studies have demonstrated that *in-vitro* treatment of macrophage with tumour promoters results in the production of O^{-}_{2} radicals (Okhawa *et al*, 1984; Lewis *et al*, 1986). The results here show that both tumour promoting and non-

Stimulation of superoxide anion, (nmol/mg protein).				
Compounds	Zymosan absent		Zymosan present	
	Max O^{-}_2	EC_{50}	Max O^{-}_2	EC_{50}
Zymosan	12.0±4	-	9±3	-
TPA	155±8	22	168±8	23
SAP-A	139±4	26	169±5	19
Rx	100±8	41	166±4	23
TxA	71±5	>80	52±11	>80
DOPPA	53±6	>80	59±4	>80
4-me-TPA	50±12	>80	51±5	>80

Table 3.1 Maximal stimulation and EC_{50} of superoxide anion by diterpene esters. Starch-elicited murine peritoneal macrophages monolayers were incubated with diterpene esters and superoxide anion production measured spectrophotometrically by SOD inhibitable reduction of cytrochrome C. Results are expressed as mean ±SEM (n=3)

promoting phorbols share the capability to stimulate O^-_2 from murine peritoneal macrophages. This suggests that correlations between O^-_2 and tumour promoters have to be treated with caution. Complete tumour promoter TPA was a strong stimulator of O^-_2 from peritoneal macrophages. Non-promoting but irritants SAP-A and Rx both stimulated superoxide anion alone and displayed synergistic interaction with zymosan resulting in maximal O^-_2 release from SEPM. However a non-promoting irritant DOPPA, was weakly active. Compounds which are partial tumour promoters but weak irritants (TxA and, 4-me TPA) were also weakly active.The results seem to agree with the data obtained from an experiment using tumour promoters to stimulate O^-_2 from peritoneal exudate cells following *in-vivo* administration (Witz and Czerniecki, 1989). They reported 4-me TPA to be 50 times less potent than TPA and, the results obtained here show that 4-me TPA was substantially weaker than TPA.

It is evident that SAP-A and Rx fail or weakly stimulate RPM, but SAP-A does produce significant response with zymosan co-treatment. Since resident macrophages clearly possess a functional superoxide generating system, the defective response could be related to some event(s) involved in the transductional pathway. It is well known that phorbol esters directly bind and activate PKC (Nishizuka, 1988). The stimulation by TPA of the oxidative burst of PMN's and macrophages results in a rapid and transient translocation of cytosolic PKC to the particulate fraction (Wolfsen *et al*, 1985; Myers *et al* , 1985). Thus PKC seems to be central component of the superoxide activation (Cox *et al*, 1985) acting probably through phosphorylation. Since it has been established that activation of PKC by SAP-A has a high Ca^{2+}

Conc. (ng/ml)	Stimulation of superoxide anion. (nmol/mg protein).		
	TPA + Zymosan	SAP-A + Zymosan	Rx + Zymosan
0	20.26±8.18	13.17±10.12	12.97±4.0
4	22.37±13.19	18.65±5.23	35.21±17.34
12	29.76±3.10	39.29±4.31	22.15±4.27
16	44.14±11.46	48.14±4.13	18.45±7.09
20	59.29±12.89	61.27±11.27	23.43±12.32
24	69.09±4.65	74.36±4.87	29.37±8.93
32	83.16±15.43	76.29±8.56	22.49±11.62
48	103.23±18.58	83.19±15.65	25.98±16.58
64	107.34±18.05	73.43±7.24	11.19±10.71
80	111.76±13.24	83.21±22.43	30.32±7.23

Conc. (ng/ml)	Stimulation of superoxide anion. (nmol/mg protein).		
	TPA - Zymosan	SAP-A - Zymosan	Rx - Zymosan
0	16.21±9.23	10.31±7.24	6.05±5.02
4	12.67±7.56	14.06±7.69	8.27±7.62
12	34.43±5.87	50.16±16.35	18.68±11.21
16	43.26±15.36	24.99±11.89	23.00±8.35
20	54.39±8.11	20.09±8.31	23.35±18.66
24	62.89±5.23	35.16±14.55	18.73±18.42
32	65.33±23.94	31.75±17.62	26.34±9.24
48	84.18±7.89	21.68±6.09	19.01±11.31
64	105.67±19.56	34.30±3.0	15.45±13.87
80	80.17±17.63	21.84±13.33	18.81±7.63

Table 3.2 Stimulation of superoxide anion by diterpene esters of resident mouse peritoneal macrophages in the presence and absence of zymosan. Diterpene esters were present in appropriate cocentration disolved in 50% EtOH and assay was the standard method described in text. Result are expressed as SEM from triplicates of 3 separate experiments.

requirement (Brooks *et al*, 1989) and Rx activates a distinct Ca^{2+}-inhibited kinase (Ryves, *et al*, 1989) (Rx-kinase), it was apparent that activation by both compounds requires the additional functioning of a separate signalling pathway, stimulated by zymosan, for maximal effectiveness. Interestingly zymosan particles have been reported to stimulate the respiratory burst by mechanisms independent of PKC (Andre *et al*, 1988) either through products of eicosanoid metabolism or by inositol trisphosphate mediated calcium mobilisation. The results also suggest that, since TPA can activate PKC and weakly activate Rx-kinase at low Ca^{2+} levels, the lack of synergy with zymosan may reflect the importance of Ca^{2+} mobilisation in mediating the action of particulate stimuli.

The difference in phorbol ester response of resident compared to elicited macrophages have been reported (Witz and Czernieki, 1989). Several tumour promoting phorbol esters including PDBu did not stimulate O^{-}_{2} production in resident compared to elicited peritoneal cells. Since there were no changes in the number of phorbol ester receptors or the binding affinity of PDBu (Weinberg and Misukonis, 1983) it was suggested that the compound directly interacts with cells but the difference in response could not be explained. The responses by macrophages of various physiological states (ie. resident, elicited) with respect to superoxide anion radical production may be the result of alterations in signal transduction mechanism, but however it may also be changes in the enzyme kinetics of the membrane bound superoxide forming NADPH-oxidase.

The response to TPA treatment of RPM were not enhanced by zymosan co-treatment, and Rx produced a weak response. Conversely TPA and Rx response in SEPM were markedly increased by zymosan co-treatment. These results suggest that in resting macrophages, stimulation of the respiratory burst was mostly dependant on PKC isozyme activation, but in partially activated macrophages, the major portion of the oxidative burst activation could be elicited by resiniferatoxin. To further investigate this possibility, the kinase isozyme profile of SEPM was determined. The stimulation with Rx demonstrated the presence of a phospholipid and Rx-stimulated kinase activity inhibited by the presence of 1mM Ca^{2+}, corresponding to the Rx-kinase previously detected in human mononuclear cells (Ryves *et al*, 1989).

Another interpretation of this complex system was derived from the ability of Rx to stimulate O^{-}_{2} in SEPM but not RPM. It seems that Rx-kinase (possibly responsible for actions of Rx) could be elicited by particulate priming of phagocytes. Additional evidence to point this fact arises from alveolar macrophages which already express this enzymatic activity (see chapter 4) due to being in constant contact with particles (such as dust). To further investigate this possibility the effect of humoral agents on Rx-sensitivity and ultimately enzyme induction was determined. The neuropeptide, substance P, agent capable of inducing oxidative stress (naphthoquinone, eg. Plm) and Rx, induced Rx sensitivity and a dramatic increase in enzyme yield from alveolar

Compound	Ca^{2+}/EGTA	^{32}P incorporation ± SD (pmol/min/assay)
TPA	1mM Ca^{2+} 1mM EGTA	18.9±5.47 12.0±0.26
Rx*	1mM Ca^{2+} 1mM EGTA	19.8±2.66 73.5±0.75
PS (alone)	1mM Ca^{2+} 5mM EGTA	17.3±0.85 8.2±0.26

Table 3.3 Analysis of the most active Rx-kinase containing HPT fraction from starch-elicited murine peritoneal macrophages. Activity is shown as pmol diterpene ester stimulated γ-^{32}P incorporation/min into histone IIIs using micellar assay described in text. Triton micelles contained diterpene esters where appropriate in a ratio of 0.003% and 20% PS (w/w). Results are expressed as SEM of triplicates (n=3) (* $p < 0.001$ on students *t*- test)

Superoxide stimulation (nmol/mg/hr)		
Compound	Alveolar Macrophages	Peritoneal Macrophages
Resting	3.81±1.07	4.16±0.27
Subs P	16.67±4.05*	14.69±3.68*
Rx	12.38±3.86*	10.88±2.14*
fMLP	5.24±1.95	3.77±1.01
4 me-TPA	5.72±1.14	4.28±1.67
PAF	4.76±1.78	3.97±0.95
Plm	18.57±5.26*	15.98±4.11*
CAPS	5.71±1.21	4.81±1.64
A23187	4.29±0.57	4.66±1.77

Table 3.4 Superoxide stimulation of macrophages by resiniferatoxin. Cells were sensitized or 'primed' by pre-treatment with various putative agonists at pre-determined doses. Then additional dose of Rx (50ng/ml) was added to cells and superoxide generation monitored over 24 h. Results are expressed as mean of duplicates ± SD (n=3) (* $p < 0.05$ compared to resting).

Conditions	Stimulated kinase activity [pmol/min/assay (Rx/PS - PS)
Unstimulated	17.56±2.16
Rx	21.37±1.45
Substance P	26.88±3.76*
Plm	30.15±2.33*
fMLP	16.19±1.87

Table 3.5 Rx-kinase yield from pre-stimulated murine alveolar macrophages. 5 x 10^8 cells in oxygen saturated medium were stimulated by various agonist for 1h prior to homogenisation. Unstimulated cells were treated identical except equal volume of buffer was added instead. Results are expressed as mean of duplicates from three separate experiments ± SD (* $p < 0.05$ compared to unstimulated)

macrophages. The experiment was however, limited by the amount of cells and resident macrophages were low in cell numbers. Hence Rx-k yield from RPM was unobtainable. Similar results were obtained from human neutrophils and monocytes (see Chapter 7, section 7.3). These effects provide further evidence that the extreme irritancy of resiniferatoxin are mediated through mechanisms distinct from those of tumour promoting irritants.

Resiniferatoxin is a weak activator of mammalian brain PKC (Ellis *et al*, 1987), yet is a hundred times more potent than TPA at mouse ear erythema test (Evans and Schmidt,1979). However the extreme potency of this compound is not at present explained on the basis of a single target (Rx-kinase), but exhibits features of mixed aetiology (Evans *et al*, 1992). Rx is also known to induce pain response which appears to have a neurogenic basis and it has been proposed that Rx acts as an ultrapotent analogue of the neurotoxin Capsiacin. (Szallasi *et al*, 1989; Szallasi and Blumberg, 1989; De Veries and Blumberg , 1989). Interestingly, in our assay, Capsiacin differed from Rx, not merely in efficacy (maximal response 30% that of Rx at 0.26M) but in that the response was weakly antagonised by zymosan co-treatment. These results suggest that Rx action on neurones may represent a special mechanism which is not applicable to the fuller pharmacological spectrum of this complex molecule.

CHAPTER 4 Purification and Properties of Rx-kinase, A Resiniferatoxin Receptor

4.1 Introduction.

Resinferatoxin (Rx) is a daphnane orthoester isolated from toxic plants of the family Euphorbiaceae (Hergenhahn *et al*, 1975; Schmidt and Evans, 1979). The daphnanes are related in chemical structure to the phorbol esters which are well known as tumour-promoting and irritant toxins (Hecker and Schmidt, 1971). Daphnanes are found in similar plant species and have a range of biological activities in common with phorbol esters (Schmidt, 1987). The best known derivative of the daphnane group is the second stage tumour promoting agent mezerein (Slaga *et al*, 1980).

The Ca^{2+} and phospholipid dependent protein kinase C (PKC) is believed to be the major receptor for phorbol esters (Castagna *et al* , 1982). Molecular cloning and biochemical analysis have revealed that PKC exists as family of multiple subspecies having a closely related structure; initially four cDNA clones α-, β_I-, β_{II}- and γ-PKC were isolated (Parker *et al* , 1986; Ono *et al*, 1986). This has been extended to include Ca^{2+}-independent forms δ-, ε-, ζ-, η- and θ- (Ono *et al*, 1987; 1988; Osada *et al*, 1990; 1992). Therefore it was not surprising that small chemical changes to the phorbol nucleus would lead to partial selectivity for activation of PKC isotypes. (Ryves *et al*, 1991). In a similar manner the daphnane derivatives are capable of PKC activation. For example mezerein was shown to activate mixed preparations of PKC (Miyake *et al*, 1984). TxA has demonstrated partial selectivity for stimulation of certain PKC isotypes and activates as yet unidentified kinase from rat brain tissue (Evans *et*

al, 1991). Significantly, Rx was unable to activate purified PKC isotypes α- , γ-, δ-, and ε- (Ryves *et al*, 1991) except β-PKC at high dose levels. Rx is of interest because unlike mezerein, TxA or TPA it is not a tumour-promoting agent (Adolph *et al*, 1982), but is the most potent pro-inflammatory of the group being 100-fold more potent than TPA on mouse skin (Schmidt and Evans, 1979). The mechanism of action of Rx remains unknown but the toxin is of importance as a probe to study the inflammatory response. Rx has been suggested to act as an ultrapotent analogue of homovallinate derivative Capsaicin and that its irritancy is of a neurogenic component (Szallasi and Blumberg, 1991a). However analysis of potency, inhibition by different antagonists, time course of irritancy and other pharmacological properties of Rx induced inflammation suggests a partial phorbol ester mechanism of action (Evans *et al*, 1992). The recent observation that Rx stimulated a peak of histone-kinase activity from human mononuclear cell, distinct from α- and β-PKC by immunological analysis is of interest in this respect (Ryves *et al*, 1989). The enzyme activity discovered was termed Rx-kinase. In this chapter the isolation and characterisation of Rx-kinase, a Rx receptor protein, is described.

4.2 Methods

4.2.1 Isolation of Human Monocytes and Neutrophils.

Whole blood was collected from healthy donors by venous puncture and mixed in a ratio 9:1 with citrate buffer (3.6% trisodium citrate). Human monocytes and neutrophils were isolated by density centrifugation. Equal volumes of histopaque 1077 was layered over histopaque 1119 and whole blood poured onto upper histopaque 1077 medium. This was centrifuged (700 x g x 30min) and granulocytic cells (>95% neutrophils) were found at the 1077/1119 interface. Neutrophils were recovered by aspiration and washed by centrifugation (400 x g x 10min x 2) and incubated in ice cold supplemented RPMI 1640 (see Chapter 2, section 2.3.1) medium containing 10 ug/ml leupeptin and 1mM PMSF for 30min at 4°C prior to homogenisation. Mononuclear cells were recovered from 1077/plasma interface and as previously, washed by centrifugation (400 x g x10min x 2), cultured for 2hr at 37°C. Non adherent cells were aspirated off to leave monocytes.

4.2.2 Isolation of Murine Alveolar Macrophages and starch elicited Peritoneal Macrophages.

Alveolar macrophages were isolated from the lungs of CD-1 mice (20g body weight). The lungs were dissected into thin strips and placed in 10mls of oxygen saturated supplemented culture medium (see section 2.3.1). The chopped lungs were then agitated for 30min and the cells were recovered by filtration, washed by centrifugation as before, resuspended in medium and cultured for 2hr at 37°C. Non-adherent cells were aspirated off to produce 95% macrophage as judged by cell morphology in

Giemsa smears.

Peritoneal cells were obtained by aseptic lavage of the peritoneal cavity of male CD-1 mice (20g) with 10mls of culture medium. The mice were untreated or had been injected 2mls of 2% (in PBS) starch solution i.p. three days prior to harvesting. After washing by centrifugation the cells were resuspended in PBS. Starch elicited peritoneal macrophages were enriched by incubation on a Sephadex G-25 column for 30min at 30°C. Following elution of non-adherent cells, the macrophages were recovered by washing with ice-cold 5mM EGTA in PBS.

4.2.3 Isolation of rat tissues for distribution studies.

3-5 rats (200g) were killed by cervical dislocation and exanguation. The tissues, brain, thymus, heart, lung, liver and spleen were removed immediately and placed in supplemented RPMI 1640 culture medium. The tissues were washed in 2-3 volumes of culture medium. All the tissues were sliced into thin strips and cultured in equal volumes (50mls) of oxygen saturated medium with or without 0.1 ug/ml plumbagin and agitated for 1hr at 37°C. The tissues were removed by filtration and their wet weight measured prior to homogenisation.

4.2.4 Protein kinase purification

Cells, fresh or cultured were suspended in ice-cold homogenisation buffer (see Chapter 2, section 2.3.6 a) and were lysed by probe sonication (3 x 10s burst) at 4°C to prepare homogenates. Tissues were homogenised by Ultra-turrax tissue homogeniser on ice for 30s. Both sets of preparation were subsequently identically treated. After centrifugation at 25000 x g x 15min at 4°C, the supernatant was loaded into a 10ml superloop and pumped onto HPT column by protein kinase separation chromatography buffer A (see section 2.3.6 b). The column was washed with buffer A to remove unbound proteins and then various gradients of 20mM to 500mM phosphate buffer were used at a flow rate of 1 $ml.min^{-1}$. Fractions of 1ml were collected on ice and immediately mixed with 25% storage buffer (see section 2.3.6 d). The fractions were immediately frozen in liquid nitrogen and stored at -70°C until analysis.

4.2.5 Further purification of Rx-kinase.

Rx-kinase was further purified by utilising gel filtration Superose chromatography. A Superose 12 column was calibrated with protein standards 29 to 205 kDa. 1ml of histone kinase activity collected from hydroxyapatite chromatography was applied to the column. Using 500mM phosphate buffer (protein kinase separation buffer B (see section 2.3.6 c) fractions were eluted at a flow rate of 0.4 $ml.min^{-1}$. Fractions were either assayed immediately or frozen as above before analysis at a later date.

4.2.6 Assay of Kinase activity

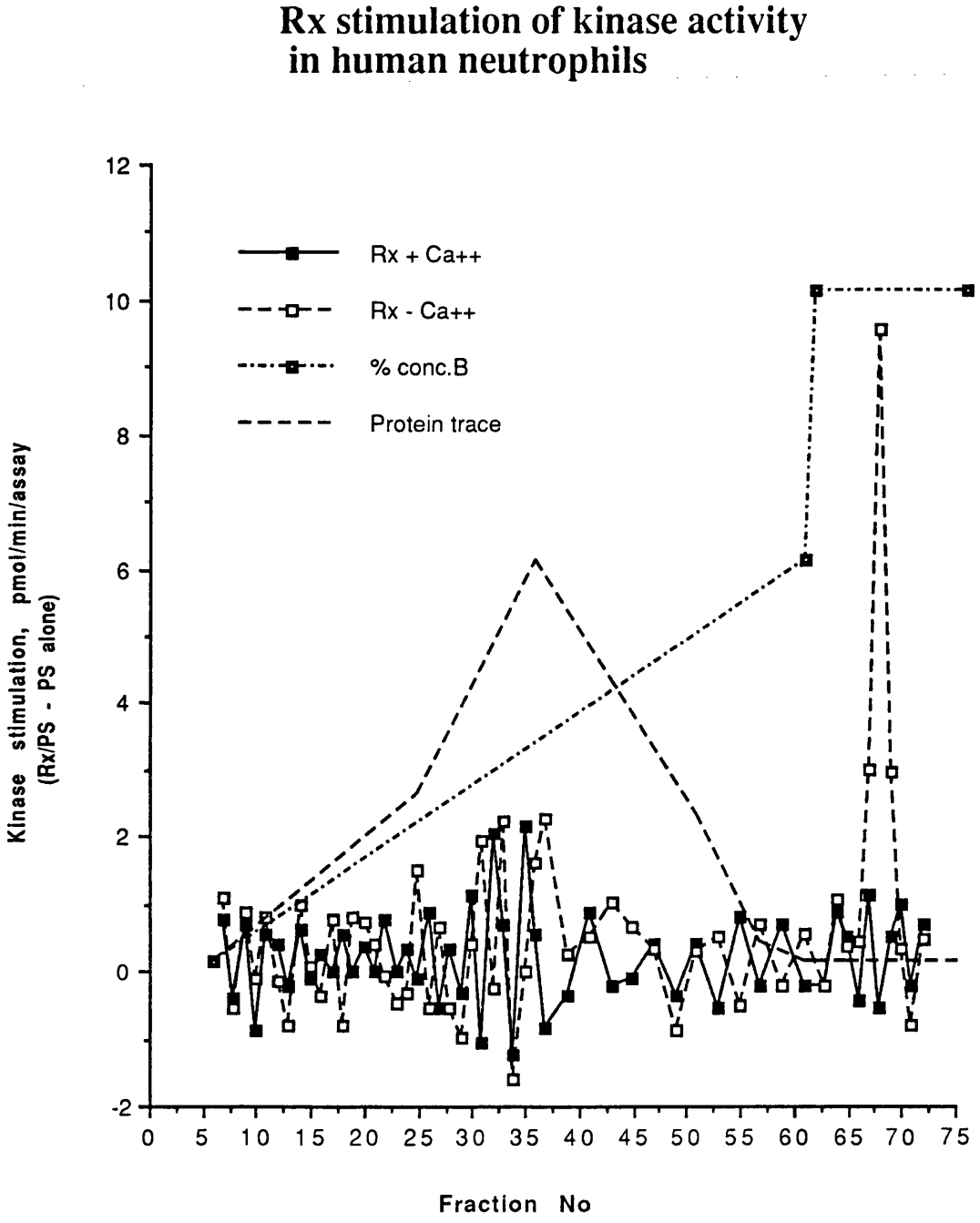


Figure 4.1 Rx-stimulation of kinase activity of human neutrophils. Approximately 5 x 108 cell homogenates wwas subjected to FPLC HPT chromatography and bound PKC was eluted by 20-300 mM phosphate buffer over 60min at 1ml/min. Rx-kinase containing fractions were eluted by increasing the phosphate gradient to 500mM. Protein trace is shown as the typical profile generated by measuring absorbance at 280nm. Results are expressed as a representative from three separate preparations.

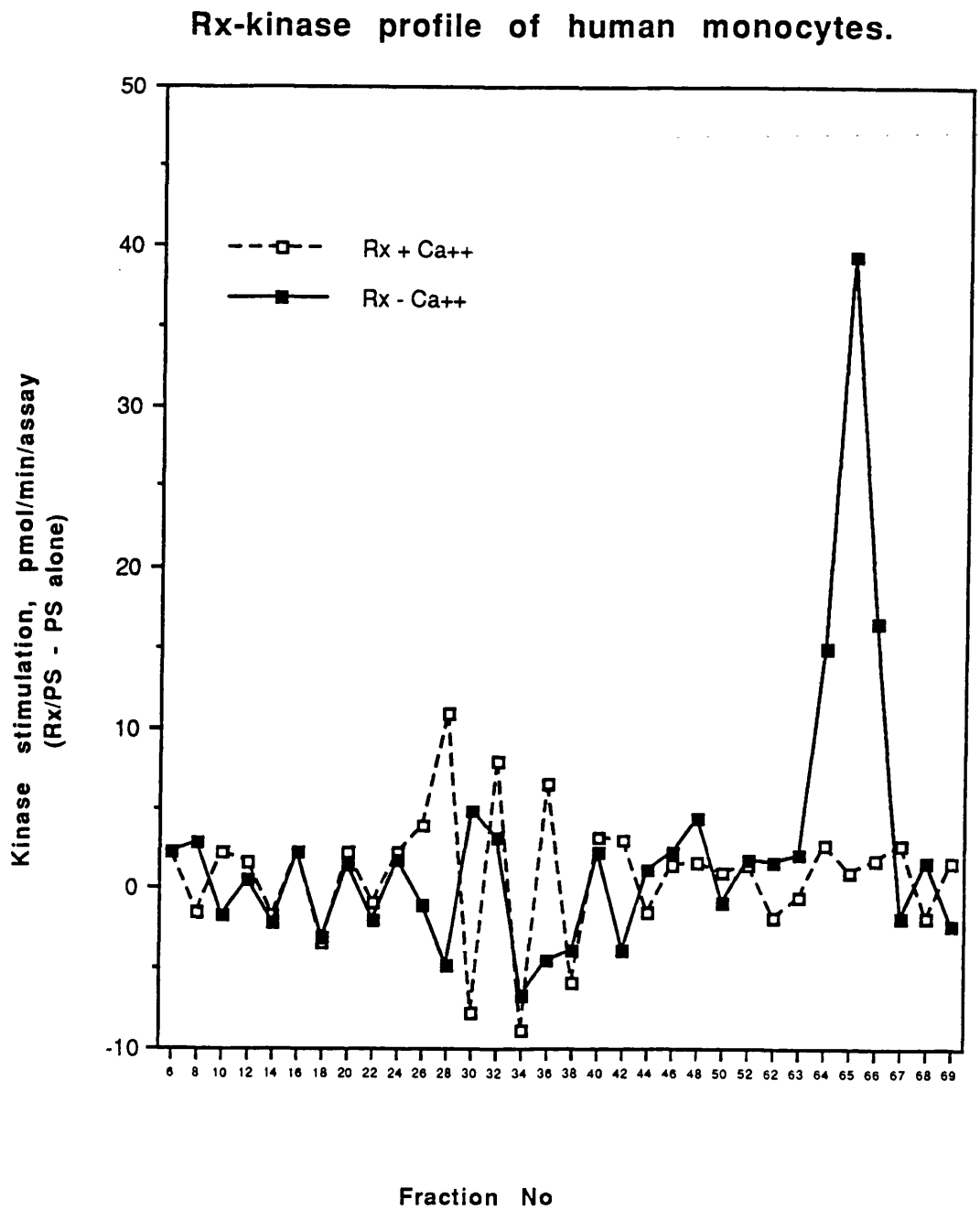


Figure 4.2 Rx-stimulation of kinase activity in human monocytes. Approximately 3 x 10^9 cells were subjected to FPLC HPT chromatography and bound PKC was eluted by a 20-300mM phosphate gradient over 60min at $1ml min^{-1}$. Rx-kinase containing fractions were eluted by increasing the phosphate gradient to 500mM. Kinase assays were the standard mixed micellar assay as described in the Methods, containing $100ng ml^{-1}$Rx/ $0.75mg ml^{-1}$ PS in the absence of Ca^{2+}. Results are expressed as Rx/PS minus Rx control and are a representative from three separate preps.

Kinase activity was determined on all fractions from hydroxyapatite or Superose 12 by measuring the transfer of ^{32}P from [$\gamma^{32}P$]-ATP to histone IIIs using essentially the micellar assay of Hannun *et al* (Hannun *et al.*, 1985) and as described in Chapter 2 (section 2.3.7.1). Assays employing the pseudosubstrate used the slightly modified procedure (Ryves *et al*, 1991) as previously described in Chapter 2, (section 2.3.7.2).

4.2.7 Ca^{2+}- dependency studies of Rx-kinase

Assays determining the effect of Ca^{2+} ions, various concentrations of Ca^{2+} (starting at a maximal final concentration 5.1mM as previously described (Ryves, 1991)) was serially diluted. To each of the Ca^{2+} solutions was added a stock solution of ATP mix buffer (5mM EGTA, 10mM $MgCl_2$, 100uM ATP and appropriate volume of γ-^{32}P-ATP). This made sure that ATP mix concentration remained identical and only the Ca^{2+} levels were altered. This was proved by counting the total radioactivity of each solutions which were similar with only 5-7% variations (data not shown). Assays were conducted as before and the concentration of free Ca^{2+} ions per assay was calculated from 'Steinhardt's Chelate' computer programme.

4.2.8 Phorbol ester binding to Rx-kinase.

Binding assays were carried out by essentially the method of Parker *et al* (1984) with a slight modification. The initial incubation with ^{3}H-phorbol esters, phorbol esters,

buffers and enzymes were at 4°C but all subsequent procedures were identical. Briefly, a stock solution of PS was prepared by drying 140ul PS (20 $mg.ml^{-1}$) under nitrogen and further adding 60ul PS before probe sonicating with 2ml Tris-Cl 20mM (pH 7.5). Assay solutions were made by mixing 10ul BSA ($5mgml^{-1}$), 5ul EGTA (40mM), 5ul Tris-Cl (20mM, pH 7.5), 5ul PS (stock solution), 5ul competitor or solvent or Tris-Cl and 10ul enzyme. The reaction was started by adding 10ul ^{3}H-phorbol ester at appropriate concentration. The whole assay mix was incubated for 15min at 4°C and the reaction was stopped by adding 0.5ml 20% DE52 in 20mM Tris-Cl. After a further incubation for 15min at 4°C (whirlimixing twice) the mixtures were decanted onto filters and washed (3 x 2ml of Tris-Cl 20mM, pH 7.5). The filters were placed in scintillation vials, 4mls of cocktail-T scintillant added and the radioactivity counted. The phorbol esters used were ^{3}H-PDBu (18 Ci/mmol) and ^{3}H-Rx (0.6 Ci/mmol).

4.2.9 Immunological Analysis.

Western blotting for α-, β-, γ-, ε- were conducted by Dr W J Ryves and has given kind permission for use of his results. 15ul of selected fractions of Rx-stimulated kinase activity as recovered in 100% 500mM phosphate buffer were subjected to immunological analysis by Western method. Samples were boiled 1:1 in double strength Laemmli buffer (see Chapter 2, section 2.3.4 a) immediately following elution from HPT. Anti-sera to PKC isotypes were isolated from rabbit blood previously immunised with synthetic peptides corresponding to V_5 region of each isotypes (Marias and

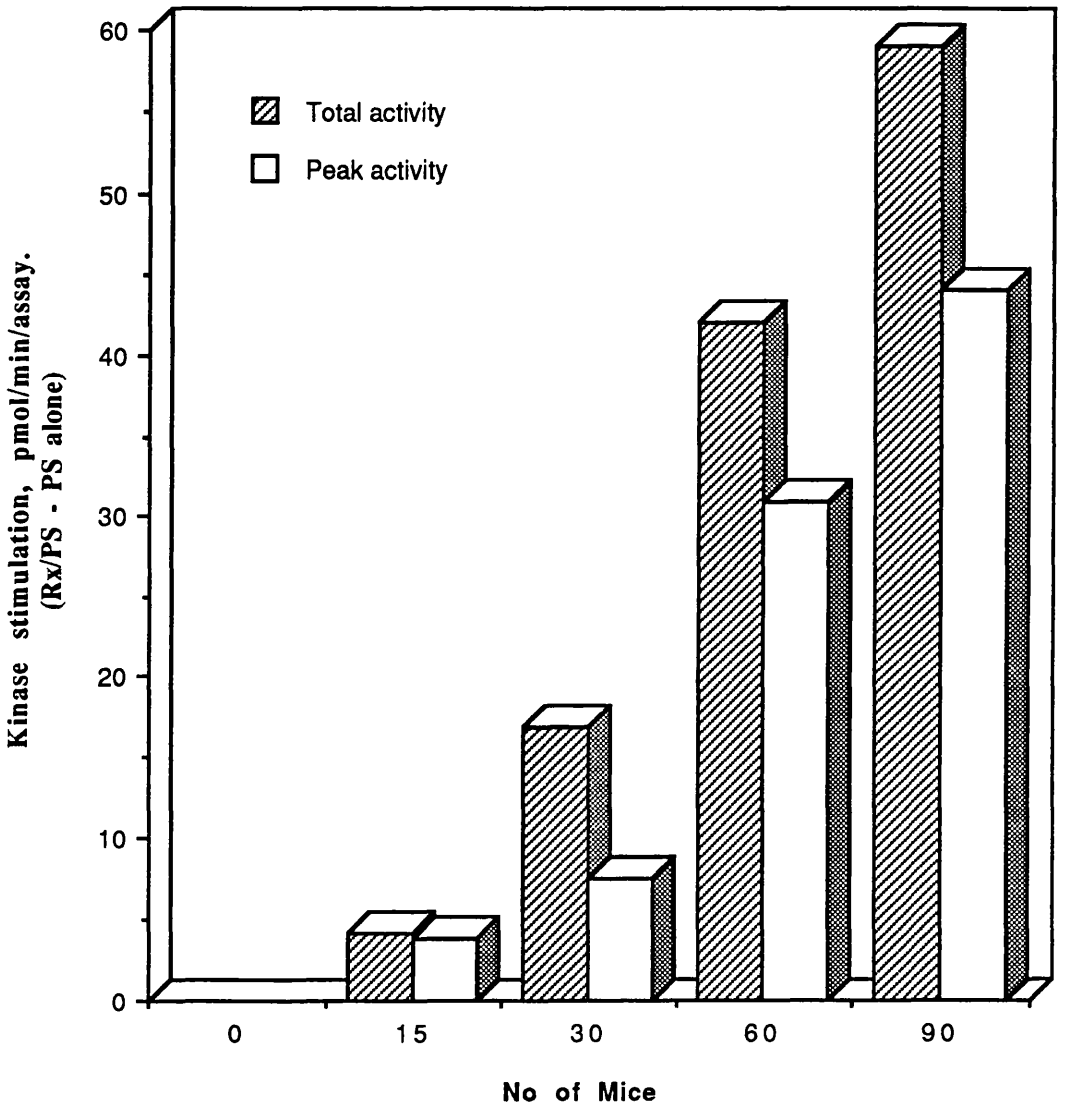


Fig 4.3 Peak and total Rx-kinase activity in mouse macrophages. Rx (100ng/ml and corresponding to 0.003 mol% of Triton/PS micelles) was assessed for stimulation of histone kinase activity in late eluting fractions from several preparations of starch elicited mouse peritoneal macrophages.

Stimulated kinase activity (pmol/min/assay) (Rx/PS - PS control)		
	Monocytes	Neutrophils
Peak Rx-kinase	13.37±2.46	10.13±1.41
Rx-kinase + Staurosporine	1.56±0.28	0.88±0.16

Table 4.1 Effect of staurosporine on Rx-kinase activity. Cell (1 x 10^8) homogenates were subjected to HPT chromatography and PKC fractions were eluted off on 60% phosphate gradient. Rx-kinase fractions were eluted at 100% phosphate gradient. Activities represent stimulation of Rx-kinase by Rx (100ngml^{-1}; 0.003 mol % of triton micelles) with basal levels (PS) subtracted in the absence and presence of staurosporine (1 μM). Results are expressed as mean ± SD (n=3).

Neutrophils (pmol/min/assay; Rx/PS - control)		
5 x 10^8 (MD)	1 x 10^8 (MD)	1.5 x 10^7 (SD)
9.40±0.37	3.3±0.65	0.32±0.08
Monocytes (pmol/min/assay; Rx/PS - control)		
3 x 10^9 (MD)	1 x 10^9 (MD)	2 x 10^8 (SD)
39.44±6.7	14.17±1.98	0.75±0.87

Table 4.2 Peak Rx-kinase in neutrophils and monocytes. Cell homogenates were subjected to HPT chromatography and PKC fractions were eluted off by 60% phosphate gradient. Rx-kinase containing fractions were eluted at 100% phosphate gradient. Activities represent stimulation of Rx-kinase by Rx (100ngml^{-1}, 0.003 mol% of Triton/PS micelle) with basal levels (PS alone) subtracted. MD represents cells taken from several or mixed donors and SD represents single donors. Results are expressed as mean of triplicates ± SD (n=3).

Parker, 1989; Schaap *et al*, 1989; Schaap and Parker, 1990). The peptides used were α- (PQFVHPILQSAV), β- (SEFLKPEVKS), γ- (PDARSPISPTPVPVM), δ- (NQEEFKGFSYFGEDLMP), and ε- (MNRRGSIKQAKI). All antisera to PKC-isozymes and synthetic peptides were generous gifts from Dr P J Parker (ICRF, London). These same peptides were used in competition experiments to define specific interactions against non-specific interactions. Samples were analyzed by SDS-PAGE on 7.5% SDS polyacrylamide gels with a electrophoretic running buffer (see section 2.3.4). Known molecular markers and PKC isotypes (where available) standards were included as controls. Separated samples were transferred and electroblotted onto nitro-cellulose using Western transfer buffer (see section 2.3.5). Non-specific binding sites were bound by blotto buffer and washed with washing buffer (WB, see section 2.3.5). The nitro-cellulose was incubated overnight at 4°C with anti-PKC antibody diluted 1:1000 in WB containing 3% BSA. In competition studies antibody was mixed with epitope before dilution and incubation. Immunoblots were washed for 3 x 15min as above and incubated with ^{125}I-labelled donkey anti-rabbit IgG at a dilution of 1:1000 for 1hr, followed by further washing and dried on paper. Bands of radioactivity were visualised by autoradiography.

4.3 Results.

4.3.1 Rx-kinase isolated from human and mouse inflammatory cells.

The results are reported as kinase activity with the basal (PS alone) activity subtracted as pmol/min/assay (assay was equivalent to 25ul enzyme used). In the profile generated from human neutrophils and monocytes the peaks of PKC activity were eluted off by 60% of the phosphate buffer gradient (300mM) and Rx-kinase recovered on increasing the phosphate concentration to 100% (Fig 4.1 and Fig 4.2) in fractions 65-70 as a clearly distinct peak of activity. The activities were entirely inhibited by 1uM staurosporine added to the micelle mix (Table 4.1). These activities were obtained from cells with mixed donors (ie after isolation of cell types, the cells were mixed from more than one donor), and were proportional to the number of cells used prior to homogenisation (Table 4.2). However, for both types of phagocytes, Rx-kinase activity was not present if only a single donor was used (see Table 4.2)

The detection of Rx-kinase from human neutrophils and monocytes prompted an investigation of other inflammatory cells. In these experiments macrophages were elicited in peritoneal cavity of mice for 3 days following injection of 2% starch solution (see Chapter 3). In the supernatant of the peritoneal mouse macrophages Rx-kinase was detected as before but was absent from resident macrophages. The activity from the elicited cells increased in direct proportion to the number of mice used in the experiment and maximum activity of 60 pmol/min/assay was obtained when 90 mice were sacrificed (Fig 4.3).

This investigation was extended further to mouse alveolar macrophages on the basis

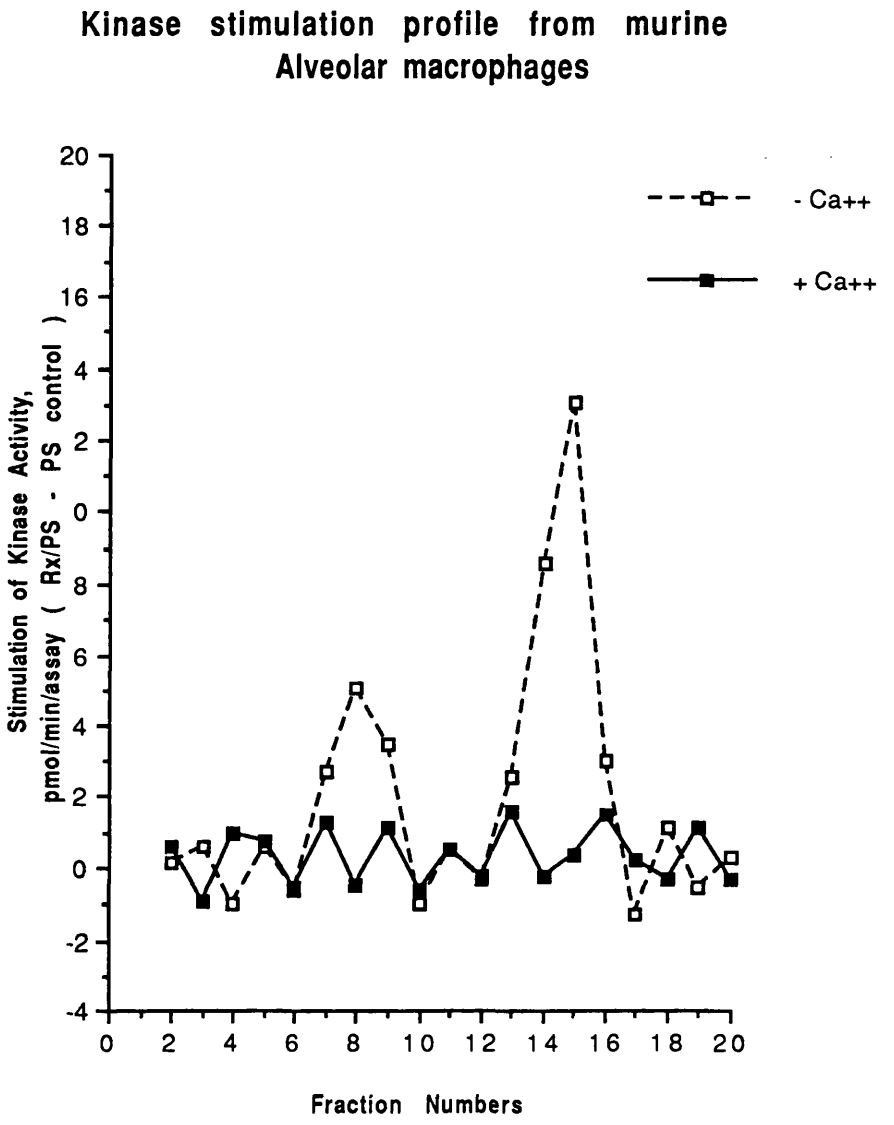


Figure 4.4 Rx-kinase stimulation profile from murine alveolar macrophages. 1 x 10^8 cells were subjected to FPLC HPT chromatography and bound PKC was eluted by 20-350mM phosphate over 30min at flow rate $1mlmin^{-1}$. Rx-kinase containing fractions were eluted by linearly increasing the phosphate gradient to 500mM over 20min at $1mlmin^{-1}$. Kinase assays were the standard mixed micellar assay as described in the Methods, containing $100ngml^{-1}$Rx/ $0.75mgml^{-1}$ PS in the absence of Ca^{2+}. Results are expressed as Rx/Ps minus PS control and are a representative from three separate preps.

that these cells previously might have been 'primed' for Rx-kinase production. The hydroxyapatite profile of these cells differed from that of the peritoneal macrophages, human neutrophils and monocytes in that two peaks of Rx-stimulated histone-kinase activity were recorded. The first, and minor of these activities, peak I eluted before (~410 mM phosphate gradient) the activity previously detected above whilst the major peak II (~460 mM) corresponding to Rx-kinase (Fig 4.4).

4.3.2 Determination of Assay conditions for Rx-kinase.

Murine alveolar macrophage Rx-kinase (peak II as above) was used as a convenient source of enzyme for experiments to optimise the assay conditions. 25ul of this purified Rx-kinase was shown to phosphorylate Histone IIIs in a concentration dependent manner upto to a maximal phosphorylation of between 2-4 mg/ml of histone (Fig 4.5) only in the presence of PS and absence of added Ca^{2+} (and presence of 5.0mM EGTA). Rx-kinase was further shown to phosphorylate Histone IIIs (1 $mgml^{-1}$) in a linear manner with time (Fig 4.6) reaching a maximum between 15-20mins. Interestingly phosphorylation with PS (0.75 $mgml^{-1}$) alone was low but steady up to 12-15mins but was rapidly increased with further time. The enzyme concentration (5 ug to 30 ug) demonstrated similar relationship and the maximal response was reached by 30 ug purified enzyme added to the assay. Phosphorylation with PS alone was again low (Fig 4.7). From these results the assay conditions were optimised for further experiments where 9 units of Rx-kinase activity corresponding to

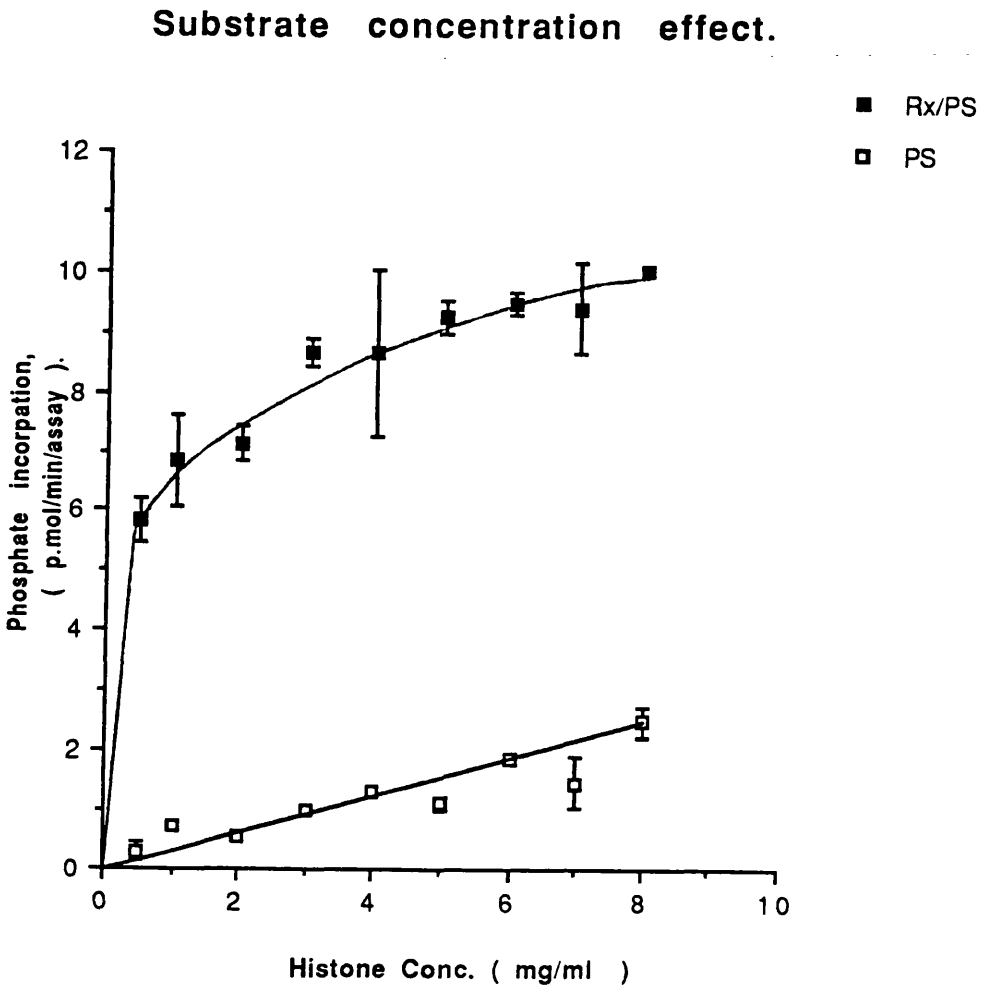


Figure 4.5 Substrate concentration effects Rx-kinase. Peak II Rx-kinase activity of murine alveolar macrophages were subjected to increasing concentration of Histone IIIs and phosphate incorporation are measured with Rx ($100ngml^{-1}$)/PS ($0.75mgml^{-1}$) or PS alone. Results are expressed as mean of triplicates ±SD.

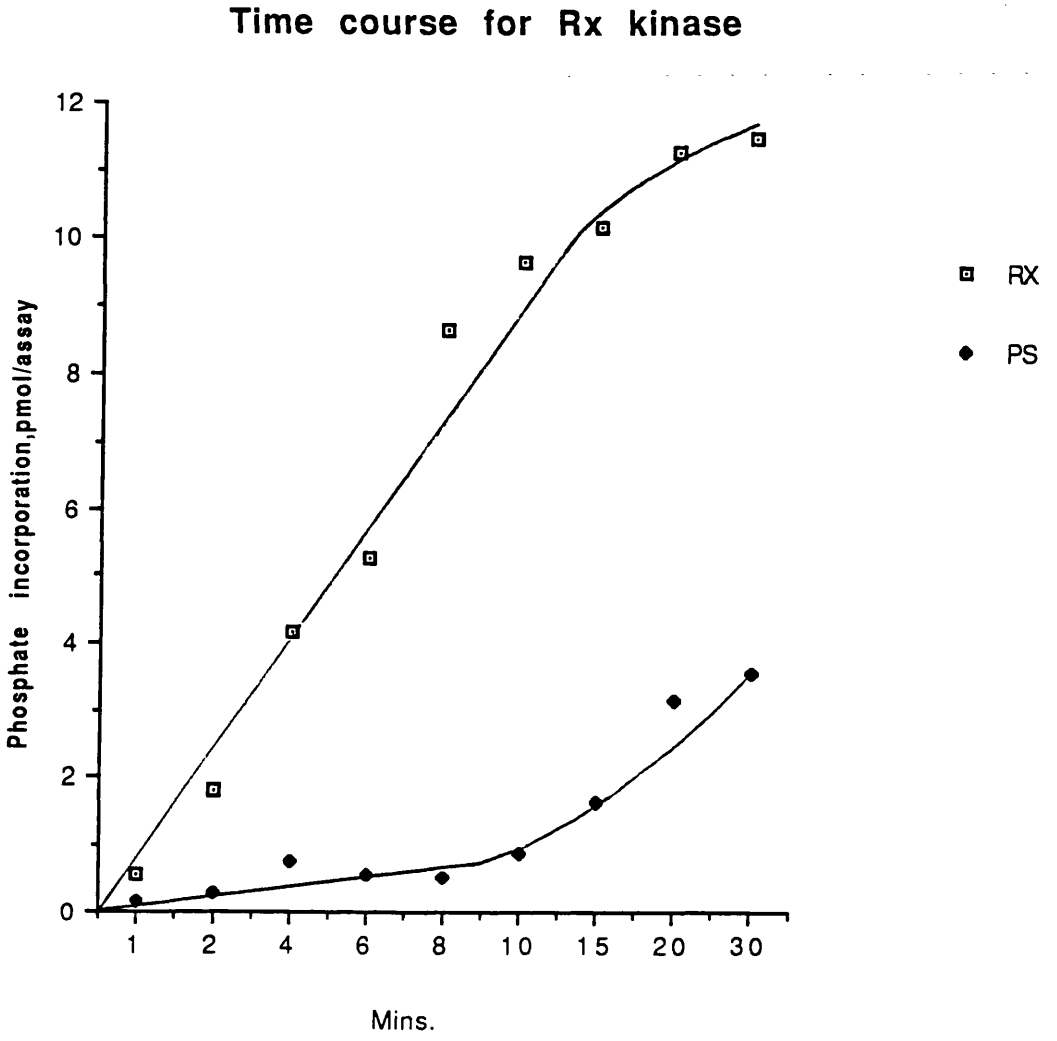


Figure 4.6 Time course for Rx-kinase. Peak II Rx-kinase activity of murine alveolar macrophages were assayed at various times upto 30min with Histone IIIs at $1mgml^{-1}$ and phosphate incorporation are measured with Rx ($100ngml^{-1}$)/PS ($0.75mgml^{-1}$) or PS alone. Results are expressed as mean of triplicates ±SD.

Enzyme concentration effects.

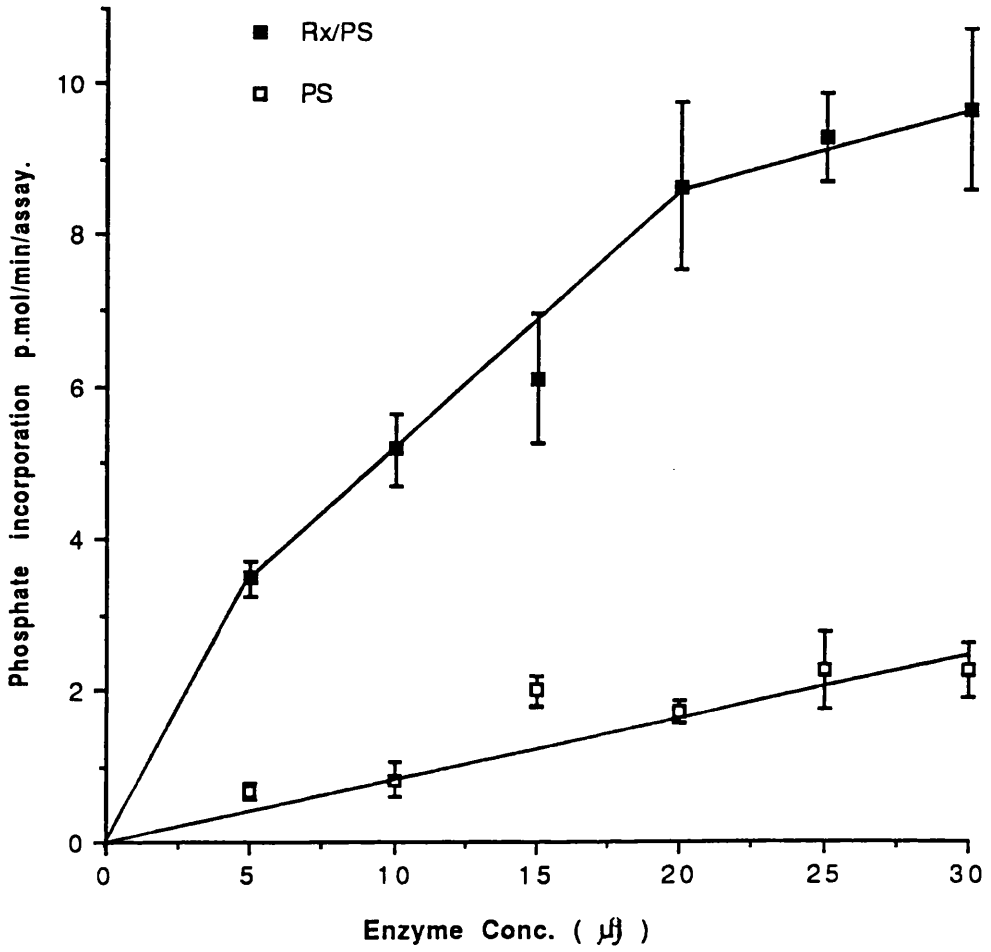


Figure 4.7 Enzyme concentration effects of Rx-kinase. Peak II Rx-kinase activity of murine alveolar macrophages were assayed using various volumes upto 30ul for 10min with Histone IIIs at $1mgml^{-1}$ and phosphate incorporation are measured with Rx ($100ngml^{-1}$)/PS ($0.75mgml^{-1}$) or PS alone. Results are expressed as mean of triplicates ±SD.

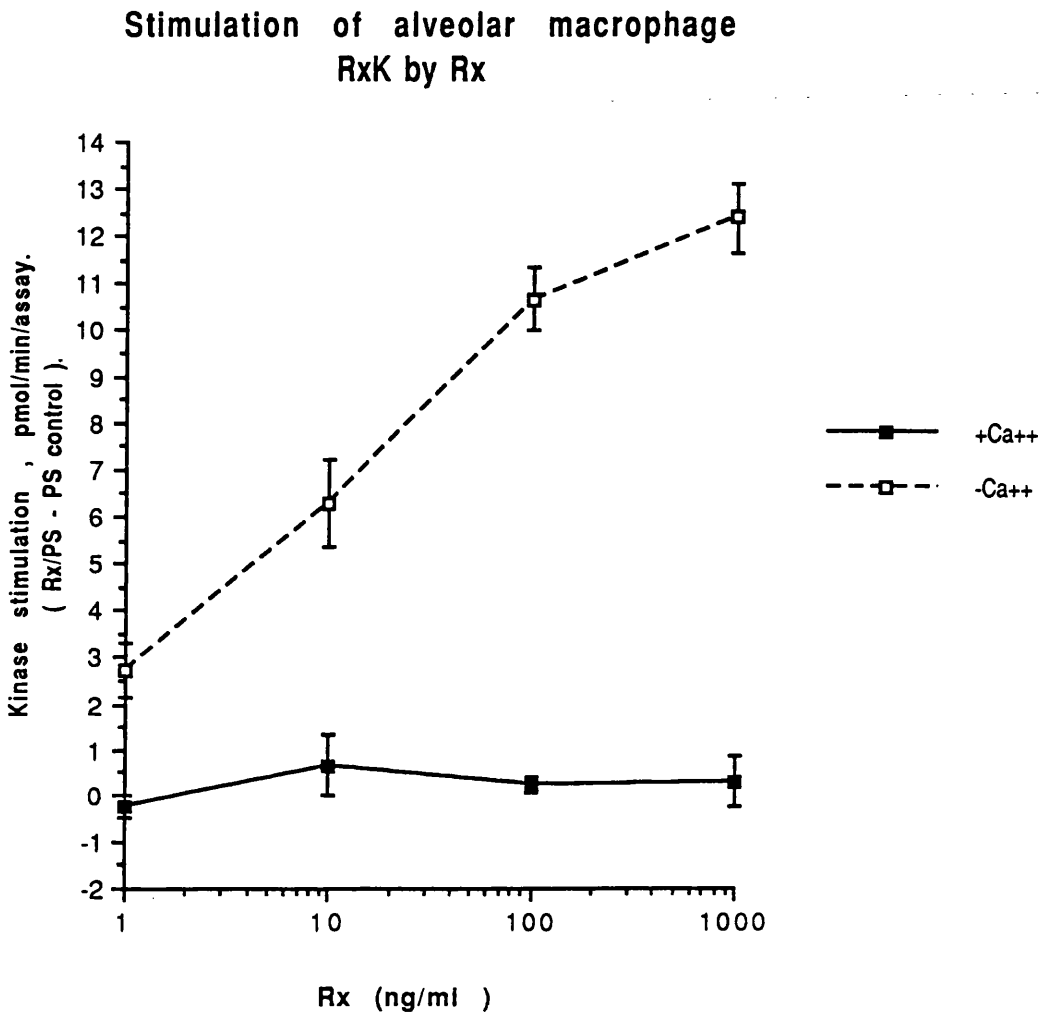


Figure 4.8 Stimulation of murine alveolar macrophage Rx-kinase by Rx . Peak II Rx-kinase activity of murine alveolar macrophages were assayed using increasing concentration Rx for 10min with Histone IIIs at $1mgml^{-1}$ and 25ul enzyme and phosphate incorporation are measured as rate of kinase activity with Rx/PS ($0.75mgml^{-1}$) minus PS alone in the presence and absence of added Ca^{2+} (100μM). Results are expressed as mean of triplicates ±SD.

about 25 ul of purified enzyme (1 unit was equivalent to the stimulation of 1 pmole of phosphate transferred/min/assay by Rx) were used together in the assay with 1mgml^{-1} Histone IIIs for a period of 10mins in the presence of PS (0.75mgml^{-1}) but absence of Ca^{2+}. Using the assay conditions dose response curves were established (Fig 4.8). From these results the apparent K_a for Rx on Rx-kinase was calculated to be 10 ngml^{-1} (9 -12 ngml^{-1} range) from which 100 ngml^{-1} was chosen as the optimum concentration of Rx for activation studies.

4.3.3 Studies of Tissue Distribution.

Studies of distribution of Rx-kinase were investigated on various tissues isolated from rat. Since Rx-kinase can be purified from 'priming' of cells (see section 4.3.1) it may be possible to induce this enzyme by causing oxidative stress to various tissues. Naphthoquinone, plumbagin was used as the oxidative stress agent because of earlier observed result (see Chapter 3, section 3.3)

Tissues were isolated and supernatants were loaded onto hydroxyapatite column connected to FPLC. PKC was eluted off in phosphate buffer gradient up to 70%. Then a linear phosphate gradient (70% -100%) was applied and elutions collected over 20 fractions. However, initially the supernatants of the various tissues were tested. Plumbagin treatment of lungs, thymus, heart and spleen all increased the enzyme activity (Fig 4.9). The activity was most significantly increased in lung Rx-kinase.

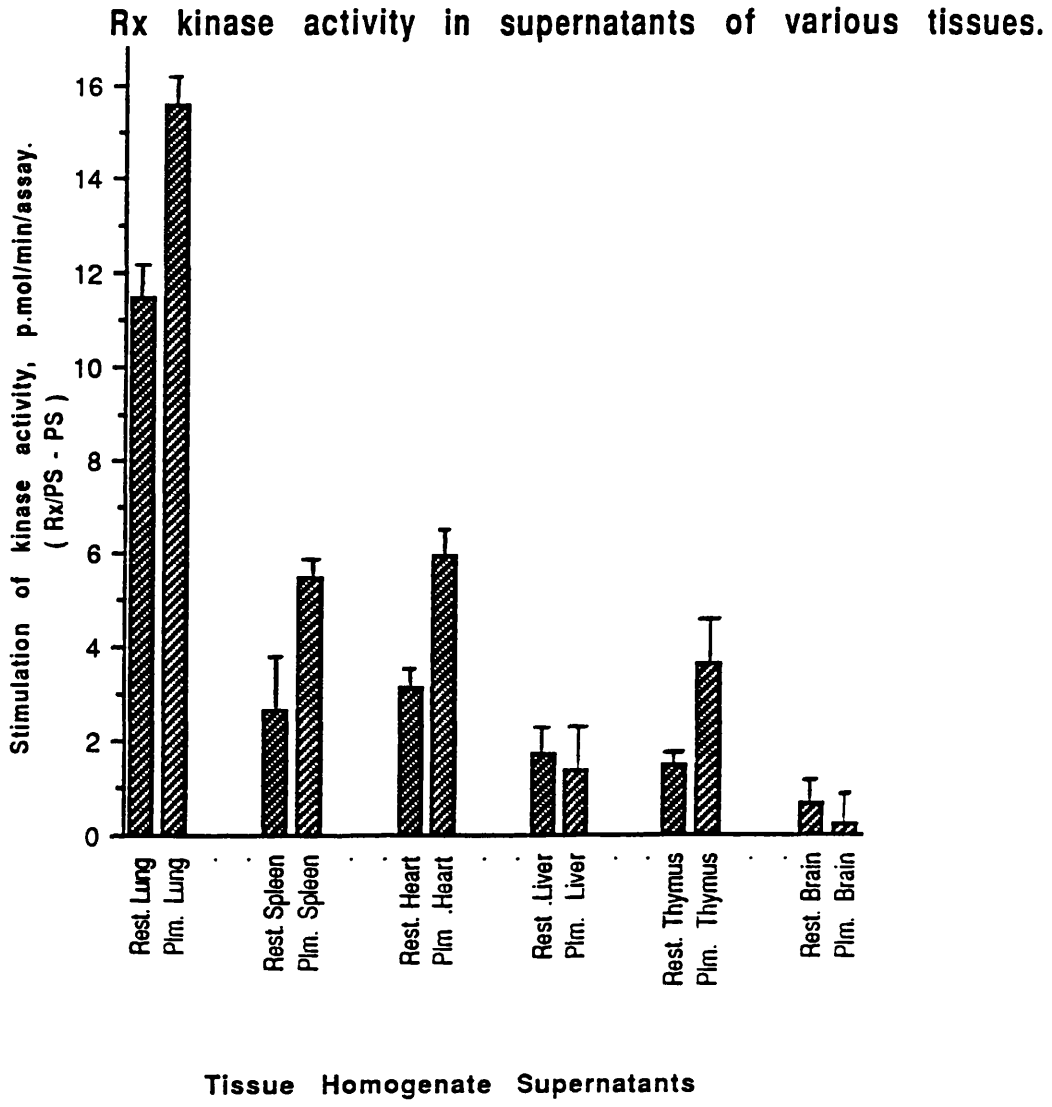


Figure 4.9 Rx-kinase activity in supernatants of various tissue. 5 g wet weight of tissues were homogenised immediately or after pre-treatment with plumbagin (0.1μg/ml) and after centrifugation the supernatants were assayed for kinase activity with Rx(100ngml^{-1})/PS (0.75mgml^{-1}) minus PS alone in the absence of added Ca^{2+} (EGTA 10mM)). Results are expressed as mean of triplicates per 5g weight.

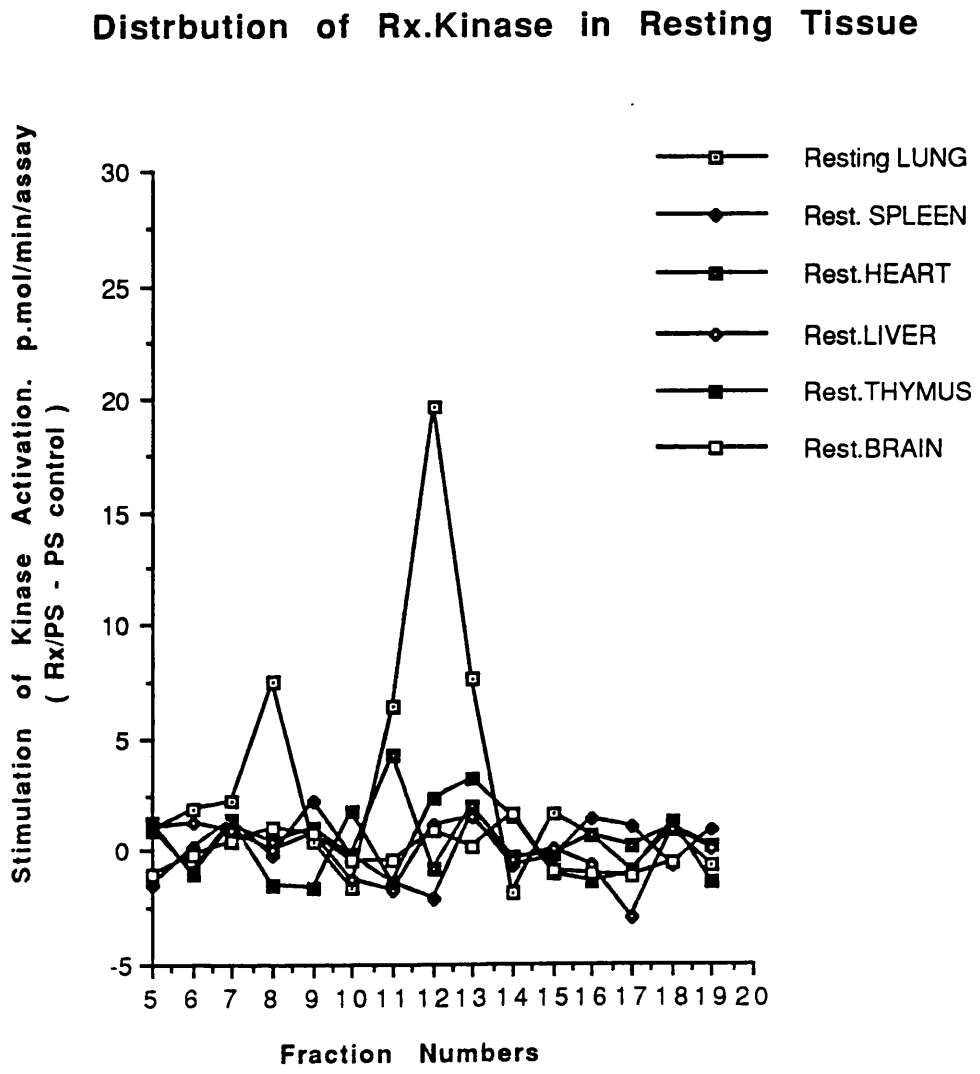


Figure 4.10 Rx-kinase activity of various tissue. 5 g wet weight of tissues were homogenised and after centrifugation the supernatants were subjected to FPLC HPT chromatography and assayed for kinase activity with Rx(100ngml^{-1})/PS (0.75mgml^{-1}) minus PS alone in the absence of added Ca^{2+} (EGTA 10mM)). Results are expressed as mean of triplicates per 5g weight and are a representative of three separate experiments.

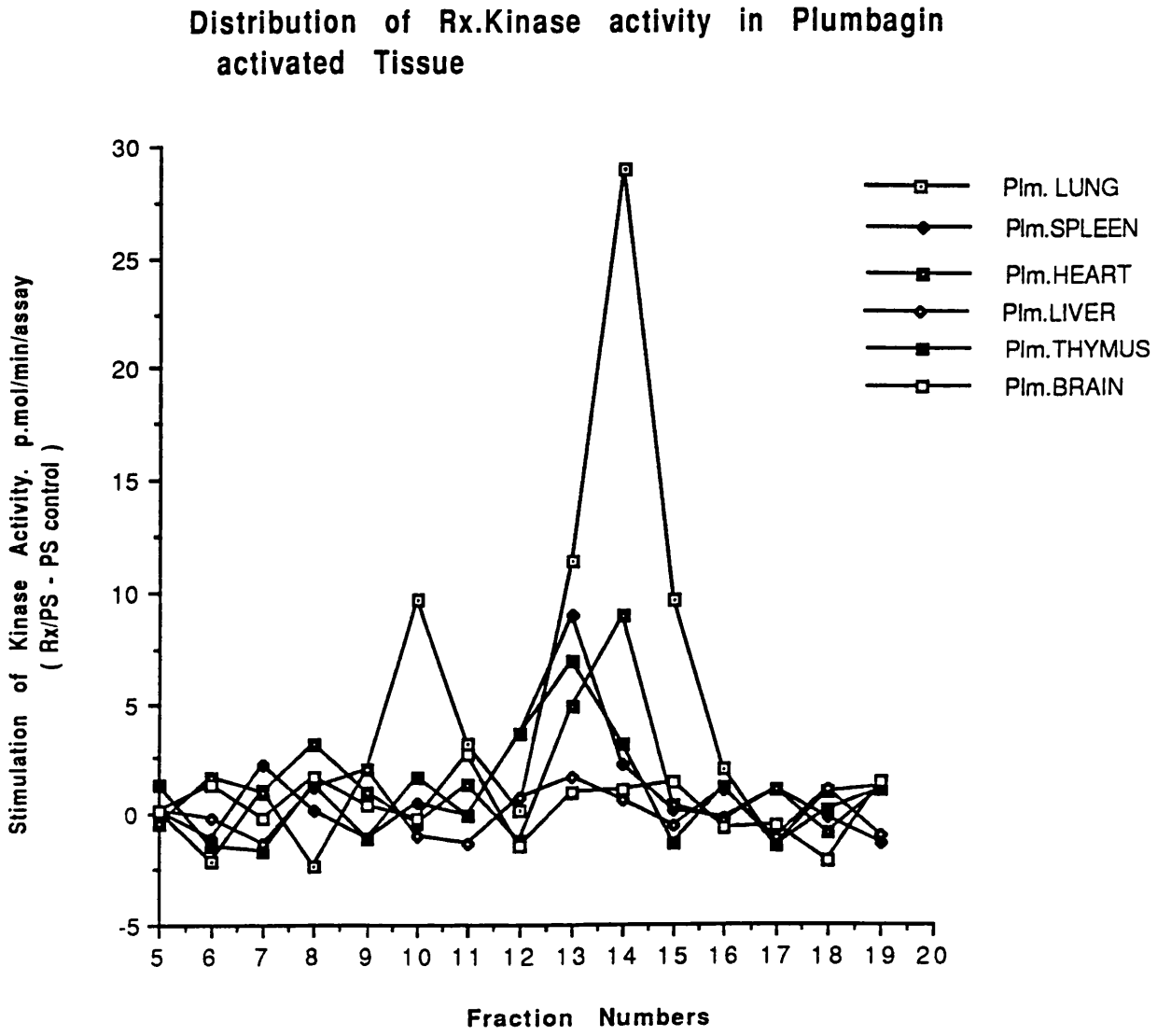


Figure 4.11 Rx-kinase activity of various tissue pre-treated with plumbagin. 5 g wet weight of tissues were pre-treated with plumbagin (0.1µg/ml), homogenised and after centrifugation the supernatants were subjected to FPLC HPT chromatography and assayed for kinase activity with Rx(100ngml^{-1})/PS (0.75mgml^{-1}) minus PS alone in the absence of added Ca^{2+} (EGTA 10mM)). Results are expressed as mean of triplicates per 5g weight and are a representative of three separate experiments.

Rat lungs (Fig 4.10) produced a profile similar to the one observed with alveolar macrophage with two distinct peaks of activity. The latter peak (II) corresponded to Rx-kinase previously detected. All other rat tissues tested (spleen, heart, liver, thymus, brain) contained no activity of Rx-kinase in elution fractions. However once the tissues were induced by plumbagin, small but distinct peaks of activity were eluted (450 - 465mM phosphate buffer) in heart, spleen and thymus (Fig 4.11). The peaks of activity corresponded to Rx-kinase previously detected. However no activity was observed from either liver or brain tissues. Rx-kinase elution profile of the lung was dramatically increased (>50%) by plumbagin stimulation (Fig 4.11).

4.3.4 Binding of (^{3}H) -phorbol derivatives to Rx-kinase

Rx-kinase activity isolated from human neutrophils, monocytes or mouse macrophages were potently activated by Rx and to determine the ability of Rx to bind to this receptor, experiments were conducted on purified Rx-kinase (peak II) prepared from murine alveolar macrophages as before. 9 units of Rx-kinase were used for saturation curve of total and specific ^{3}H-PDBu binding (Fig 4.12). Non-specific binding was in the range of 10-15% of total binding. Subsequently a Scatchard plot of ^{3}H-PDBu binding was analyzed. Fig 4.13 illustrates the analysis for the binding of ^{3}H-PDBu to Rx-kinase as a linear relationship between the ratio of bound to free versus bound PDBu (fmol/unit of kinase) with K_d= 230 fmol/U and B_{max} = 11.6 fmol/U.

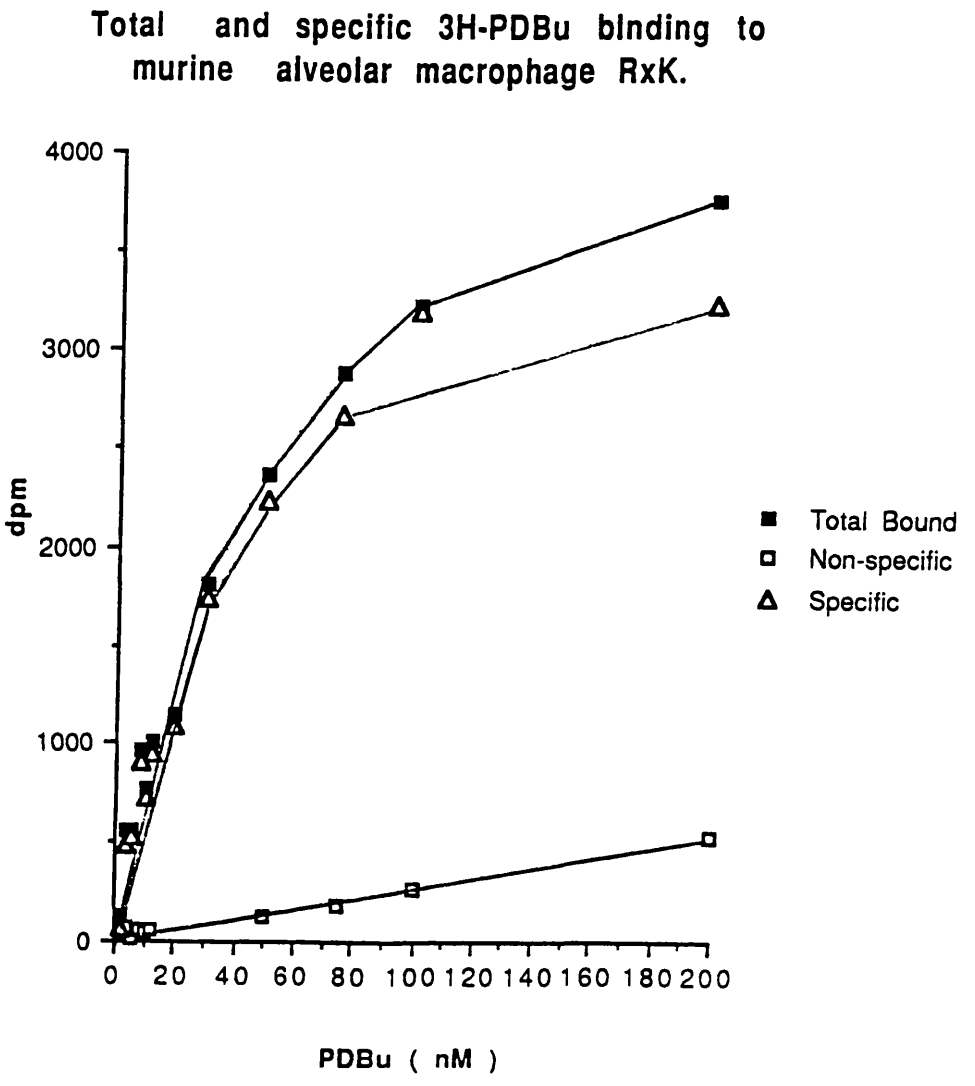


Figure 4.12 Total and specific ^{3}H-PDBu binding to murine alveolar macrophage Rx-kinase. 9 units of peak II alveolar macrophage Rx-kinase previously isolated was used to study the total and specific binding of PDBu.

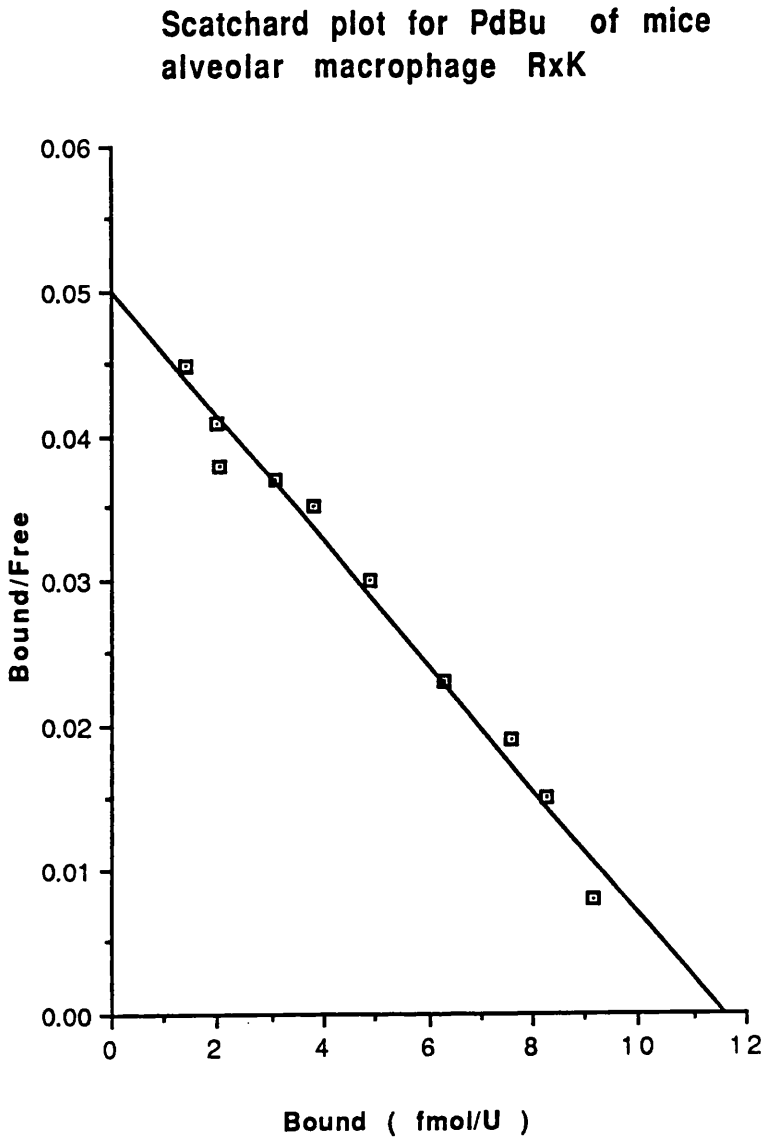


Figure 4.13 Scatchard plot for PDBu of mice alveolar macrophage Rx-kinase. 9 units of Rx-kinase previously isolated was used to study the specific binding of PDBu and from this data Scatchard analysis was established.

A comparison was made between the ability of Rx and TPA to inhibit ^{3}H-PDBu (100 $ngml^{-1}$) binding to the kinase in an identical manner. EC_{50} evaluation found Rx to be approximately 1000 times more effective than TPA in these experiments (Fig 4.14) which measures % specific binding of ^{3}H-PDBu versus concentration of agonist. Binding studies of radiolabelled Rx (1 $mgml^{-1}$) were also conducted on Rx-kinase (9 units) using varying amounts of cold Rx as inhibitor of ^{3}H-Rx specific binding (Fig 4.15). Non-specific binding was found to be larger than for PDBu at 50-60% of total bound. These results are the first reported for specific binding of Rx to purified Rx-kinase *in-vitro* and confirm Rx-kinase as a putative Rx-receptor protein isolated from inflammatory cells.

4.3.5 Bivalent cation dependence of Rx-kinase.

Rx-kinase was originally described as a Ca^{2+}-independent kinase activity (Ryves *et al*, 1989) but here we have investigated the ability of Ca^{2+} to inhibit Rx-kinase in an optimised assay previously described. Rx-kinase (9 units) for the experiments was purified from human neutrophils and as shown in Fig 4.16 the enzyme activity was clearly inhibited by the addition of Ca^{2+} (100 uM free) to the assay mix in the presence of 100 $ngml^{-1}$ Rx. Interestingly the activation by PS (0.75 $mgml^{-1}$) alone was also inhibited by added Ca^{2+} . Further scrutiny of this inhibition was investigated by generating a 'Ca^{2+} curve' for Rx-kinase. The enzyme activity was dramatically inhibited by increasing the amount of Ca^{2+} added (Fig 4.17). The K_i for 50%

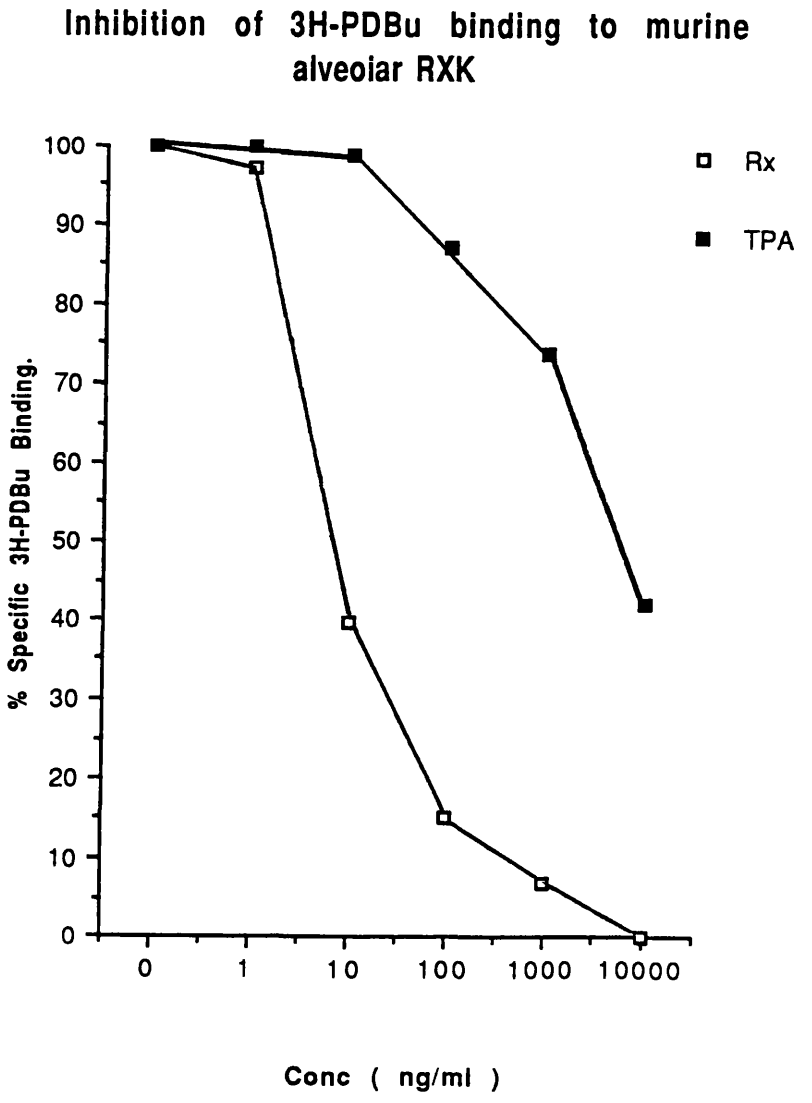


Figure 4.14 Inhibition of ^{3}H-PDBu binding to murine alveolar RxK. 9 units of Rx-kinase previously isolated was used in this study and the procedure is described in the Methods. Rx and TPA were both used at a concentration of 100ngml^{-1}.

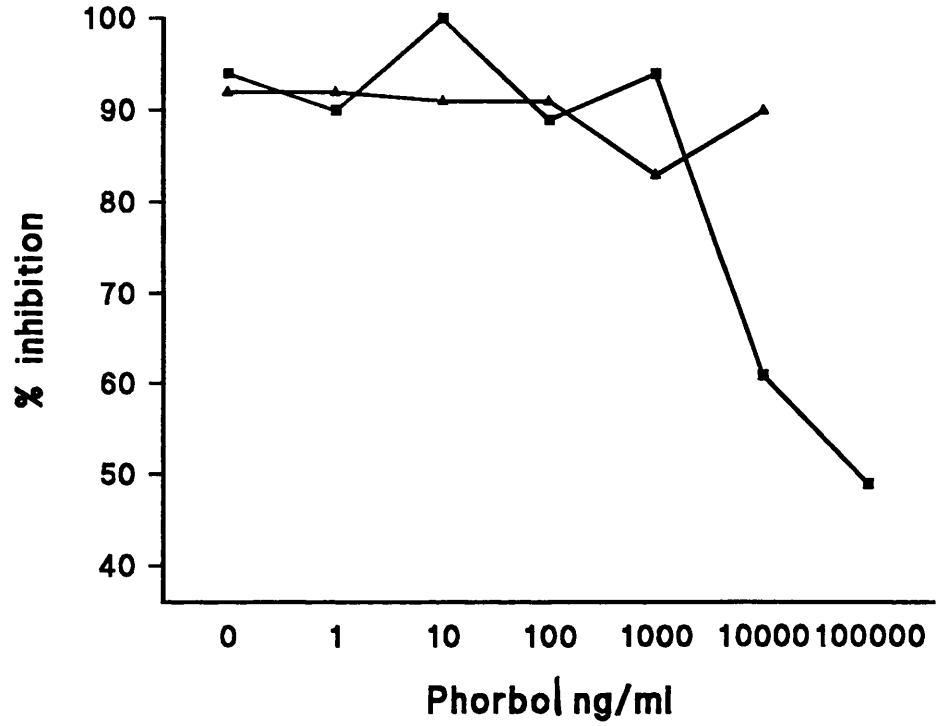


Figure 4.15 Competition of ^{3}H-Rx binding on peak Rx-kinase activity. TPA (▲) and Rx (■) were dissolved in 50% EtOH. ^{3}H-Rx (1mg/ml) used had specific activity of 600mCi/mmol.

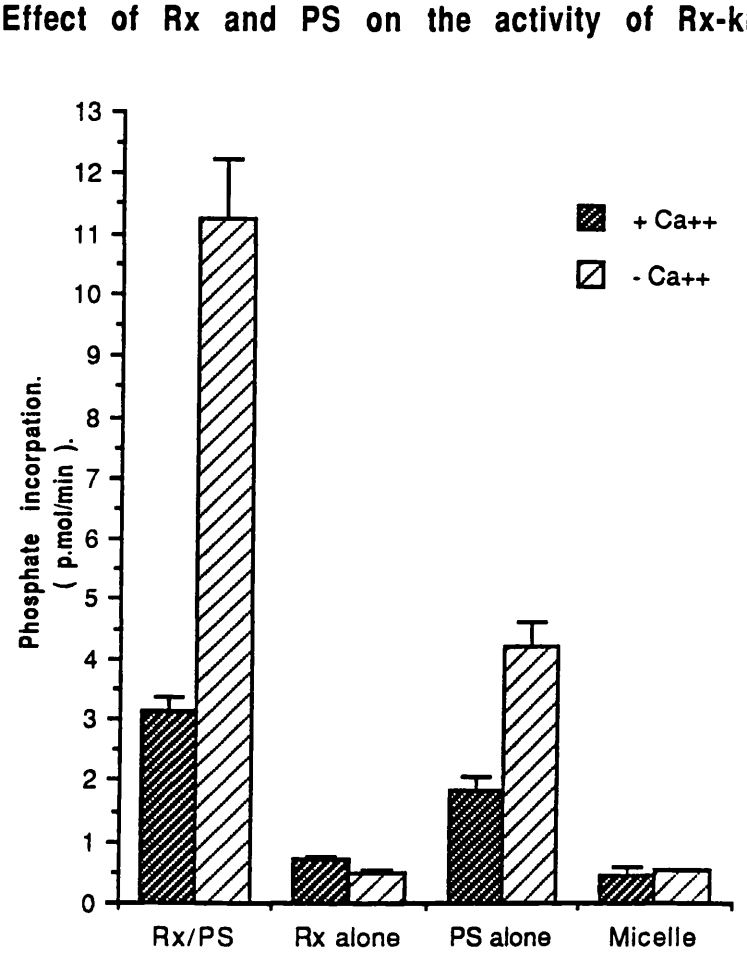


Figure 4.16 Effects of Rx and PS on the activity of Rx-kinase. Rx-kinase (9 units) used in this study was isolated from human neutrophils. Assays were conducted as the mixed micellar assay as described in the Methods with concentrations of Rx at 100ng/ml and PS at 0.75 mg/ml in the presence and absence of added Ca^{2+}. Results are expressed as mean of triplicates ±SD (n=3).

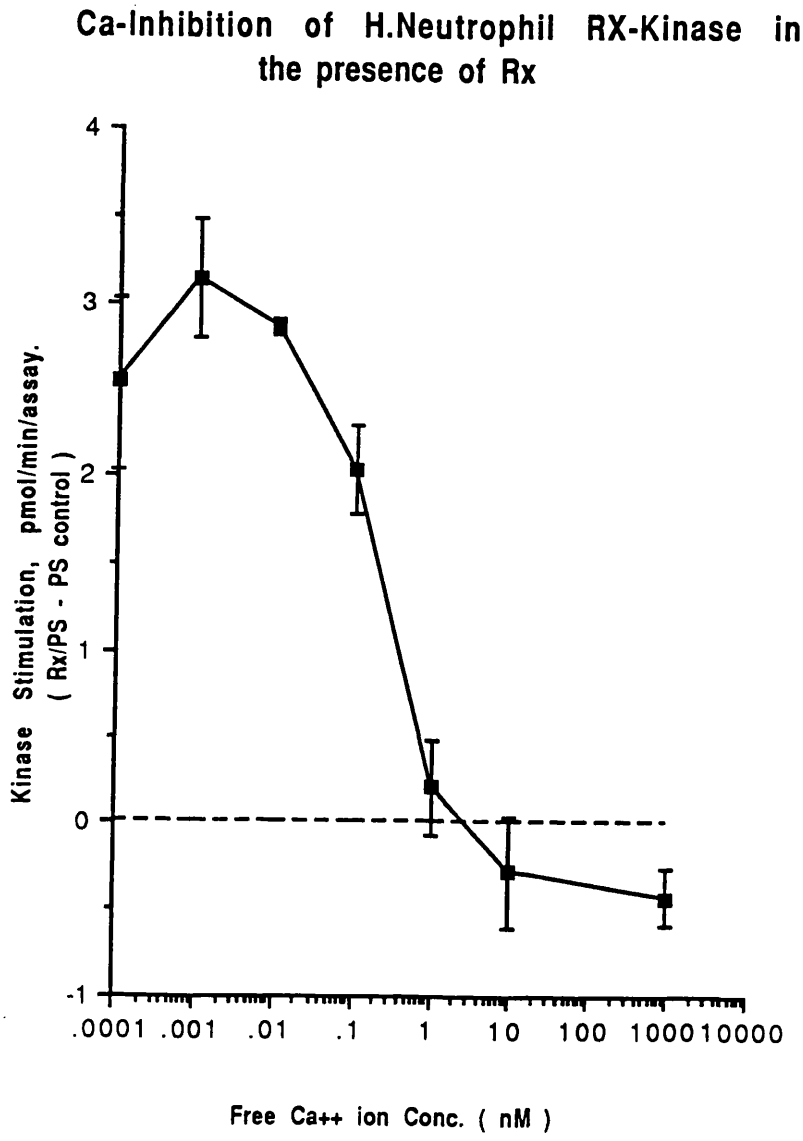


Figure 4.17 Calcium inhibition of activity of Rx-kinase. Rx-kinase (9 units) used in this study was isolated from human neutrophils. Assays were conducted as the mixed micellar assay as described in the Methods with concentrations of Rx at 100ng/ml and PS at 0.75 mg/ml. Results are expressed as mean of triplicates ±SD (n=3).

inhibition was calculated from a computer programme to be in the range 0.1 - 0.5nM free Ca^{2+} concentration in the presence of PS. The activity stimulated by Rx in the absence of Ca^{2+} was found to be absolutely dependent on Mg^{2+} ions (Fig 4.18).

4.3.6 Comparison of Rx-kinase activation by Rx, other phorbol esters and capsaicin.

To compare the abilities of a number of phorbol and daphnane derivatives to activate the kinase, samples of Rx-kinase (9 units) were prepared from alveolar macrophages. The phorbol esters TPA, SAP-A, DOPP and DOPPA together with daphnanes TxA and Rx all at 100 $ngml^{-1}$ were used to stimulate Rx-kinase in the presence and absence Ca^{2+} in the optimised assay previously described. These results were compared to stimulation induced by Capsaicin (CAPS) and diacylglycerol analogue, $DiC_{18:1}$ (DAG), both at concentration of 10 $ugml^{-1}$. Using peak II of alveolar macrophages Rx-kinase, Fig 4.19 shows that Rx clearly activated the kinase in the absence of Ca^{2+} whilst the other daphnane and phorbol esters were unable to induce a stimulation above background. It is clear that neither CAPS nor DAG stimulate Rx-kinase even at this high concentration. A sample of kinase activity collected as peak I from these cells responded to Rx in the absence of Ca^{2+} but in this instance the phorbol, DOPPA, also induced activation but was lower in efficacy than Rx (Fig 4.20).

4.3.7 Immunological analysis of Rx-kinase.

Rx-kinase was purified by HPT chromatography from starch-elicited peritoneal macrophages of 90 mice. This Rx-stimulated kinase activity equivalent to the peak of Rx-kinase activity (9 units) was subjected to immunological analysis by SDS-PAGE, Western blotting and probed with PKC isotypes anti-sera for α- , β-, γ-, δ- and ε-, and with anti-sera previously incubated with its competing peptide. The peak of Rx-kinase activity failed to respond to the probes and was distinct from PKC isotypes on that basis (Fig 4.22 and Fig 4.23).This was in contrast to the reaction produced by dilutions of purified isotypes α-, β- and γ- with the histone kinase activity equivalent to the peak of Rx-kinase (Fig 4.21).

An antibody for PKC-η was not available for these studies but purified η-PKC was compared to Rx-kinase by direct stimulation with phorbol esters. When histone IIIs was used as the substrate neither TPA nor Rx stimulated the isotypes, either in the presence or absence of added Ca^{2+} (Fig 4.24). However, when the pseudosubstrate was used in place of histone TPA induced a maximal stimulation of about 9.5 pmol/min/assay whilst Rx failed to stimulate η-PKC at concentration of 100ngml^{-1} and even at 1000 ngml^{-1} stimulation was only slightly greater (Fig 4.25). On the basis of this analysis Rx-kinase differed from η-PKC because the latter enzyme preferentially phosphorylated a pseudosubstrate rather than histone IIIs, exhibited a Ca^{2+}-independent activation and was stimulated by TPA rather than Rx.

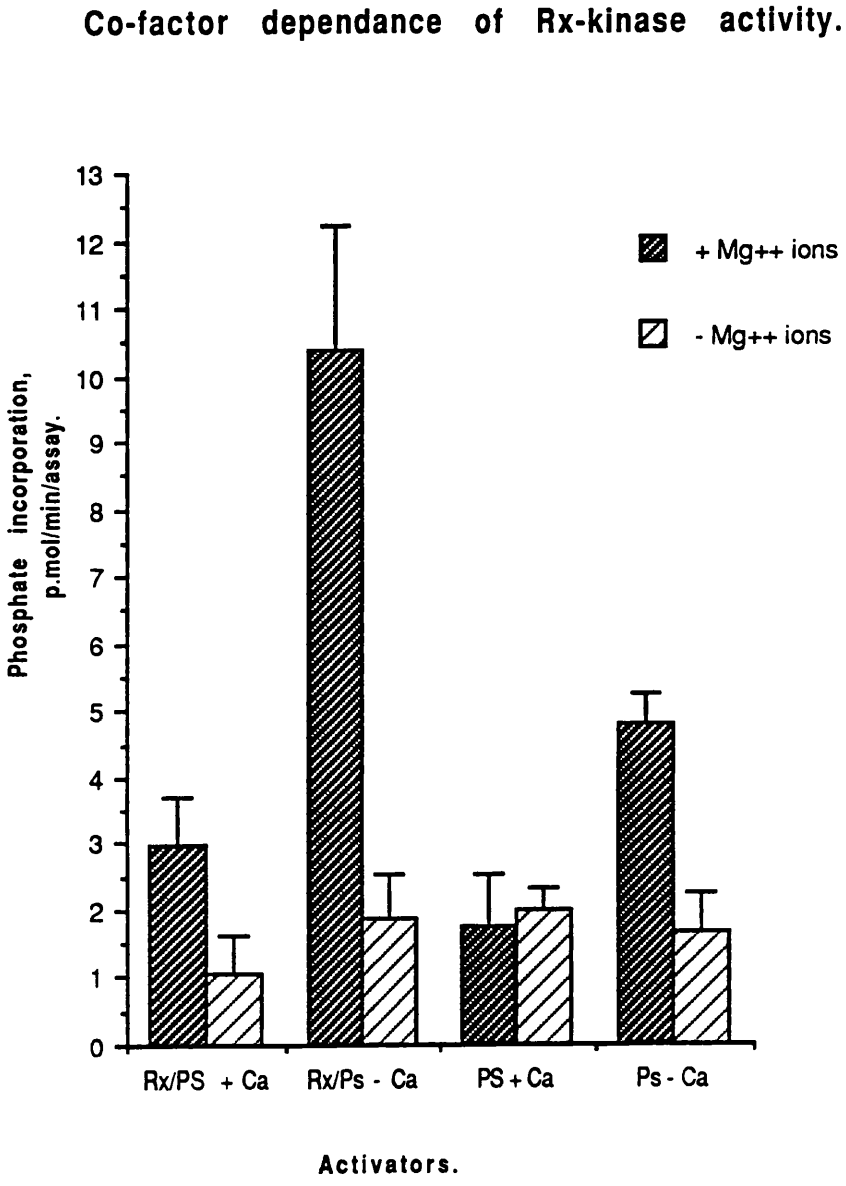


Figure 4.18 Co-factor dependence of activity of Rx-kinase. Rx-kinase (9 units) used in this study was isolated from human neutrophils. Assays were conducted as the mixed micellar assay as described in the Methods with concentrations of Rx at 100ng/ml and PS at 0.75 mg/ml in the presence and absence of added Ca^{2+} (100μM) and Mg^{2+} (10mM) Results are expressed as mean of triplicates ±SD (n=3).

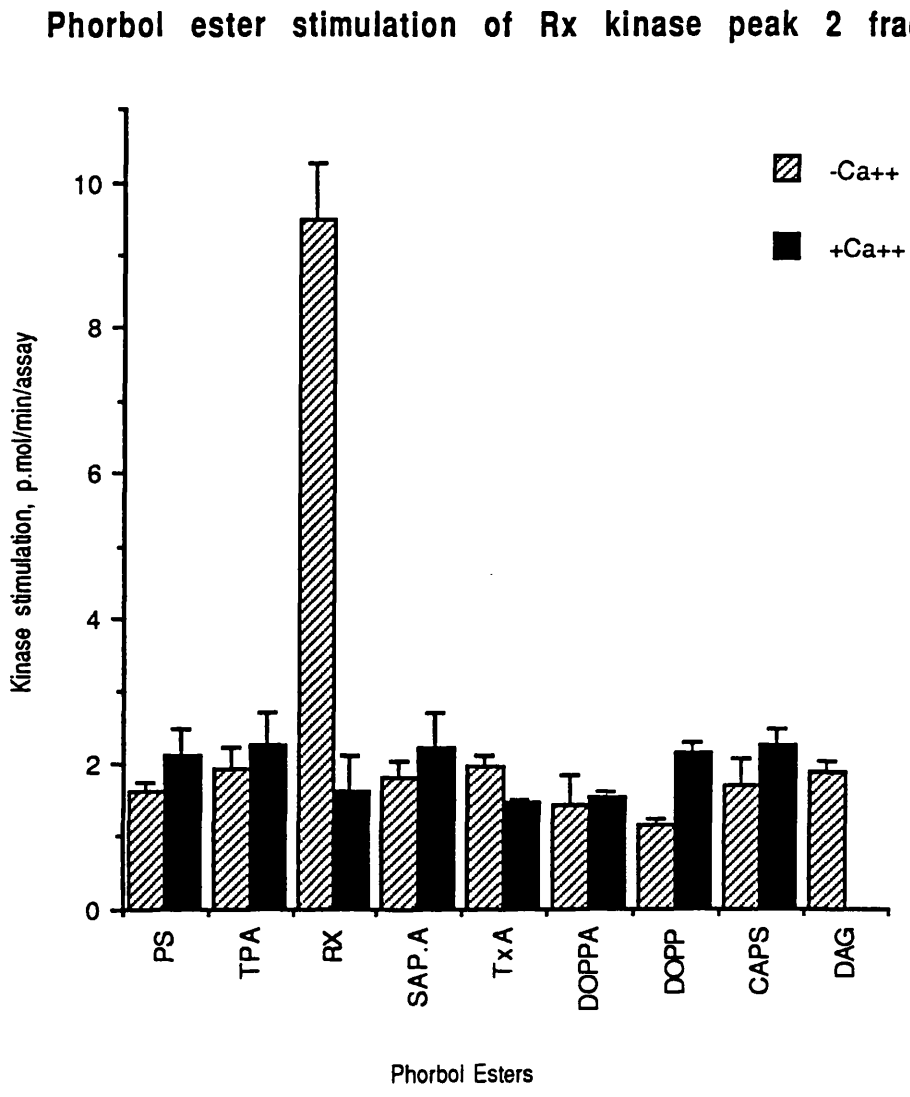


Figure 4.19 Phorbol ester stimulation of Rx-kinase peak II fraction from murine alveolar macrophages. Peak activity of previously isolated Rx-kinase was used in this study. Assays were conducted as the standard mixed micellar assay as described in the Methods with phorbol concentration at 100ngml^{-1} and CAPS and DAG at 10μgml^{-1}. Results are represented as phorbol/PS (0.75mgml^{-1}) minus PS alone and the results are expressed as mean of triplicates ±SD (n=3).

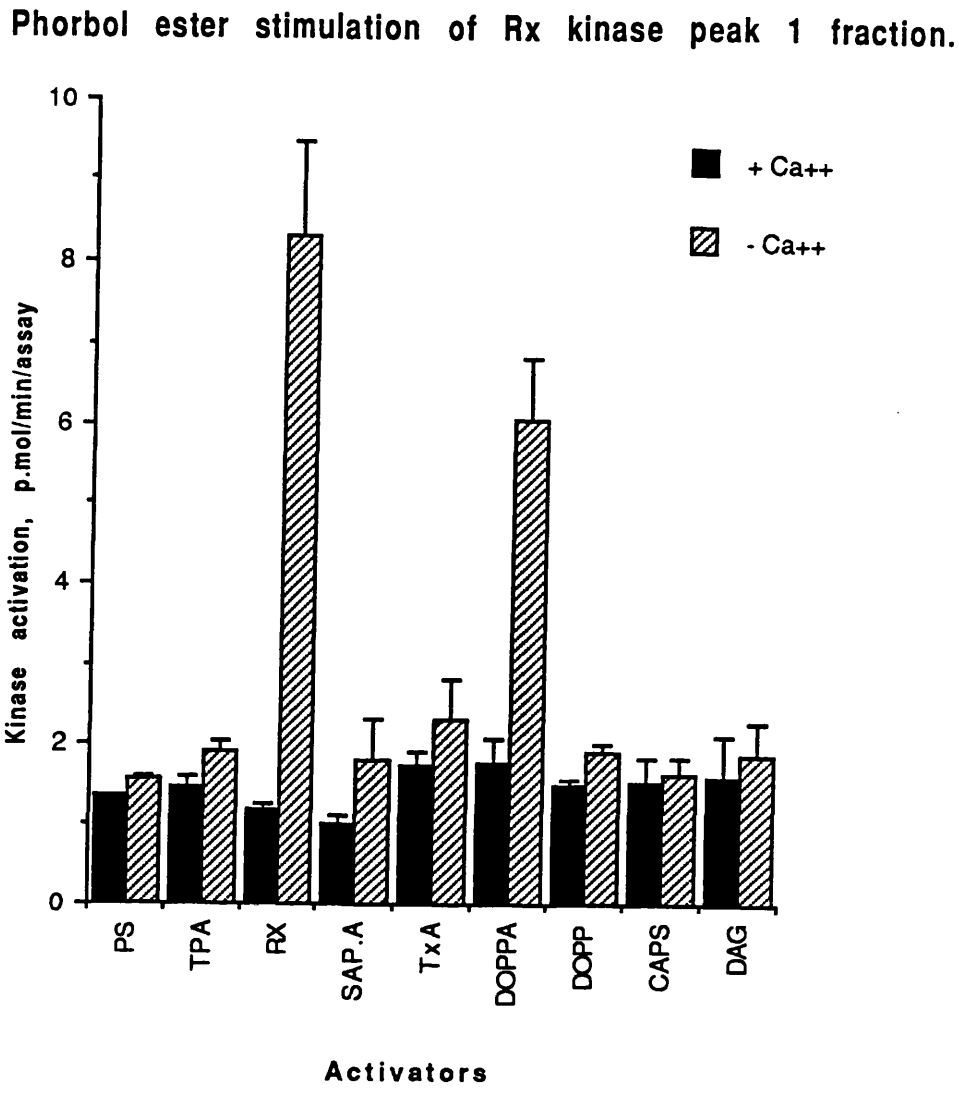


Figure 4.20 Phorbol ester stimulation of Rx-kinase peak I fraction from murine alveolar macrophages. Peak activity of previously isolated Rx-kinase was used in this study. Assays were conducted as the standard mixed micellar assay as described in the Methods with phorbol concentration at 100ngml^{-1} and CAPS and DAG at 10μgml^{-1}. Results are represented as phorbol/PS (0.75mgml^{-1}) minus PS alone and the results are expressed as mean of triplicates ±SD (n=3).

4.3.8 Further purification of Rx-kinase on Superose.

Human neutrophil Rx-kinase corresponded in terms of its elution profile from hydroxyapatite and characteristics to murine macrophage Rx-kinase. This was used for further purification on the basis that it was a readily available source than mouse macrophages. Rx-kinase activity was separated from PKC as before by removing PKC from the column in 70% phosphate gradient and then collecting at 100% phosphate (500mM). The peak of collected Rx-kinase was immediately subjected to pre-calibrated Superose 12 chromatography on a FPLC machine and eluted fractions assayed for histone-kinase activity as before. Rx-kinase was eluted from the column in fractions 17 to 20 (Fig 4.26) and corresponded to a molecular weight band between 68 to 90 kDa from a previous elution of protein standards (Fig 4.26). This Rx-kinase was extremely sensitive to freezing and thawing and loss of activity occurred over a 24 hour period.

4.4 Discussion.

Histone kinase activity, Rx-kinase, was isolated from the supernatant of inflammatory cells (and oxidative stressed tissues) and was capable of producing an oxygen burst, which on activation and binding analysis was a soluble protein receptor for the potently pro-inflammatory daphnane ortho-ester Rx (Schmidt and Evans, 1979).

Rx-kinase activity was originally detected from HMNC following HPT chromatography and was distinct from α-PKC and β-PKC on the basis of elution profile, immunological analysis and independence of Ca^{2+} as cofactor (Ryves *et al*, 1989). HMNC was discovered to be a variable source of this kinase depending upon the donor and cell incubation conditions prior to enzyme isolation (see Table 4.3; Ryves, 1991). Accordingly, human blood cells were fractionated before analysis by HPT chromatography and it was found that the histone-kinase activity was not detected in supernatants of B- and T-lymphocytes or from platelets (Ryves, 1991) but could be collected as a distinct peak of activity from human neutrophils and monocytes using a modified phosphate gradient to remove PKC isotypes before elution from HPT. In both sets of cells Rx-kinase activity was proportional to the number of cells and detected after cell purification using mixed donors (cell isolation from several donors were mixed), and seems to fit the theory that this kinase activity can be induced from cells which are 'primed' (see Chapter 3). Rx-kinase activity was not detected in cells taken from single donors but however, this may be in part due to low cell numbers. A large scale preparation using a cell separator to isolate enriched PMN and monocytes from single and multiple donors could overcome this problem and is suggested as a possible further experiment. This yield of Rx-kinase apparent under 'primed' conditions may be due to post-translational changes in a pro-kinase (eg phosphorylation, proteolysis), changes in genetic or mRNA processing, or may be compartmentalisation of the enzyme activity.

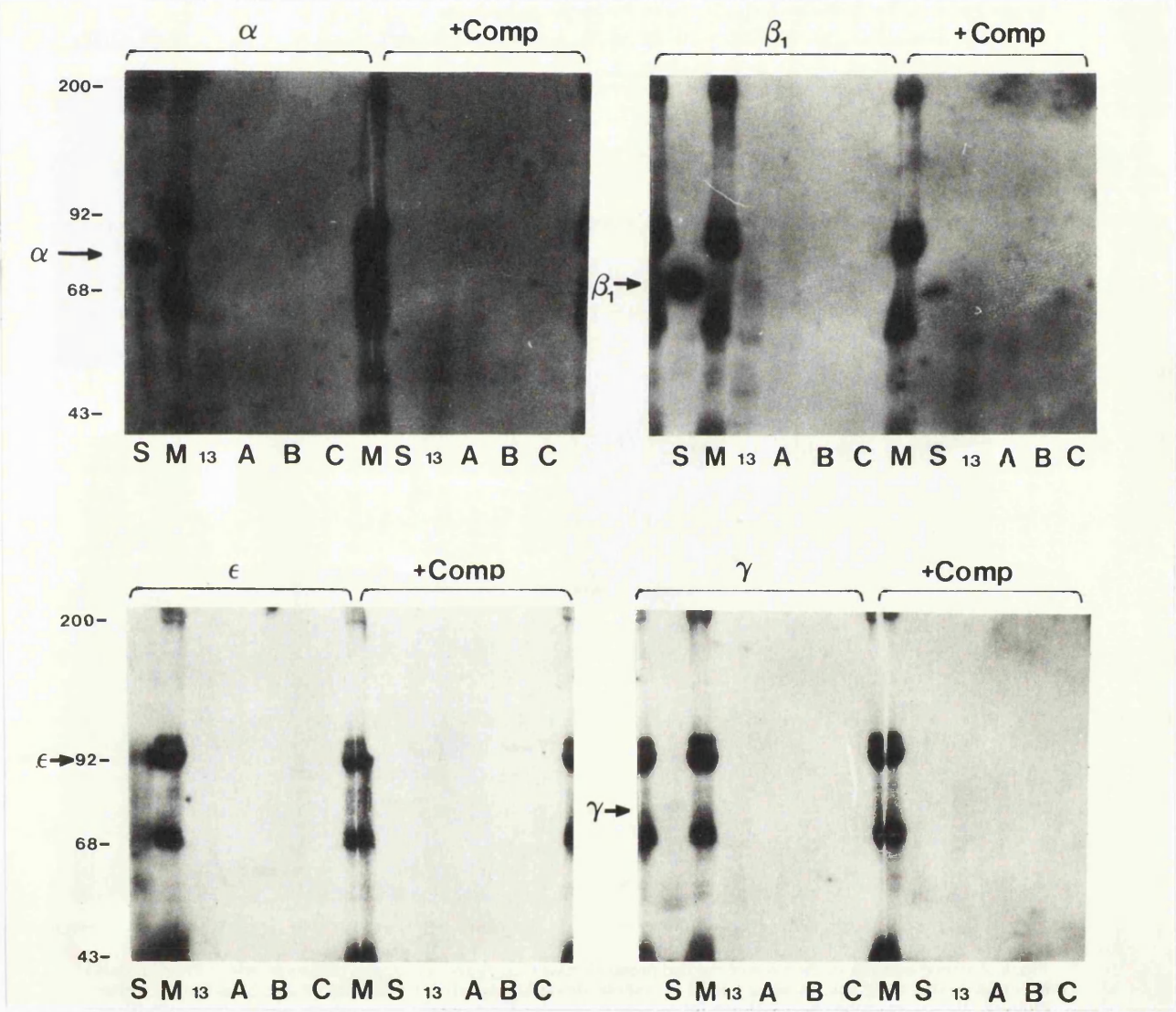


Figure 4.21 Autoradiography of dilutions of purified PKC isotypes α-, β- and γ. Lanes a, b and c represent 10-, 100, and 1000-fold dilution of PKC respectively; lane M = molecular weight markers. These data are kindly provided by Dr WJ Ryves

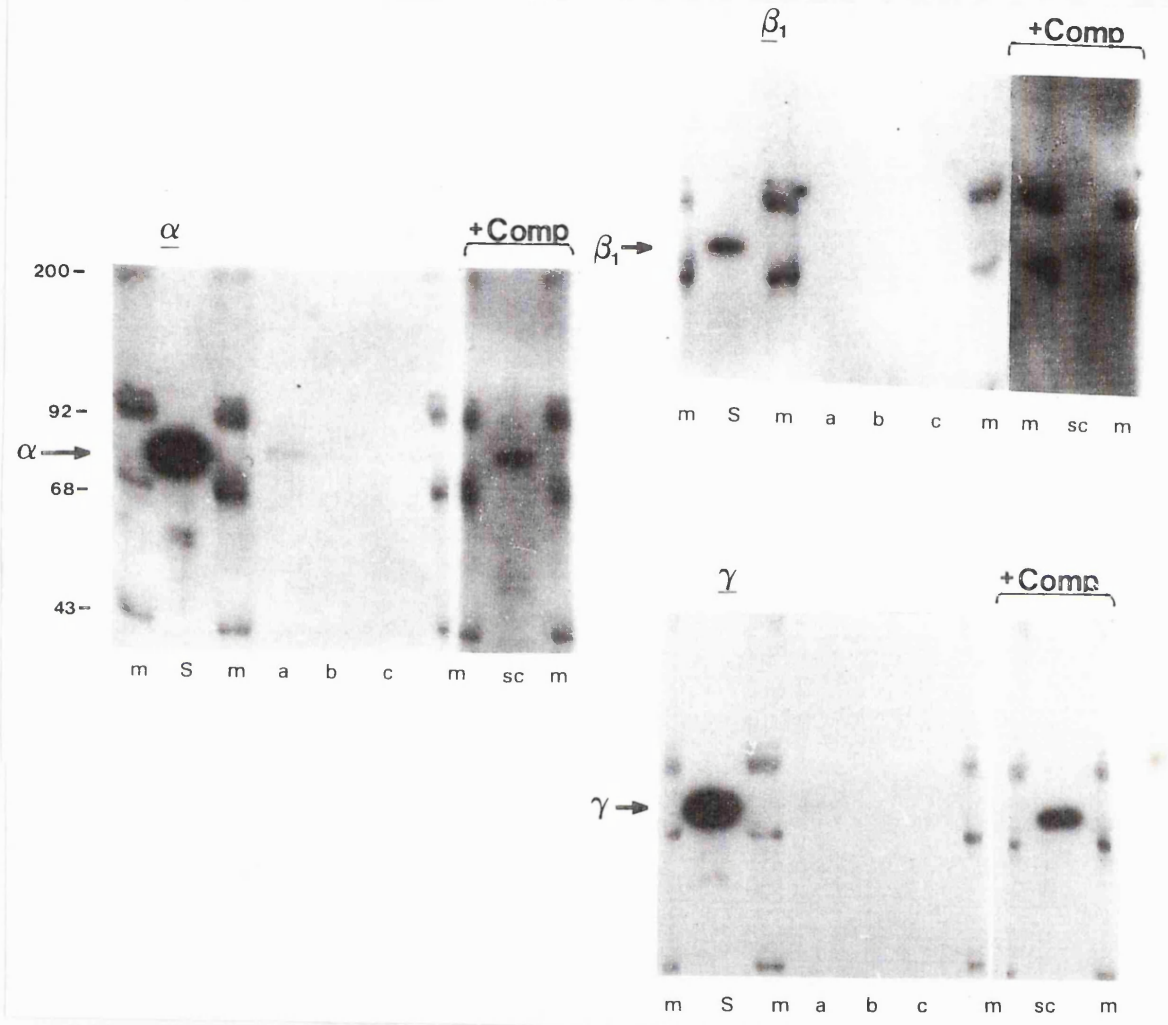


Figure 4.22 Competition autoradiography of mouse Rx-kinase samples probed for PKC α-, β-, γ- and ε. Lanes S = standards, M = molecular weight markers, 13 A, B and C = Rx-kinase fraction from 90 mice with peak activity in B. These data are kindly provided by Dr WJ Ryves.

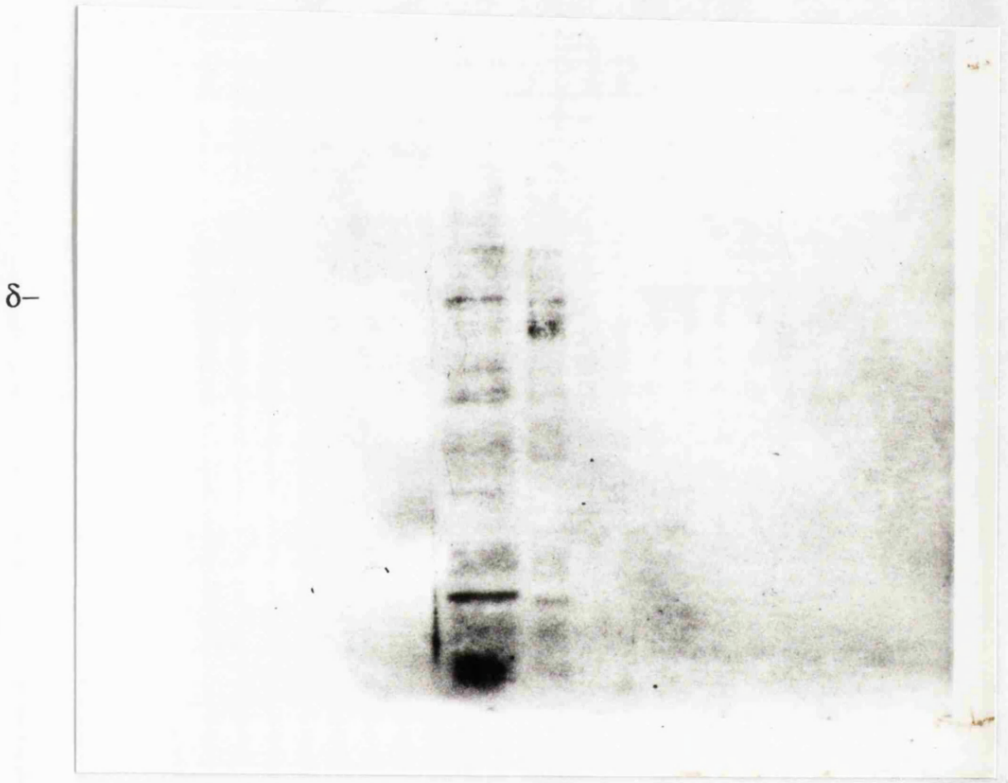


Fig 4.23 Western Blot of Rx-kinase fractions and rat brain PKC with anti-PKC δ antibody. The procedure is described in the Methods and lanes A, B, C are the most active Rx-kinase containing fractions, D and E are rat brain standards.

The occurrence of Rx-kinase in human neutrophils and monocytes was of interest in that Rx-kinase activity isolated from starch-elicited murine peritoneal macrophages has been shown to potently activate NADPH-oxidase and induce superoxide anion production (see Chapter 5; Evans *et al*, 1990). The peak of kinase activity from peritoneal macrophages was similar in its elution profile from HPT to human neutrophils and monocytes Rx-kinase and increased in total kinase activity directly in proportion to the numbers of mice used in elicitation procedures to reach a peak of 60 pmol/min/assay when 90 mice were sacrificed (Fig 4.3). The detection of the kinase in human neutrophils and monocytes and, apparent elicitation over a three day period in murine macrophages prompted an investigation of resident murine alveolar macrophages on the basis that these cells might be 'primed' for Rx-kinase activity because of being in constant contact with dust particles. In the profile of murine alveolar macrophages eluted from HPT two distinct peaks of Rx stimulated histone activity were detected (referred to as peak I and peak II, see Fig 4.4).

The latter peak was the major Rx-stimulated activity and corresponded to Rx-kinase isolated above in terms of elution profile. Peak I was encountered from alveolar macrophages for the first time and appeared to be absent from neutrophils, monocytes and peritoneal macrophages. This observation may be of interest in furthering our understanding of the mechanism of action of Rx in that it has previously been shown that Rx at concentration of $1000ngml^{-1}$ failed to stimulate the PKC isotypes α-, γ-, δ- and ε- (Ryves *et al*, 1991) in cell free assay using purified enzymes. This supports the

observation that Rx differs from phorbol esters such as TPA and the daphnane TxA in that it is not a tumour promoting agent (Adolph *et al*, 1982; Brooks *et al*, 1989). Rx has been shown to stimulate superoxide anion production in neutrophils and to inhibit agonist stimulated divalent cation influx *in-vivo* (see Chapter 6; Merrit *et al*, 1993) thereby implicating these functions in the known pronounced inflammatory effects of Rx on mammalian skin (Schmidt and Evans, 1979).

The characteristic instability of Rx-kinase activity and large numbers of cells required for large scale preparation from human neutrophils, monocytes and mouse macrophages severely restricted wider investigation of this activity. Detection of activity also depended upon pure reagents in buffer preparations since the inclusion of even small quantities of contaminating calcium could substantially influence the observed Rx-kinase activity in the assay while having little effect on rat brain PKC controls. Taking this into consideration various investigations into co-factor dependence of Rx-kinase was investigated. The Rx-kinase was consistently dependent on PS for activation. The exclusion of PS at any point in activation assay resulted in complete loss of RX-stimulatable activity.

The Rx-kinase activity as originally reported was described as Ca^{2+}-independent (Ryves *et al*, 1989). This is reminiscent of the class of nPKC isotypes of δ-, ε-, ζ- and η- (Ono *et al*, 1987; Ono *et al*, 1988; Osada *et al*, 1990; Bacher *et al*, 1991). However I have here demonstrated that Rx-kinase is in fact calcium inhibited with K_i of 0.1-

Quantity of blood (ml)	Days in Culture	No. of fractions with Rx-kinase	Peak Rx-kinase activity (pmol/min/assay)
200	8	31	9.3
450	1	31	13
450	3	34	8.9
2000	0	33-35	10.1

Table 4.3 Peak Rx-kinase activity of human mononuclear cells. Cells were cultured for various days and homogenised, assayed according to text (see Chapter 2). These results are used with the kind permisson of Dr WJ Ryves.

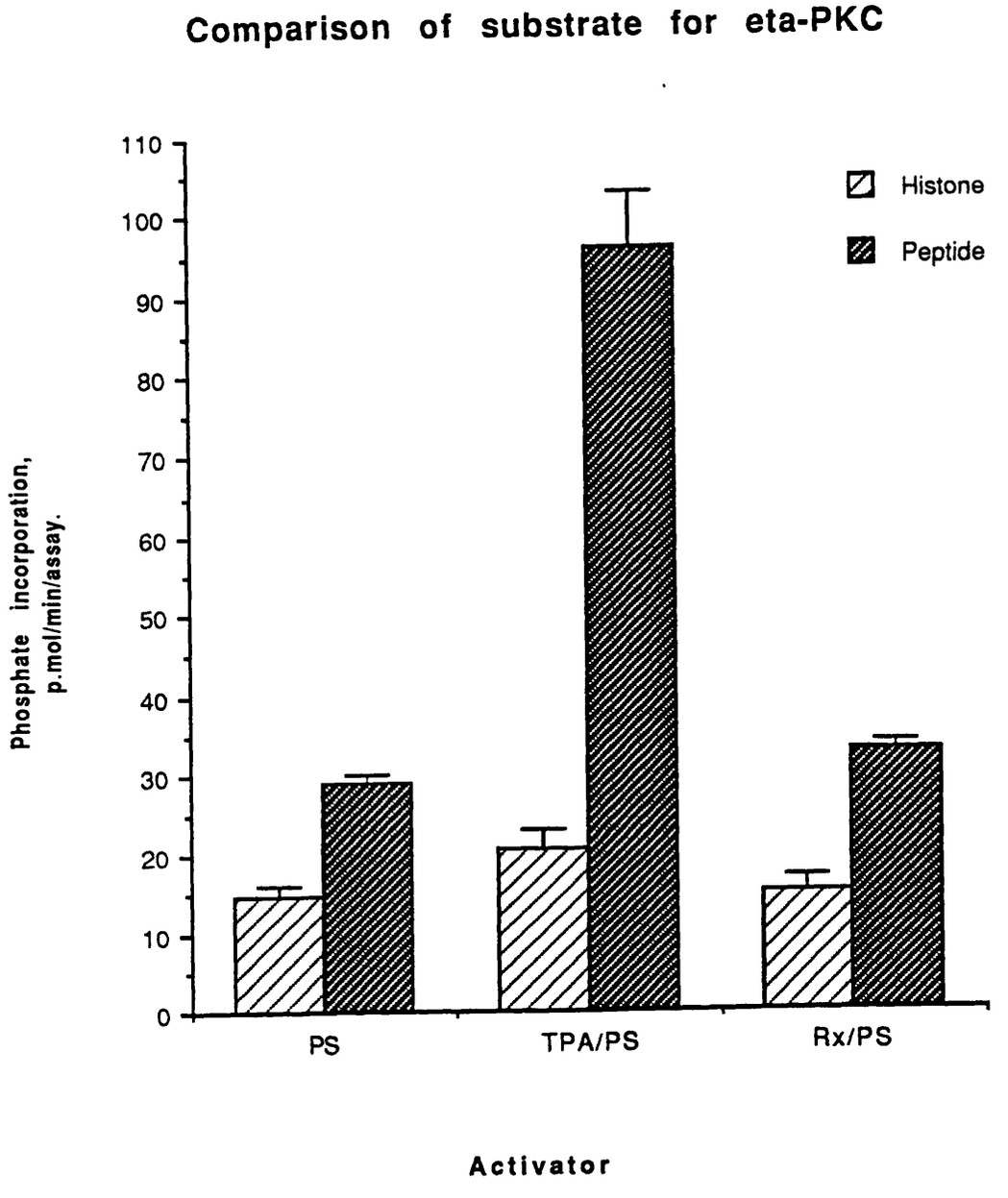


Figure 4.24 Comparison of substrate for η-PKC. η-PKC was assayed for phorbol ester activation in the presence of Histone IIIs (0.625 mg/ml) or pseudosubstrate (0.25μg/ml), PS (1.25mg/ml) and TPA (100ng/ml) or Rx (100ng/ml). Results are expressed as mean of triplicates ±SD(n=2).

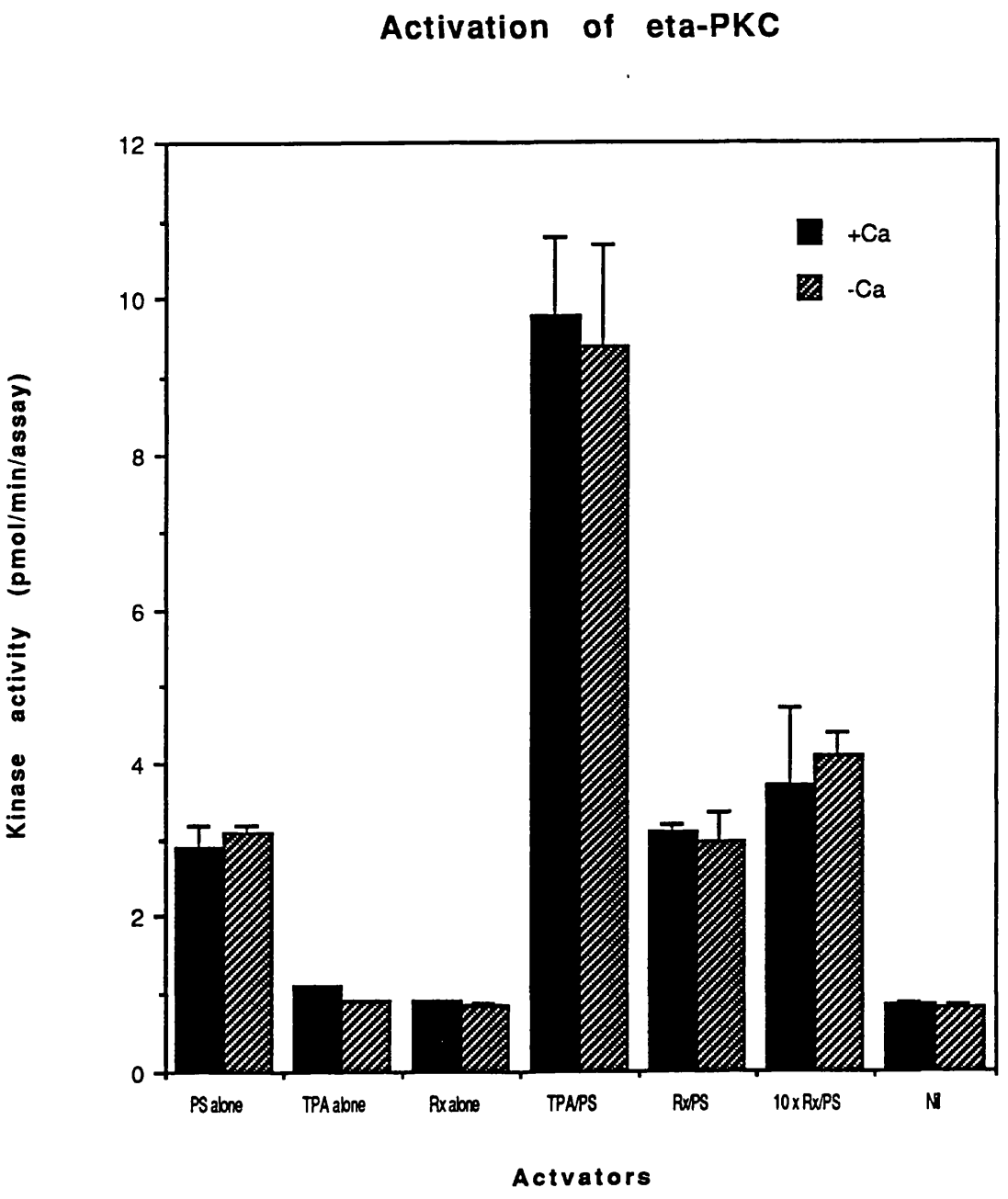
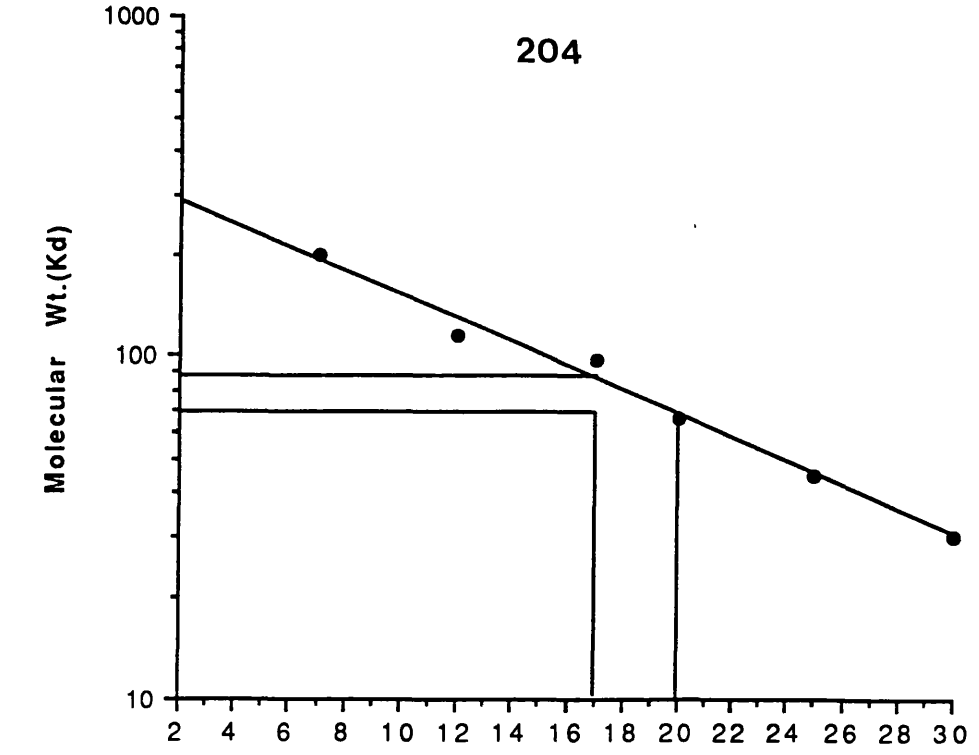


Figure 4.25 Comparison of activation of η-PKC. η-PKC was assayed for phorbol ester activation in the presence of Histone IIIs (0.625 mg/ml) or pseudosubstrate (0.25μg/ml), PS (1.25mg/ml) and TPA (100ng/ml) or Rx (100ng/ml) in the presence and absence of added Ca^{2+} (100μM free ions). Results are expressed as mean of triplicates ±SD (n=3).

0.5nM free Ca^{2+} as calculated from the 'chelate-Steinhardt's' computer programme in a cell free assay. It is likely on this basis that at resting physiological Ca^{2+} levels in cellular systems the activity could also be inhibited, but the fact that in human neutrophils at least, Rx has been reported to inhibit divalent cation influx (Merrit *et al* , 1993) together with Rx-kinase apparent Ca^{2+} inhibition may point to at least one physiological role for the kinase apart from the previously known effects of increasing superoxide production (Evans *et al*, 1990). This might offer a partial explanation for the extra irritancy of this daphnane ester when phorbol/daphnane receptors (Evans *et al*, 1991) and kinase cascades have been more fully defined (Charriaut - Marlangue *et al*, 1991).

The information on the tissues distribution of Rx-kinase would help our understanding of its role(s) in normal and possibly more importantly in pathophysiological circumstances. Therefore tissue distribution studies were conducted on Rx-kinase by Rx-stimulation of HPT elution fraction in both activated and resting tissue (Fig 4.10 and Fig 4.11). Rx-kinase activity from untreated tissues was only observed in rat lungs. This activity was similar to alveolar macrophages with distinctive peaks (I and II) and the latter peak (II) corresponding to previously described Rx-kinase in terms of elution profile. All possible precautions were taken to limit alveolar macrophage contamination but the effect of interstitial macrophages cannot be ruled out. No activity was observed in rat brain tissue of both untreated or plumbagin treated conditions. This was significant due to the fact that only θ-PKC has previously been reported not to be



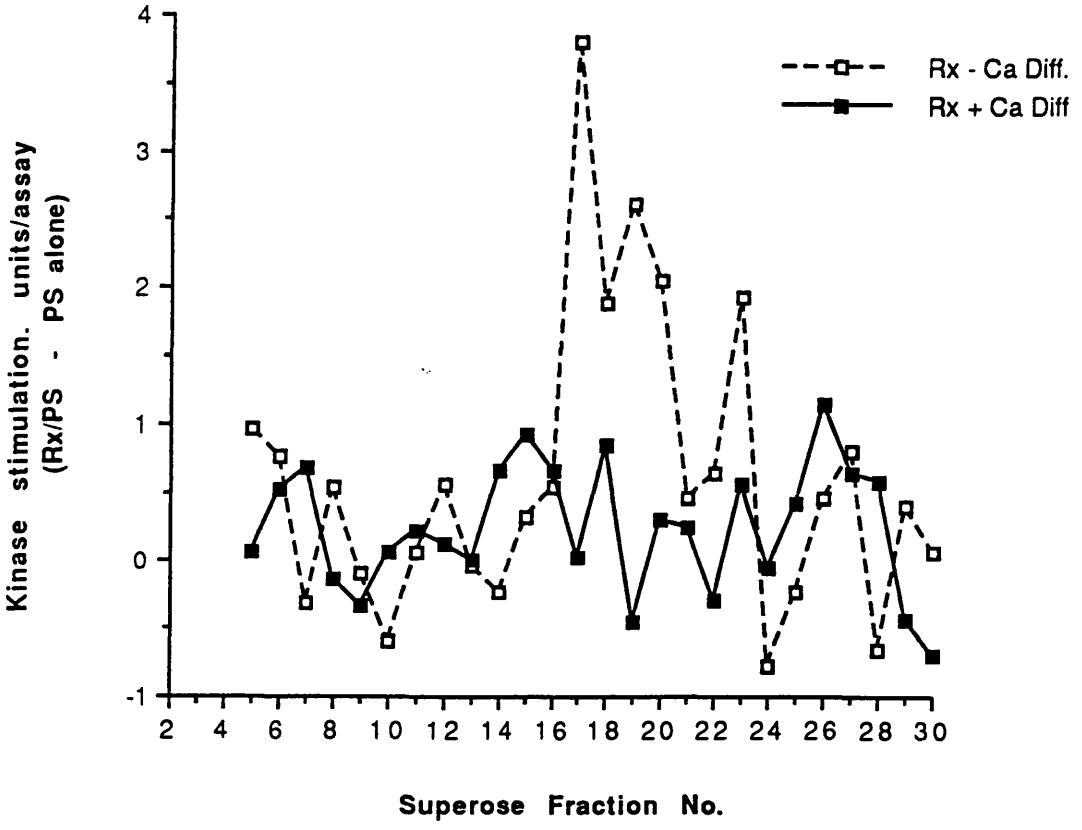


Figure 4.26 Superose-12 chromatography of Rx-kinase. 1 x 10^8 human neutrophil homogenates was subjected to HPT chromatography. After elution of bulk PKC with 350mM phosphate buffer the gradient was increased to 500mM phosphate and eluents were immediately subjected to pre-calibrated superose column. Fraction collected were assayed according to the mixed micellar assay described in Methods. These data are a representative of two separate experiments.

Table 4.4 Sources of Rx-kinase and its activity.

Cells	Observed activity
Peritoneal Macrophage	+++
Alveolar Macrophage	+++
Monocytes	+++
HMNC	+++
Neutrophils	++
Thymocytes	+
Tissue	
Lung	+++
Heart	+
Thymus	+
Liver	-
Brain	-

+++ Strong activity
++ Activity
+ Weak activity
- No activity

expressed in brain (Osada *et al*, 1992). The importance of this has to be further characterised before any conclusions can be reached.

Plumbagin treatment of phagocytes have produced Rx-sensitivity (see chapter 3) and ultimately enzyme induction. Plumbagin treated tissues expressed Rx-kinase in terms of activity from thymus, heart and spleen (Fig 4.11). These activities were low but nevertheless significant. Liver possessed no observable activities from both conditions of treatment. As a Ca^{2+}-inhibited activity expressed in several tissues this has major implications for a comprehensive physiological role of cellular Ca^{2+} levels in homeostatic and signalling processes. The Rx-kinase activity expressed in various cells and tissues are all summarised in Table 4.4.

The Rx binding receptor has not previously been isolated although in rat and pig dorsal root ganglion cells (Szallasia and Blumberg, 1990a) and particulate preparation of pig dorsal root horn (Szallasia and Blumberg, 1991) a binding analysis involving ^{3}H-Rx indicated specific binding despite the high reported levels of non-specific binding. Using purified alveolar macrophage Rx-kinase (peak II) I attempted to determine a Scatchard analysis using ^{3}H-PDBu. The binding of phorbol esters to soluble PKC receptors was successfully demonstrated elsewhere (Driedger and Blumberg , 1980) using ^{3}H-PDBu as an optimum derivative to reduce non-specific effects. The assay has since been used to demonstrate marked differences between phorbol esters (Ellis *et al*, 1987) to inhibit ^{3}H-PDBu binding to pool PKC isolates. In these latter experiments

Rx was found to be a weak inhibitor of ^{3}H-PDBu binding compared to TPA thereby confirming the comparative inability of Rx to activate PKC isotypes (Ryves *et al*, 1991). This observation led us to suppose that the use of ^{3}H-PDBu in a binding analysis on purified Rx-kinase would reduce non-specific binding. The strategy proved to be useful in that following analysis, non-specific binding was recorded as only 10 -15% of total binding. The Scatchard plot exhibited a linear relationship between the ratio of bound to free versus bound suggesting a reversible binding to a single population of receptors possessing a single affinity for the ligand with B_{max}=11.6 fmol/U and K_d = 230 fmol/U (41.6nM) (Fig 4.13).

In a competitive binding study of the ability of Rx and TPA to inhibit ^{3}H-PDBu binding to purified Rx-kinase, Rx was shown to be approximately 1000-fold more effective than TPA in this respect . These experiments indicate a distinct difference between the phorbol ester binding sites of PKC and Rx-kinase in that TPA preferentially binds to PKC whilst Rx binding was largely to Rx-kinase. Rx contains an aromatic moiety in its structure together with a strained orthoester function and this increased electronegativity of the molecule may account for non-specific binding when ^{3}H-Rx is used *in-vitro*. Small quantities of ^{3}H-Rx was available from a parallel synthetic programme (F.J. Evans, in preparation) and with this radioligand a binding analysis was attempted on purified Rx-kinase. Rx-kinase binding to ^{3}H-Rx specifically was demonstrated despite the high but expected non-specific binding encountered. The competitive inhibition of ^{3}H-Rx binding Rx-kinase was studied using unlabelled Rx.

Since the concentration of ^{3}H-Rx was high (1mgml^{-1}) but specific activity relatively low (0.6mCi/mmol) the competitive binding required high doses of unlabelled competitor. Nevertheless unlabelled Rx added in increasing concentration was able to inhibit this binding with 50% inhibition occurring at a 10-fold dilution.

The ability of Rx to activate the enzyme was compared to other phorbol derivatives at the concentration of 100ngml^{-1}. The results of this experiment confirmed the data from binding studies in that TPA failed to significantly stimulate alveolar macrophage Rx-kinase in comparison to Rx and neither did other phorbol derivatives DOPP, DOPPA, SAP-A and the daphnane TxA. This was in agreement with previous results using Rx-kinase purified from starch-elicited murine peritoneal macrophages (Ryves, 1991). The K_a for activation of Rx-kinase by Rx was calculated to be 10ngml^{-1} and thus would indicate specific structural requirements in the daphnane phorbol ester series of derivatives for activation of PKC or RX-kinase. DAG at concentrations which activate PKC in a Ca^{2+}-dependent manner was not capable of activating Rx-kinase. The biological actions of Rx have been claimed to be that of an 'ultrapotent capsaicin analogue' (the inflammatory agent of red peppers (Szallasia and Blumberg, 1989)), whereupon a proportionally high dose (10ugml^{-1}) of capsaicin was tested for activation of Rx-kinase. Capsaicin failed to stimulate any Rx-kinase activity suggesting that while the compounds may share some pathways in exertion of biological activity, they cannot be classed together in all facets.

An immunological analysis was carried out on Rx-kinase from murine peritoneal macrophages using anti-sera prepared against PKC isotypes α-, β-, γ-, δ- and ε- (see methods, section 4.2). These results confirm that Rx-kinase is distinct from c- group of PKC isotypes and from Ca^{2+}- independent δ- and ε- PKC. By using dilution of PKC preparations of known kinase activity in subsequent immunological tests it was established that PKC activity equivalent 1/10th the Rx-kinase activity was still detectable. In these studies an antibody for PKC-η was not available but a direct comparison was carried out between purified PKC-η and purified Rx-kinase. PKC-η has been reported from lung tissue (Osada *et al*, 1990) but here I have demonstrated that using a PKC-η pseudosubstrate TPA is a potent activator of PKC-η in the presence or absence of Ca^{2+} whilst Rx failed to stimulate the isotype denoting a significant difference between the two enzymes. Furthermore PKC δ- and ε- members of the Ca^{2+}-independent PKC group are known to elute from HPT in the region of other PKC isotypes (Ways *et al*, 1992) well before Rx-kinase elution.

Rx-kinase is unstable and suffers a loss of enzyme activity on freezing and re-thawing to a greater extent than PKC isotypes when kept under identical conditions (data not shown). However, when Rx-kinase taken directly form HPT elution was further chromatographed on Superose 12 a peak of Rx sensitive activity of molecular weight 68 to 90KDa was recovered. This purified kinase was within the molecular weight range of the n-PKC group of isotypes but from Ca^{2+} cofactor requirements, immunological analysis and direct comparison for activation differs from other

members of this expanding family of kinases. Peak I Rx-kinase from alveolar macrophage was not further investigated in this study but it was activated by the phorbol ester DOPPA although to a lesser extent than by Rx. DOPPA has been shown to activate PKC-β_I in cell free assays (Ryves *et al*, 1991) but not other PKC isotypes up to a concentration of 1000 $ngml^{-1}$. It has recently been implied that PKC subjected to hydrogen peroxide stress can change its conformation and elute from DE52 as a distinct peak of Ca^{2+}- independent activity, reminiscent of the n-PKC group of isotypes (Palumbo *et al*, 1992). However this peak of activity still immuno-reacted for PKC without molecular weight change by Western blotting. It is possible that peak I from alveolar macrophages (and possibly from rat lung tissues) represents a stressed PKC isotype or a proteolytic fragment but is distinct from the major Rx receptor described here.

The mechanism of action of Rx is unknown but the purification of a major receptor for the toxin as described here may explain the high potency of this plant secondary metabolite and provide a novel target for the development of anti-inflammatory agents.

CHAPTER 5 Partial Purification and Activation of NADPH-oxidase

5.1 Introduction.

Among their microbicidal mechanism, phagocytes such as neutrophils initiate a 'respiratory burst' (Babior, 1978), during which there is a marked increase in non-mitochondrial oxygen consumption with a generation of oxygen derived species such as superoxide anion (O^-_2) and H_2O_2. The pivotal importance of the respiratory burst in combating infectious disease is illustrated by the inherited conditions chronic granulomatous disease (CGD) wherein the neutrophils fail to generate oxidants, and afflicted individual suffer frequent and severe infections (Smith and Curnutte, 1991). Oxidant generation is initiated by the activation of the superoxide anion-generating respiratory burst oxidase or NADPH-oxidase (Babior, 1984). The oxidase, dormant in unstimulated cells, can be activated by a variety of stimuli including opsonised particles (eg bacteria, zymosan), protein kinase activators such as diacylglycerol and phorbol esters and chemoattractants such as formylmethionylleucylphenylalanine (fMLP) (for review see Lambeth, 1988).

Complete characterisation of NADPH-oxidase has been hampered by rapid loss of activity following detergent solubilization (Babior and Peters, 1981; Tamura *et al*, 1989) but, using gel filtration chromatography this area has looked promising (Bellavite *et al*, 1985). However a cell free system obtained from non-activated phagocytic cells has been developed (Curnutte *et al*, 1987; McPhail *et al*, 1985) and has allowed recent advances in understanding the protein components and regulatory factors which participate in the respiratory burst. The system consists of cytosol,

plasma membranes and presence of an anionic amphiphile such as sodium dodecyl sulphate (SDS) or arachidonic acid. The use of molecular cloning, together with studies using cell fractions from CGD neutrophils in the cell free system, has led to the definitive identification of several components of the respiratory burst oxidase. The plasma membrane associated cytrochrome b_{-245} consists of two subunits of apparent molecular weight by SDS-PAGE of 92 kDa and 22 kDa, both of which have been cloned and sequenced (Royer-Pokara *et al*, 1986; Parkos *et al*, 1988). The cytochrome is generally assumed to be the terminal oxidase which univalently reduces oxygen to generate superoxide anion ($O^{\cdot}_2$)(Cross *et al*, 1984). cDNAs encoding two cytosolic components, p47-phox and p67-phox have been cloned and sequenced (Lomax *et al*, 1989, Volpp *et al*, 1989; Leto *et al*, 1990), but the absolute functions of these proteins remains unclear. Specifically, they do not show sequence homology to known electron transport or nucleotide binding proteins. In addition, there are number of suspected components and/or activators including a NADPH binding species and/or a flavoprotein (Gabig, 1983), and GTP binding proteins (Segal and Abo, 1993).

The mechanism by which the oxidase is activated is controversial. A role for DAG/PKC is supported by the following : (a) PKC activators phorbol esters (De Chatelet, *et al*,1976) and some diacylglycerol (Cox *et al*, 1986; Bass *et al*, 1989) mimic some aspects of activation by receptor-linked agonist such as fMLP; (b) fMLP induces hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) resulting in the

generation of diglyceride (DG) plus calcium messenger Inositol 1,4,5 -trisphosphate (IP_3) (Ohta *et al*, 1985; Smith *et al*, 1986); (c) for a variety of agonists (fMLP, ionomycin, concanavalin A), elevation in the concentration of DG correlates with activation of 0^{-}_2 generation (Rider and Niedel, 1987); (d) long chain bases (Sphinganine and Sphingosine) and staurosporine, which are known inhibitors of PKC block the activation of the respiratory burst by variety of agonists (Wilson *et al*, 1986; Dewald *et al*, 1989). On the other hand, fMLP and other receptor linked agonists generate multiple second messengers (DG, AA, Ca^{2+}, cAMP), and additional pathways which do not require PKC have been proposed (McPhail, *et al*,1984). In particular, agonist induced phosphoinositide hydrolysis cause an increase not only in DG but also Ca^{2+}, so that activation usually correlates with an increase in both putative mediators (Korchak *et al*, 1984). An obligatory role for PKC has also been contested, since some compounds which act as PKC inhibitors under *in-vitro* conditions (isoquinolinesulphanomides, polymyxin B) fail in whole cells to antagonise neutrophil activation by fMLP (Gerard *et al*, 1986; Naccahe, *et al*, 1985). In cell-free systems activation by DAG has been shown to act in concert with SDS and a G protein to markedly enhance activation of the NADPH-Oxidase in human neutrophils by PKC-independent mechanism (Ulhinger *et al*, 1991). In light of such conflicting reports it was decided to examine NADPH-Oxidase at both the active state (ie. isolated after pre-stimulation of cells) and reconstituted but stimulatable cell-free system.

5.2 Methods.

5.2.1 Isolation of Human Neutrophils and Murine Macrophages.

200 mls Neutrophil enriched blood were obtained from healthy donors using a cell separator unit (courtesy of Department of Haematology, UCH, London) and neutrophils isolated by density centrifugation as before (see chapter 4). Briefly equal volumes of blood was layered onto a histopaque 1119 medium and centrifuged at 700 x g x 30 mins. The plasma / histopaque interface containing the neutrophils were recovered and washed by centrifugation (400 x g x 10 min, x2) in supplemented RPM1 1640 medium (see chapter 2, section 2.3.1) and incubated in ice-cold culture medium containing 10 $ugml^{-1}$ leupeptin and 1mM PMSF for 30 mins at 4°C prior to homogenisation. Starch-elicited murine peritoneal macrophages were isolated as described in chapter 3 (section 3.2.1)

5.2.2 Isolation of Cytosolic and Membrane Fractions.

Approximately 10^9 cells were collected and lysed by sonication (3 x10 s) at 4°C in buffer-1 (see section 2.3.2a). The homogenate was initially centrifuged (1100 x g x10 mins) and the post-nuclear supernatant layered onto a discontinuous sucrose gradient (10% /40%). After centrifugation (100,000 x g x 1hr) the band between 10% and 40% sucrose gradient (membrane fraction) was stored at -70°C. The cytosol

recovered from 10% sucrose was re-centrifuged at 100,000 x g x 1 hr and supernatant stored at -70°C (cytosolic fraction).

5.2.3 Partial Purification of NADPH-Oxidase

1 - 5 x 10^9 cells were treated with agonist (eg TPA at 100 $ngml^{-1}$) for five mins, washed in buffer-1 followed by sonication (3 x 10 s) at 4°C. The homogenate was initially centrifuged as above and layered onto a discontinuous sucrose gradient. After centrifugation (100,000 x g x 1hr) the membrane fractions were recovered and further solubilised with 0.4% lubrol Px (v/v) and 0.4% Na deoxycholate (w/v) in buffer-2 (see section 2.3.2 d). The solubilised membrane was further ultracentrifuged at 100,000 x g x 1hr and the supernatant (4 ml) was loaded onto sephacryl S-300 column (1.6 x 30 cm) connected to FPLC machine pre-equilibrated with buffer-2 containing 0.1% Lubrol Px, 0.1% Na deoxycholate and eluted with same buffer . Fractions of 1ml were collected at a flow rate 0.4ml/min. Wherever possible assays were analyzed immediately, otherwise stored in Lq N_2 (however enzyme was susceptible to degradation with loss of activity even over a 24 hr period).

5.2.4 Assay of NADPH-oxidase.

Simple detection of oxidase partially purified by phorbol ester (or other agonist, eg fMLP) stimulated phagocytes were analyzed using the SOD inhibitable reduction of

Superoxide production (nmol/min/mg)				
	Post nuclear supernatants	Membrane fractions	Solubilised extracts	Peak activity
NADPH-oxidase activity	3.94	30.73	31.91	60.65
Purification factor	1	7.8	8.1	15.4

Table 5.1 NADPH dependent superoxide forming activity. Neutrophils (5 x 10^9) were pre-treated with 100ngml^{-1} TPA prior to homogenisation. At each stage of the purification stage, aliquots were assayed for NADPH-oxidase activity. Total protein was determined by Sigma Total Protein Lowry kit.

cytochrome C as described in chapter 2 (section 2.3.3.1). All reagents were dissolved in buffer-2 and the reaction was started by the additions of NADPH-cofactor (100uM) and continuously monitored on dual beam spectrophotometer at 25°C with change in absorbance measured at 550nm. For studying the effects of phorbol ester (PE) on the oxidase complex, PE was diluted in 50% ethanol and final volumes were balanced by adjusting the volume of buffer-2. For kinetic analysis of various sources of oxidase, NADPH-cofactor at concentration of 20 - 200 uM was used as a substrate in equal volumes (100 ul) dissolved in buffer -2.

5.2.5 Effect of kinases on reconstituted oxidase complex.

For studying the effect of kinases on reconstituted enzyme complex employed the method described in chapter 2. The assay was prepared by mixing the constituents (see section 2.3.3.2) and, the mixture was then incubated at room temperature for 5 mins. The reaction was started by adding NADPH cofactor (100uM) and continuously monitored on a spectrophotometer with change in absorbance measured at 550nm.

5.3 Results.

5.3.1 Partial purification of active NADPH-oxidase.

Plasma membranes of TPA activated neutrophils were purified by sucrose density centrifugation as described in Methods. Solubilisation of the membranes was then

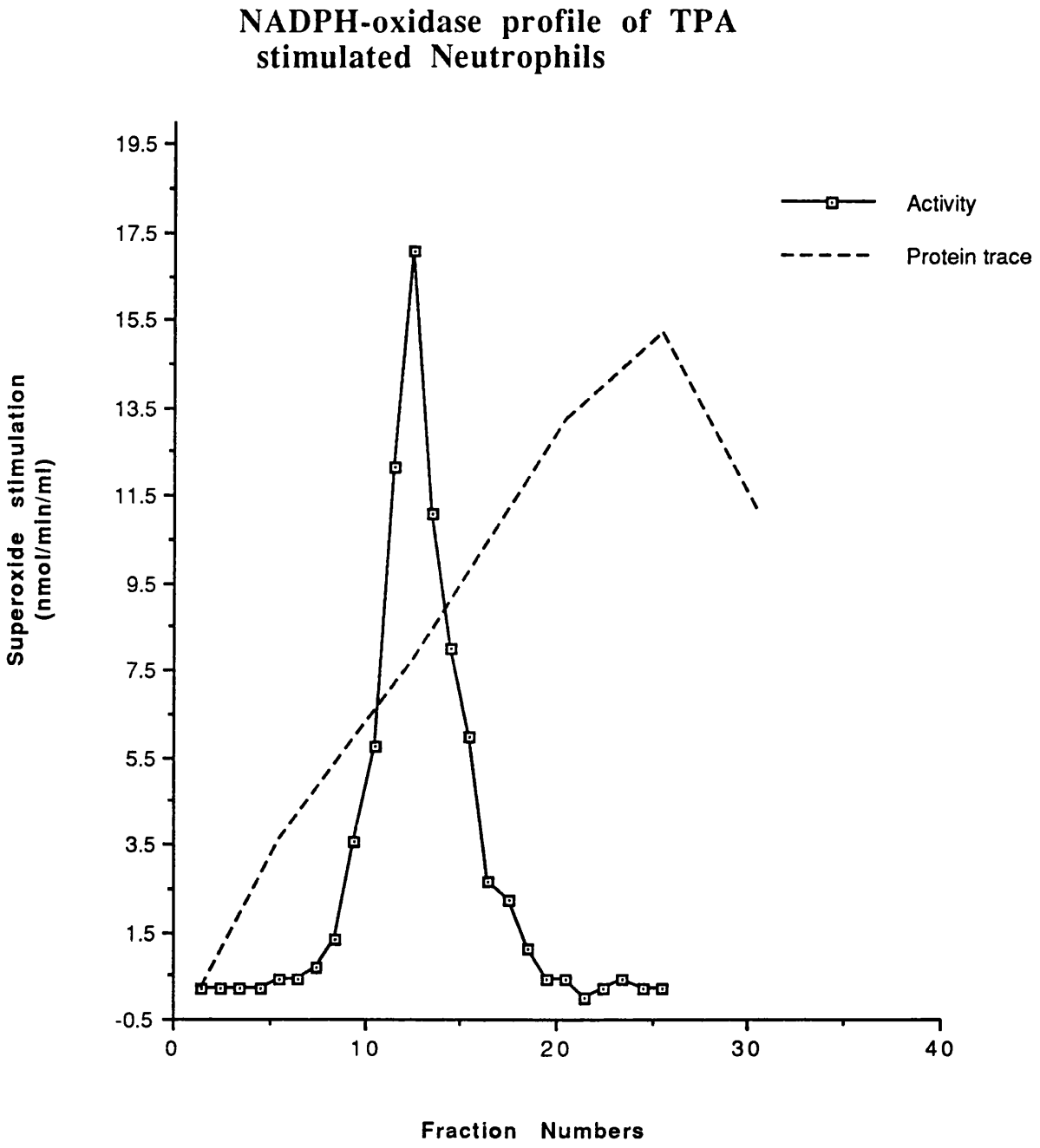


Fig 5.1 NADPH-oxidase profile of TPA stimulated neutrophils. Approximately 1 x 10^9 cells were treated with TPA (100ngml^{-1}) prior to homogenisation. After isolation (as described in methods), the membrane was solubilised with 0.4% lubrol PX and 0.4% Na deoxycholate. The resultant supernatant was chromatographed through sephacryl S-300 and 1ml fractions were collected at a flow rate of 0.4mlmin^{-1}. The protein trace shown is a typical profile generated by measuring absorbance at 280nm. Result expressed is a typical profile generated by TPA stimulated neutrophils.

performed with a mixture of deoxycholate and Lubrol Px, which is thought of as a 'mild' treatment and preserves the NADPH dependent O_2^- forming activity as previously reported (Bellavite *et al*, 1984). Initially a series of known molecular weight markers were standardised through the column (data not shown). Void volume (Blue dextran) eluted at fraction 11 (Fig 5.1). The elution profile of the gel filtration chromatography revealed a single peak of NADPH-oxidase activity (Fig 5.1). This peak corresponded to the void volume of the column, indicating an aggregated material with molecular weight > 205 kDa.

Table 5.1 reports the specific activity or content and the purification factors of NADPH-oxidase in various fractions obtained at different stages of purification procedure. Data refers to post-nuclear supernatant, membrane fraction, solubilised extract and pooled fractions of the peak obtained from gel filtrations (fractions 9-16). NADPH-oxidase activity increased by 8-fold in the purification step from post-nuclear supernatant to the membrane fraction and a further 2-fold by gel filtration chromatography. As expected solubilisation of the membranes by Lubrol and deoxycholate seemed to have preserved the activity.

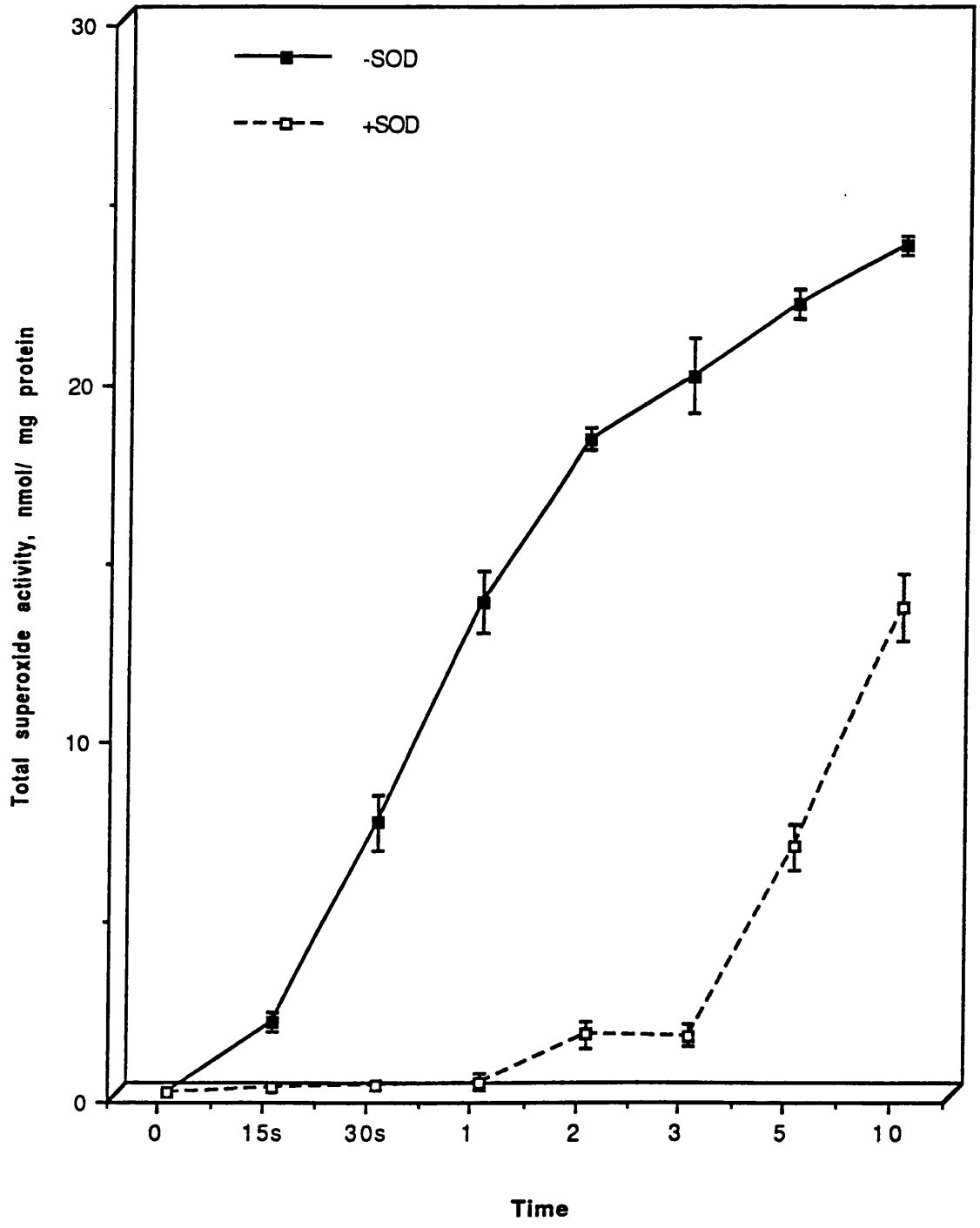
5.3.2 Time and concentration dependency of active oxidase

NADPH-oxidase was partially purified from TPA stimulated neutrophils, and assays were performed as described (see section 5.2). In the absence of superoxide

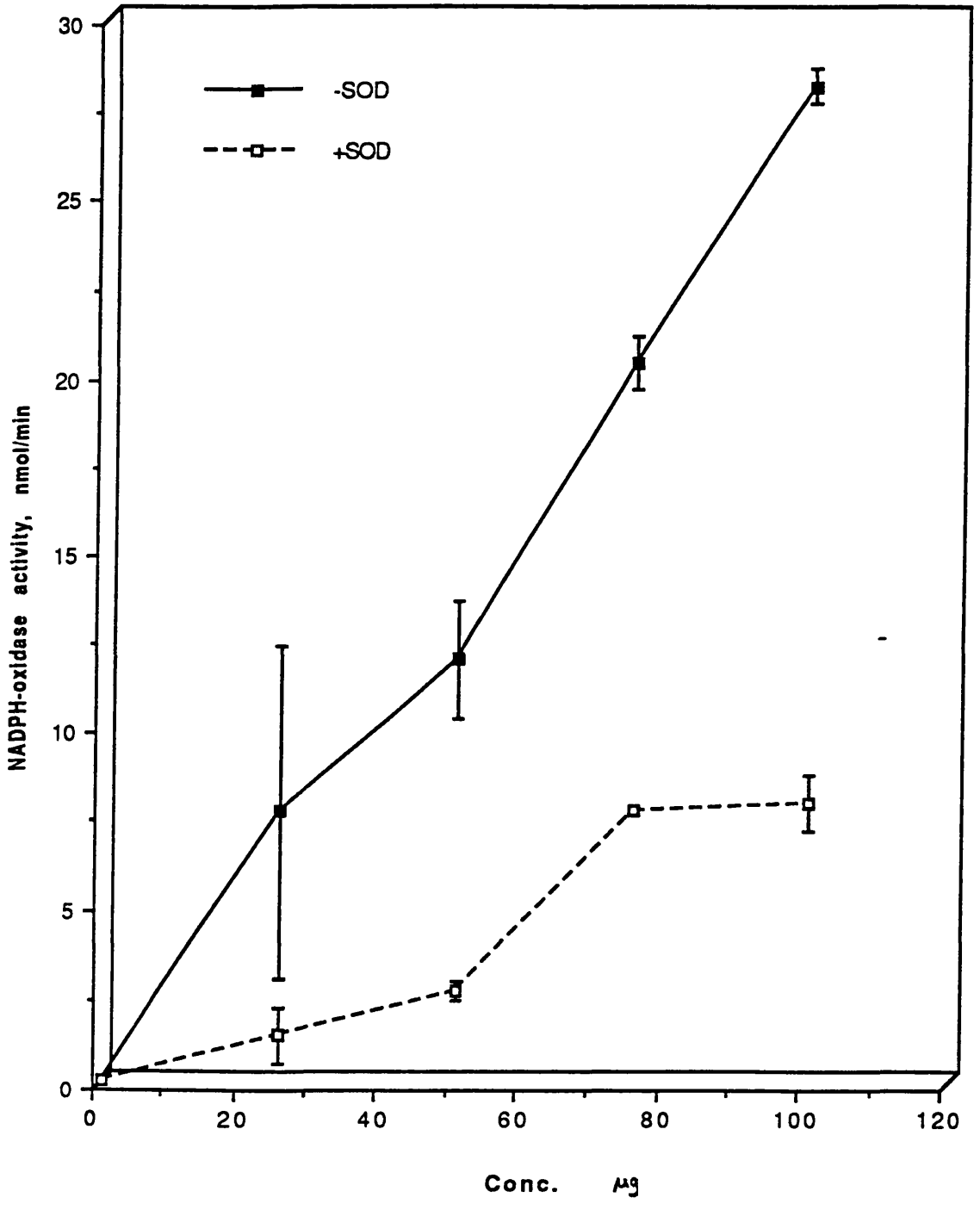
Legends for Figure 5.2 and Figure 5.3 Time and Concentration dependency of NADPH-oxidase activity.

The rate of superoxide anion generation was determined at various time (0-10 min; Fig 5.2) and concentrations (0-100 µg urified enzyme; Fig 5.3) both in the presence and absence of SOD (300U). NADPH-oxidase was partially purified by Sephacryl S-300 chromatography from TPA (100ng/ml) pre-treatment of human neutrophils and assays conducted as described in the text. Data are expressed as mean ±SEM (n=4).

Time course for NADPH-oxidase



Concentration dependent activity of NADPH-oxidase



dismutase (SOD), the reaction rapidly increased within 1 min and was steady by 2-3 mins (Fig 5.2). The presence of SOD delayed the increase of enzyme activity to 3 mins but was rising by 5 mins. For all subsequent measurements two minute recordings were used. The difference between absence and presence of SOD was calculated and this represented the amount of O^{-}_{2} generated which was consequently the activity of NADPH-oxidase.

Concentration dependent studies using variety of enzyme dilutions produced a linear response (Fig 5.3) and for all subsequent experiments 100ul dilution was chosen.

5.3.3 Kinetic analysis of TPA stimulated neutrophil NADPH-oxidase.

Using NADPH cofactor (20-200 uM) as a substrate Lineweaver-Burk double reciprocal plots were established (Fig 5.4). Under conditions used (100uM Ca^{2+}; pH 7.5 and at 25°C) K_m values for NADPH was calculated to be in the range of 59.17-67.1 uM and V_{max} 31.25-47.6 nmol/min/mg, of two separate preparations of active enzyme. However it was decided to further evaluate the kinetic analysis of active enzyme in the presence of phorbol esters (Table 5.2). The inclusion of TPA (100ngml^{-1}) in the assay lowered the K_m by 25 %. V_{max} was however increase by 7-10 fold compared to controls. Similar response was observed with SAP-A as K_m decreased by 15 - 20 % but V_{max} 6-9 fold higher than controls. K_m and V_{max} values by Rx did not significantly differ from that of controls

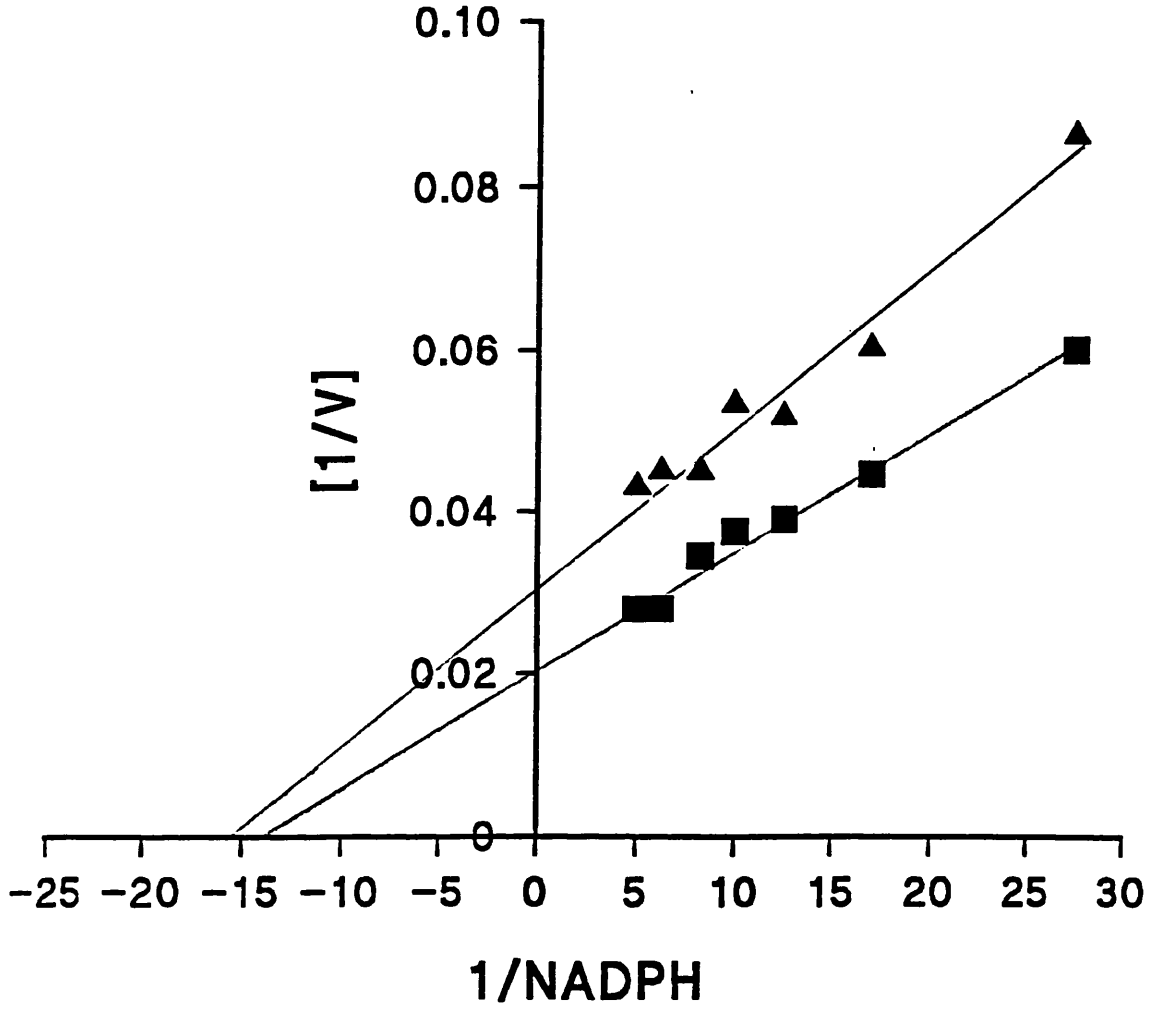


Fig 5.4 Double reciprocal Lineweaver-Burk plot of neutrophil NADPH oxidase using NADPH as substrate. NADPH-oxidase was partially purified on Sephacryl S-300 from TPA (100ng/ml) pre-treatment of human neutrophils. Plots were carried out from the mean values of two separate oxidase preparation as represented by ■ and ▲. NADPH substrate was used in the range of 20-200μM.

Conditions	K_m (μM)	V_{max} (μM)
Control	59.17-67.10	31.2-47.6
TPA	43.25-50.01	311.6-347.5
SAP-A	49.30-53.69	293.1-312.4
Rx	60.26-64.30	39.8-44.9

Table 5.2 Kinetic analysis of TPA stimulated neutrophil NADPH-oxidase in the presence of calcium. The of superoxide generation was determined at various concentrations of NADPH (20 - 200μM) in the presence of 100μM Ca^{2+} and 100ngml^{-1} phorbol esters. NADPH-oxidase was partially purified by sephacryl S-300 chromatography from TPA (100ngml^{-1}) pre-treated human neutrophils and assayed conducted as described in text. Data expressed are range of values from two separate enzyme preparations.

5.3.4 NADPH-oxidase profile of neutrophils pre-treated with phorbol esters .

Preliminary experiments with TPA activated cells produced significant peak of activity with gel filtration chromatography. Though this activity resolved with the void volume, its strong activity prompted a closer investigation using various probes of phorbol ester to stimulate neutrophils prior to homogenisation. NADPH-oxidase activity was again resolving in fractions corresponding to void volume (Fig 5.5) (Void volume in this particular column was fraction 12.). TPA (100ngml^{-1}) pretreatment of cells generated high oxidase activity as measured by O^{-}_{2} production. This activity correlated with the previous experiment. SAP-A (100ngml^{-1}) treatment of cells produced a similar response, with the efficacy in response being 85% of that achieved by TPA. Conversely Rx (100ngml^{-1}) pretreatment of cells generated a significantly lowered rate of activity. This rate was only 30% of the response produced by TPA. Unstimulated neutrophils (cells treated with equal volume 50% ethanol; the solvent used to dilute the phorbols) produced little or no activity of oxidase in the fractions of gel filtration chromatography. All chromatographic procedures were carried out on same column. The sephacryl S-300 column was re-generated by extensively washing in elution buffer (See Methods section 5.2) and the total protein eluted was monitored by u.v spectra, attached to the FPLC machine. Peak rate of activity achieved by each phorbol ester eluted at an identical position (fraction 13, Fig 5.5) suggesting the recording of the activities were specific.

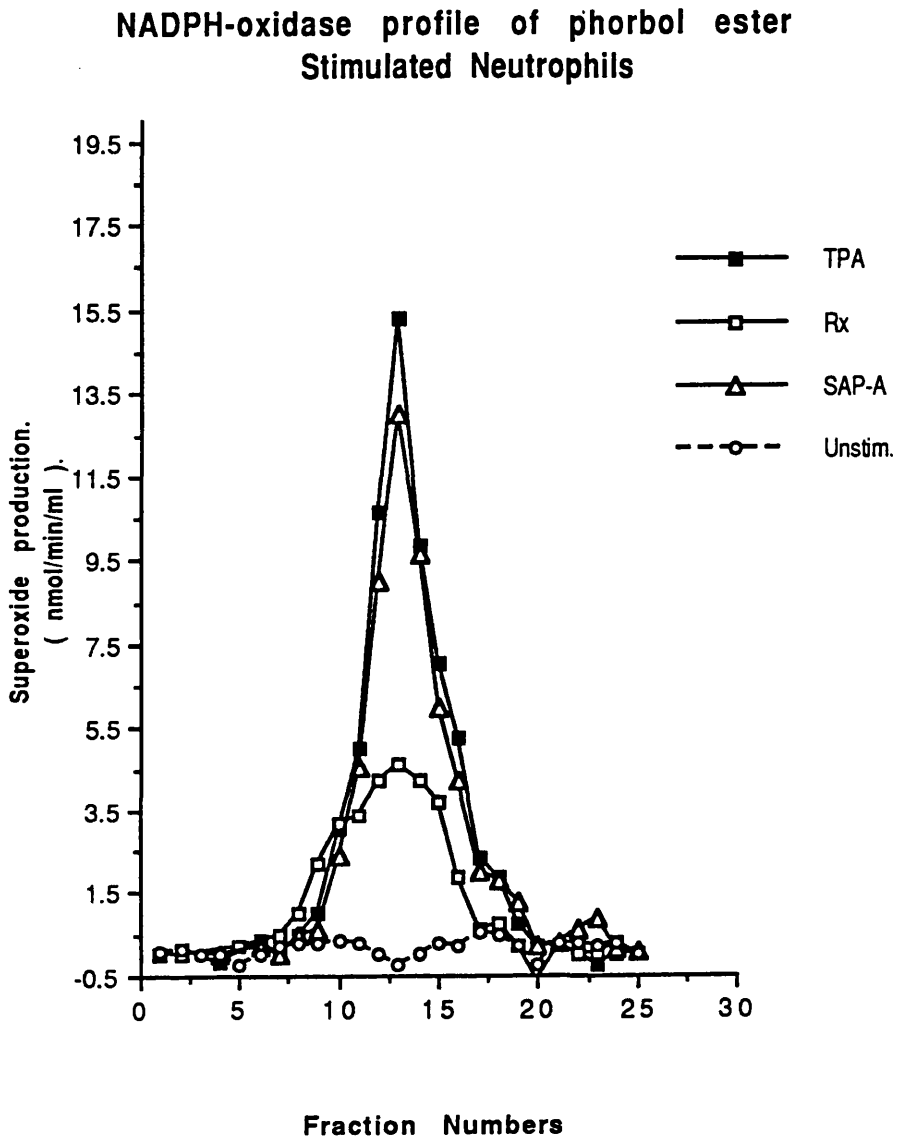


Fig 5.5 NADPH-oxidase profile of phorbol ester stimulated neutrophils. Approximately 1 x 10^9 cells were treated with phorbols esters (100ngml^{-1}) prior to homogenisation. After isolation (as described in methods), the membrane was solubilised with 0.4% lubrol PX and 0.4% Na deoxycholate. The resultant supernatant was chromatographed through sephacryl S-300 and 1ml fractions were collected at a flow rate of 0.4mlmin^{-1}. Result expressed is a typical profile generated and similar results were obtained from two separate experiments.

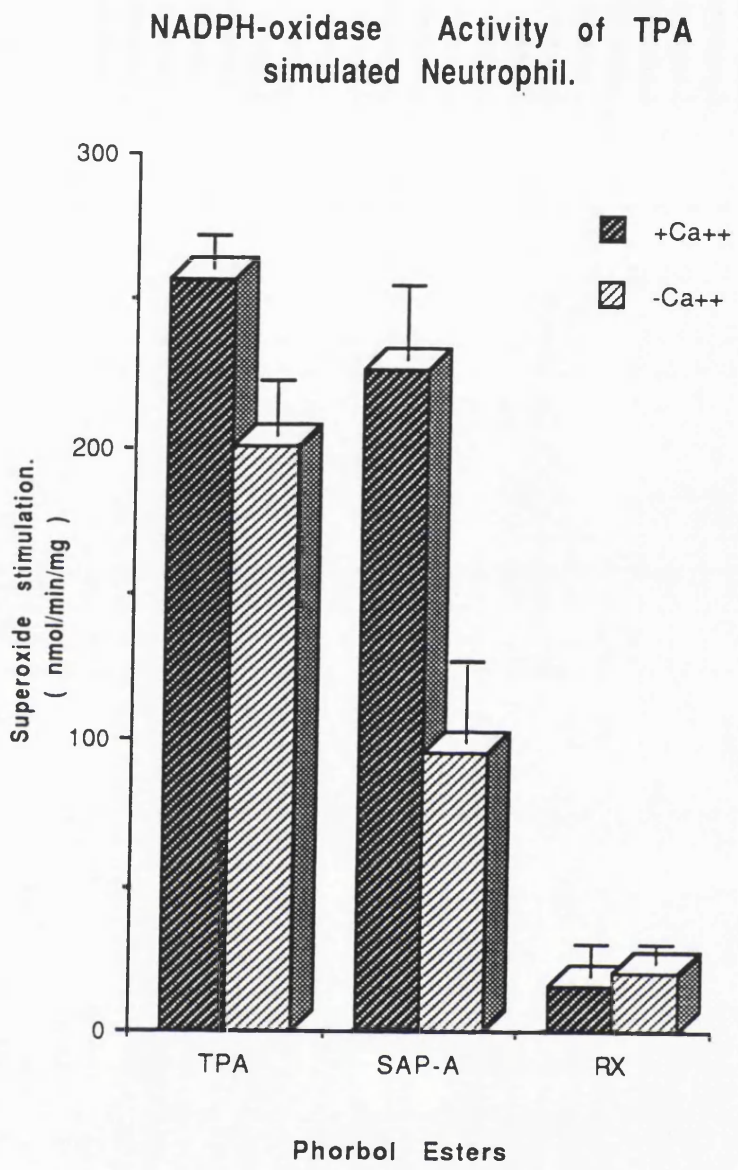


Fig 5.6 NADPH-oxidase activity of TPA treated neutrophils. 1 x 10^9 cells were subjected to TPA (100ngml^{-1}) pre-treatment. The oxidase was partially purified by Sephacryl S-300 from 0.4% lubrol PX and 0.4% Na deoxycholate solubilised membranes. The oxidase was stimulated *in-vitro* TPA (100ngml^{-1}), Sap-A (100ngml^{-1}) and Rx (100ngml^{-1}) in the presence and absence of 100μM Ca^{2+}. Results are expressed as phorbol ester stimulated oxidase activity minus oxidase only activity ±SEM (n=3).

5.3.5 Effect of phorbol esters on NADPH-oxidase Activity of stimulated neutrophils.

5.3.5.1 Oxidase activity of TPA stimulated neutrophils.

Approximately 10^9 cells (neutrophils) were subjected to 100 $ngml^{-1}$ TPA pretreatment prior to partial purification of the oxidase. NADPH-oxidase was pooled from sephacryl S-300 chromatographs and were further stimulated by phorbol ester probes in the presence and absence of added Ca^{2+} (100uM) or EGTA (5mM). TPA (100$ngml^{-1}$) stimulation *in-vitro* produced a maximal response which was greater than that observed with activity profile. In the presence of exogenous Ca^{2+} ions the rate increased by greater than 16-fold compared to the original elution (Fig 5.6) and the activity was more than 250 nmol/min/mg of protein. Absence of added Ca^{2+}, TPA stimulation of the rate was lowered by 20% to approximately 200 nmol/min/mg. *In-vitro* stimulation by SAP-A (100$ngml^{-1}$) produced a similar results in the presence of added Ca^{2+}. However without Ca^{2+} this activity was lowered by more than 50% to approximately 100 nmol $O^{\cdot}_2$/min/mg. Rx (100$ngml^{-1}$) was clearly weaker in this system and produced little or no stimulation above basal levels (activity of NADPH-oxidase eluting off the column.).

5.3.5.2 NADPH-oxidase activity of SAP-A stimulated neutrophils.

Approximately 10^9 neutrophils were subjected to SAP-A (100$ngml^{-1}$) pretreatment

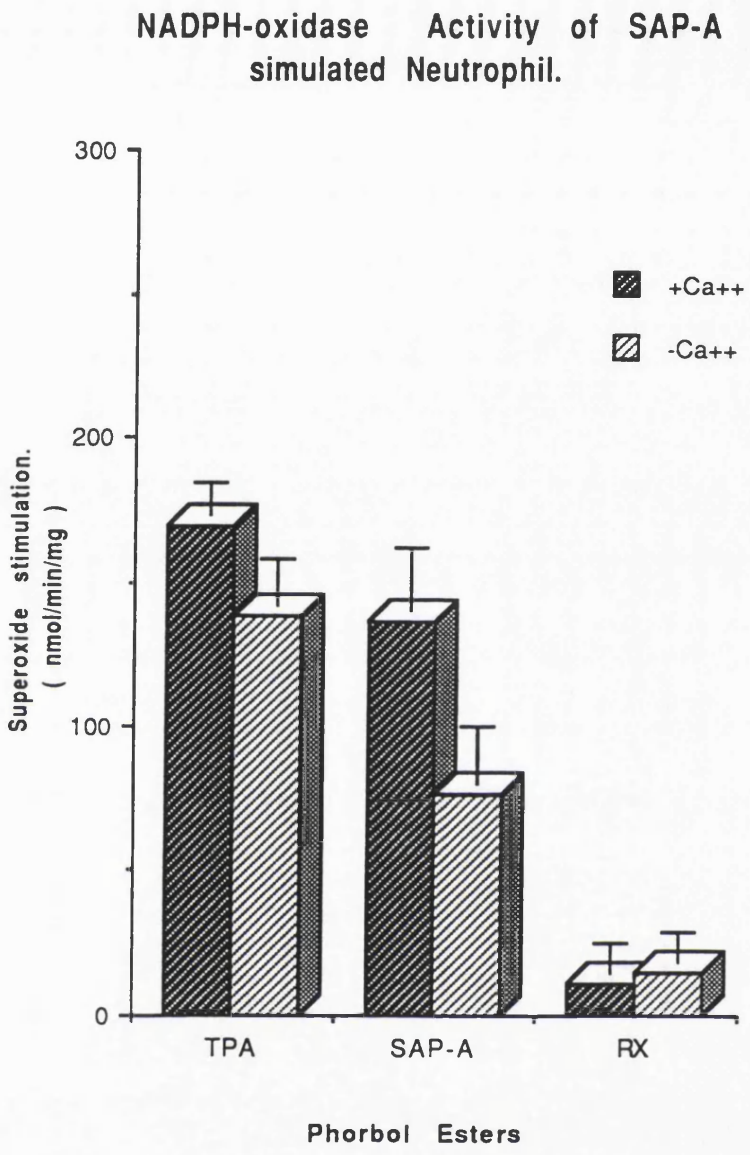


Fig 5.7 NADPH-oxidase activity of Sap-A treated neutrophils. 1 x 10^9 cells were subjected to Sap-A (100ngml^{-1}) pre-treatment. The oxidase was partially purified by Sephacryl S-300 from 0.4% lubrol PX and 0.4% Na deoxycholate solubilised membranes. The oxidase was stimulated *in-vitro* TPA (100ngml^{-1}), Sap-A (100ngml^{-1}) and Rx (100ngml^{-1}) in the presence and absence of 100μM Ca^{2+}. Results are expressed as phorbol ester stimulated oxidase activity minus oxidase only activity ±SEM (n=3).

prior to partial purification of oxidase. *In-vitro* stimulation by TPA of oxidase activity from SAP-A treated cells were again higher than that of basal levels (Fig 5.7). The stimulation was partially Ca^{2+} dependent and in the presence of Ca^{2+} the stimulated activity was 12-fold higher than original elution activity. SAP-A stimulation *in-vitro* of partially purified oxidase was Ca^{2+}-dependent. In the presence of added Ca^{2+} the rate of activity was 2-fold greater (~ 150 nmol/min/mg) than in the absence of added Ca^{2+} (~ 70 nmol/min/mg). Rx failed to stimulate NADPH-oxidase in the presence or absence of Ca^{2+} above basal levels. This non-response with Rx was similar to the results observed in neutrophils stimulated by TPA.

5.3.5.3 NADPH-oxidase activity of Rx stimulated neutrophils.

10^9 cells were subjected to Rx (1000ngml^{-1}) pretreatment prior to homogenisation. NADPH-oxidase pooled from elution fractions of sephacryl S-300 were stimulated by phorbol esters *in-vitro* with or without added Ca^{2+} (see Fig 5.8). *In-vitro* stimulation by TPA of enzyme activity markedly increased from basal values. The increase was greater than 11-fold in the presence of added Ca^{2+}. SAP-A failed to stimulate the oxidase obtained from Rx primed neutrophils. Similarly Rx also failed to stimulate the oxidase above basal levels with added Ca^{2+}. However in the absence of added Ca^{2+} Rx stimulated the oxidase by more than 6-fold. The rate was increased from 4.5 to 30 nmol $O^{\cdot}_2$ /min/mg protein. Nevertheless all the values in Rx-stimulated neutrophils were notably lower than compared to activities observed in both TPA and

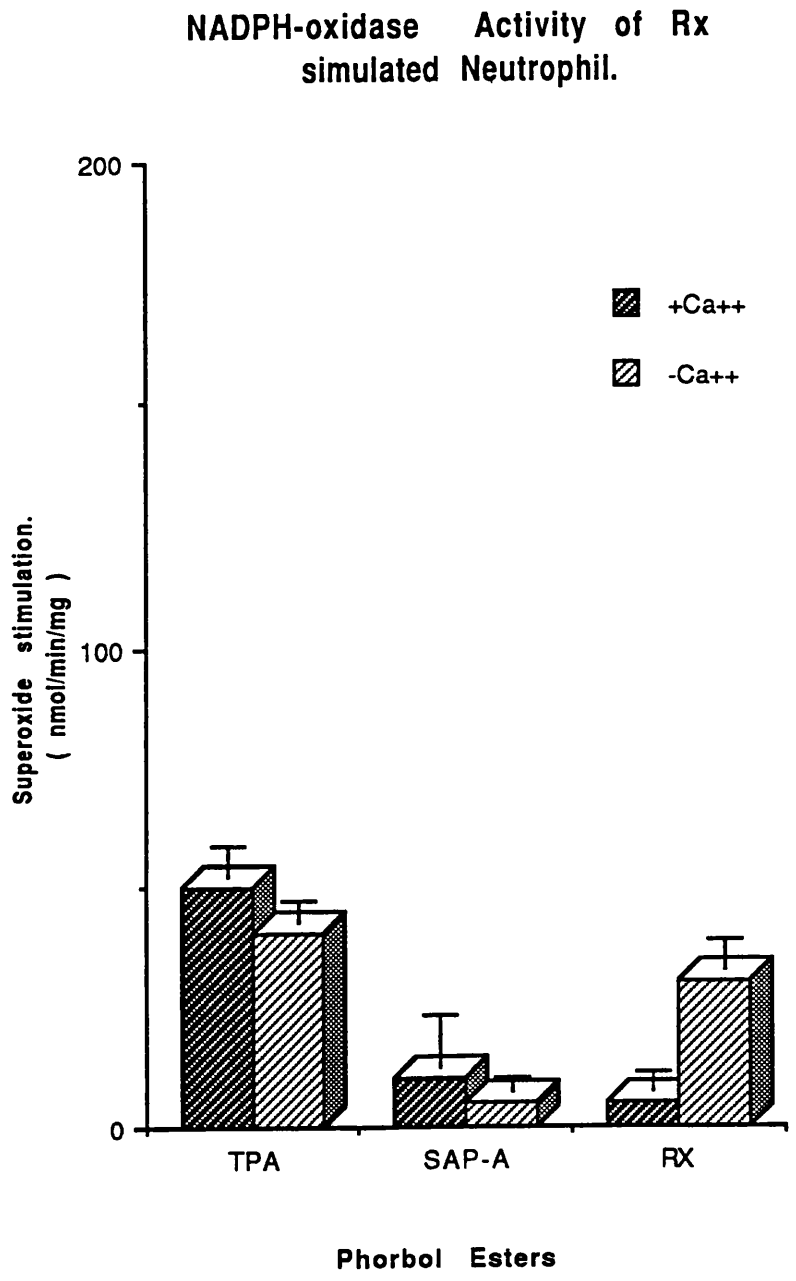


Fig 5.8 NADPH-oxidase activity of Rx treated neutrophils. 1 x 10^9 cells were subjected to Rx (100ngml^{-1}) pre-treatment. The oxidase was partially purified by Sephacryl S-300 from 0.4% lubrol PX and 0.4% Na deoxycholate solubilised membranes. The oxidase was stimulated *in-vitro* TPA (100ngml^{-1}), Sap-A (100ngml^{-1}) and Rx (100ngml^{-1}) in the presence and absence of 100μM Ca^{2+}. Results are expressed as phorbol ester stimulated oxidase activity minus oxidase only activity ±SEM (n=3).

Superoxide generation (nmol/min/mg protein)				
Stimulation *in-vitro*	Neutrophil pre-treatment			
	TPA	DAG	zymosan	fMLP
Control + Ca^{2+}	13.56±1.45	11.56±1.50	4.56±1.22	13.78±2.72
Control - Ca^{2+}	12.14±2.76	11.15±2.67	3.99±0.56	14.27±1.32
*TPA + Ca^{2+}	256.41±19.15	129.75±4.98	52.26±6.59	187.95±11.27
*TPA - Ca^{2+}	189.27±11.27	120.66±8.11	31.74±8.74	146.00±14.36
DAG + Ca^{2+}	16.55±2.54	17.59±3.43	6.21±2.43	25.19±4.48
DAG - Ca^{2+}	14.98±1.66	13.85±4.37		13.85±1.39
fMLP + Ca^{2+}	23.67±4.14	20.24±3.89	17.21±1.74	23.66±3.51
fMLP - Ca^{2+}	15.41±3.08	12.06±1.05	11.44±3.74	16.47±2.66

Table 5.3 *In-vitro* stimulation of NADPH oxidase activity from selective pre-treatment of neutrophils. The rate of superoxide anion was measured *in-vitro* by various agonist at pre-determined doses. Neutrophils were subjected to pre-treatment with TPA ($100ngml^{-1}$), a diacylglycerol analogue ($DiC_{18:1}$ at 10μM), zymosan ($1mgml^{-1}$) and fMLP (1μM). Assay of oxidase was performed after partial purification through sephacryl S-300 column as described in methods. Data expressed are SEM of triplicates from three separate enzyme preparations (* $p < 0.001$ compared to controls).

SAP-A stimulated cells.

5.3.6 Stimulation of oxidase isolated from selective pretreatment of neutrophils.

Phorbol esters, particularly TPA and SAP-A were strong 'priming' agents for NADPH-oxidase activity. This ability of TPA was compared with variety of putative priming agents, and the partially purified enzyme was further stimulated *in-vitro* by numerous agonist with or without Ca^{2+} (see Table 5.3). Selective priming by TPA produced a similar response as previously stated. A diacylglycerol analogue sn-1,2 dioleolglycerol ($DiC_{18:1}$) was used because it was reported to be cell permeant (Bass *et al*, 1989). DAG (10 uM) caused a 10-fold increase in TPA stimulatable NADPH-oxidase activity compared to controls. This activity however was approximately 50% of the value achieved by TPA pretreatment, and was not partially Ca^{2+} dependent like the TPA response. Opsonised zymosan ($1mgml^{-1}$) produced a weaker response compared to the other agonist used. fMLP (1 uM) caused a 10-13 fold increase in TPA stimulatable oxidase activity compared to its control and was partially Ca^{2+} dependent but was approximately 70% of TPA pretreatment response. When the *in-vitro* activity of the unstimulated enzyme was compared to its pretreatment agents, the results suggested that fMLP≥ TPA > DAG >> zymosan at activating NADPH-oxidase from cells. DAG (10 uM) and fMLP (10uM) failed to stimulate oxidase activity *in-vitro* even from preparations of TPA pretreated cells.

	Superoxide stimulation. (nmol/min/mg protein).
None	10.89±1.76
pooled PKC	13.67±2.14
*PKC + Co-factors	198.61±5.49
Co-factors	14.24±3.81

Table 5.4 . Effects of pooled PKC/Co-factors on activity of neutrophil NADPH-oxidase. Active fraction of rat brain PKC were pooled following FPLC hydroxyapatite chromatography prior to use. Co-factors were in a mixture giving a final concentration of 100μM Ca^{2+}, 10mM $MgCl_2$, 100μM ATP and 0.75 mg/ml PS. NADPH-oxidase was partially purified from TPA (10ng/ml) pre-treatment of human neutrophils (* $p < 0.01$ compared to none).

Conditions.	Superoxide stimulation. (nmol/min/mg protein)
None	17.65±3.59
*TPA + PKC	586.48±18.24
TPA + Buffer	218.66±2.35
*SAP-A + PKC	546.11±22.43
SAP-A + Buffer	144.44±1.98
[1]Rx + PKC	124.56±13.46
Rx + Buffer	16.89±3.99

Table 5.5 Effects of phorbol esters on activation of neutrophil NADPH-oxidase in the presence and absence PKC. Active fraction of rat brain PKC were pooled following FPLC hydroxyapatite chromatography and used in conjuction with co-factors (100μM Ca^{++} ions, 10mM $MgCl_2$, 100μM ATP and 0.75 mg/ml PS). NADPH-oxidase was partially purified from TPA (10 ng/ml) pre-treatment of human neutrophils. Results are expressed as mean of triplicates of three separate experiments ± SEM (* $p<0.001$; [1] $p<0.01$ compared to controls).

5.3.7 Effects of PKC on activity of NADPH-oxidase.

Active fractions of rat brain PKC were pooled following FPLC HPT chromatography elution (generously supplied by Dr W J Ryves). NADPH-oxidase was partially purified from TPA (100 $ngml^{-1}$) pretreatment of human neutrophils. Effect of PKC or cofactors alone (100 uM ATP, 10mM $MgCl_2$, 100 uM Ca^{2+}, 0.75 $mgml^{-1}$ PS) were minimal. However PKC co-administered with cofactors markedly increased the rate of oxidase activity. This response was nearly 20-fold higher than basal levels (see Table 5.4) and reached above 200 nmol/min/mg. The basal rate was measured by additions of equal volume of PKC storage buffer (16% glycerol / 0.02% Triton X-100) and mixed with buffer-2 (see Chapter 2, section 2.3.2 d) in appropriate ratios.

Since PKC with cofactors significantly increased the rate of enzyme activity it was of interest to study if this system could be further activated by stimulating the PKC itself by phorbol esters (Table 5.5). TPA, SAP-A and Rx responses were similar as reported earlier. However, PKC stimulated by phorbols increased the rate of oxidase activity by 3-7 fold. Maximal response was produced by TPA in the presence of PKC (~ 686 nmol/min/mg). This response was higher than 3-fold compared to TPA alone values. SAP-A with PKC was higher than 4-fold (~ 650 nmol/min/mg) compared to SAP-A alone. Rx alone produced no increment on oxidase activity but this response was larger than 7-fold in the presence of PKC. However even with this large fold

Superoxide production (nmol/min/10^7 cell eqiv.)		
	Neutrophils	Macrophages
Membrane (M)	0.96±0.21	0.32±0.16
Cytosol (C)	2.37±0.19	0.26±0.26
M + C (Enz)	1.23±0.33	0.86±0.32
Enz + SDS	19.87±2.14	18.47±2.09
Enz + TPA	2.19±1.06	1.44±0.38
Enz + TPA + SDS	26.74±2.88	28.66±4.31
Enz + TPA + SDS + PKC	53.96±7.05	48.85±3.37
Enz + TPA + PKC	51.88±3.79	55.64±5.74

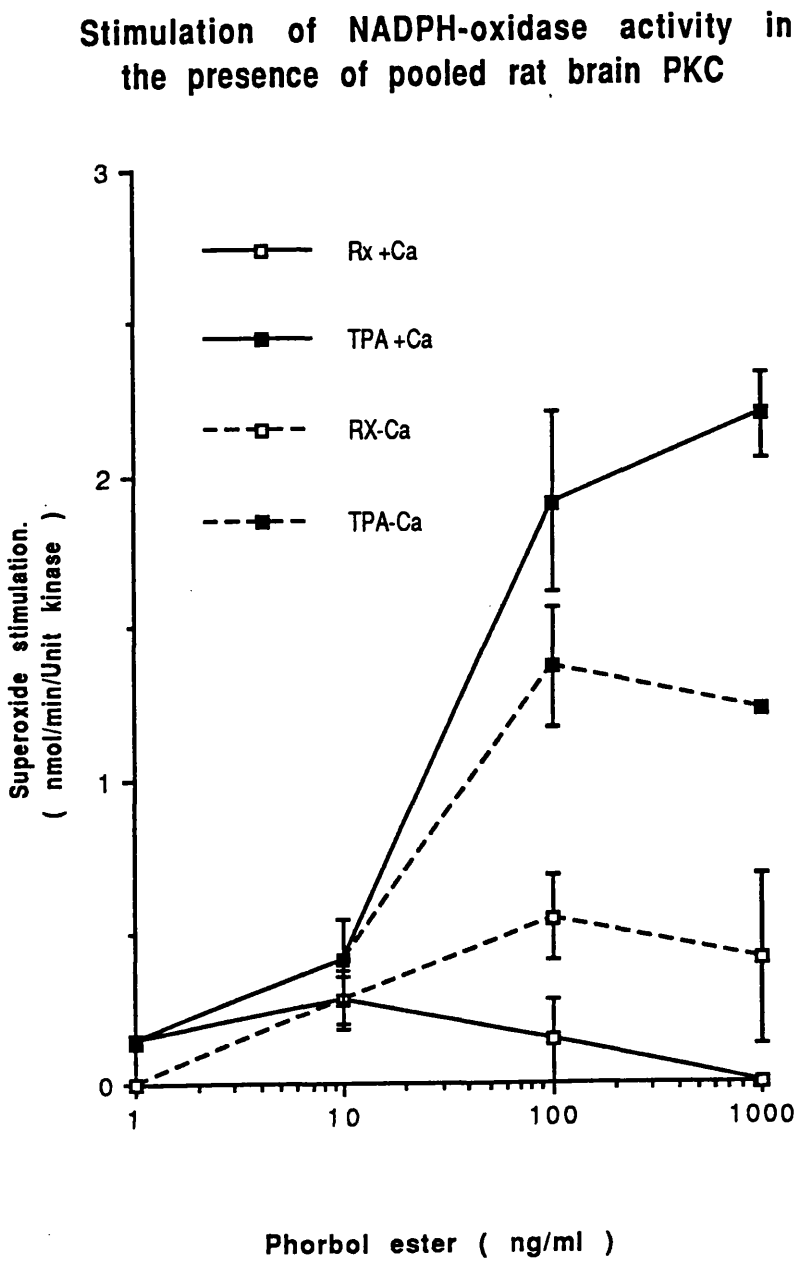
Table 5.6 Comparison of neutrophils and macrophage NADPH-oxidase. Membrane and cytosolic fractions were isolated by sucrose density centrifugation as described in the methods. 50µl cytosolic fraction (187µg protein of neutrophil preparation, 118µg macrophage protein) and 50µl membrane fraction (319µg protein neutrophil, 211µg protein macrophage) were used in the assay. 100ngml^{-1} TPA and 100µM SDS as final concentration were used in the assay. Results are expressed in terms of oxidase activity per 10^7 cell equilivent ± SEM of three determinations.

increase, Rx with PKC response was only 20% (~ 125 nmol/min/mg) of that achieved by TPA .

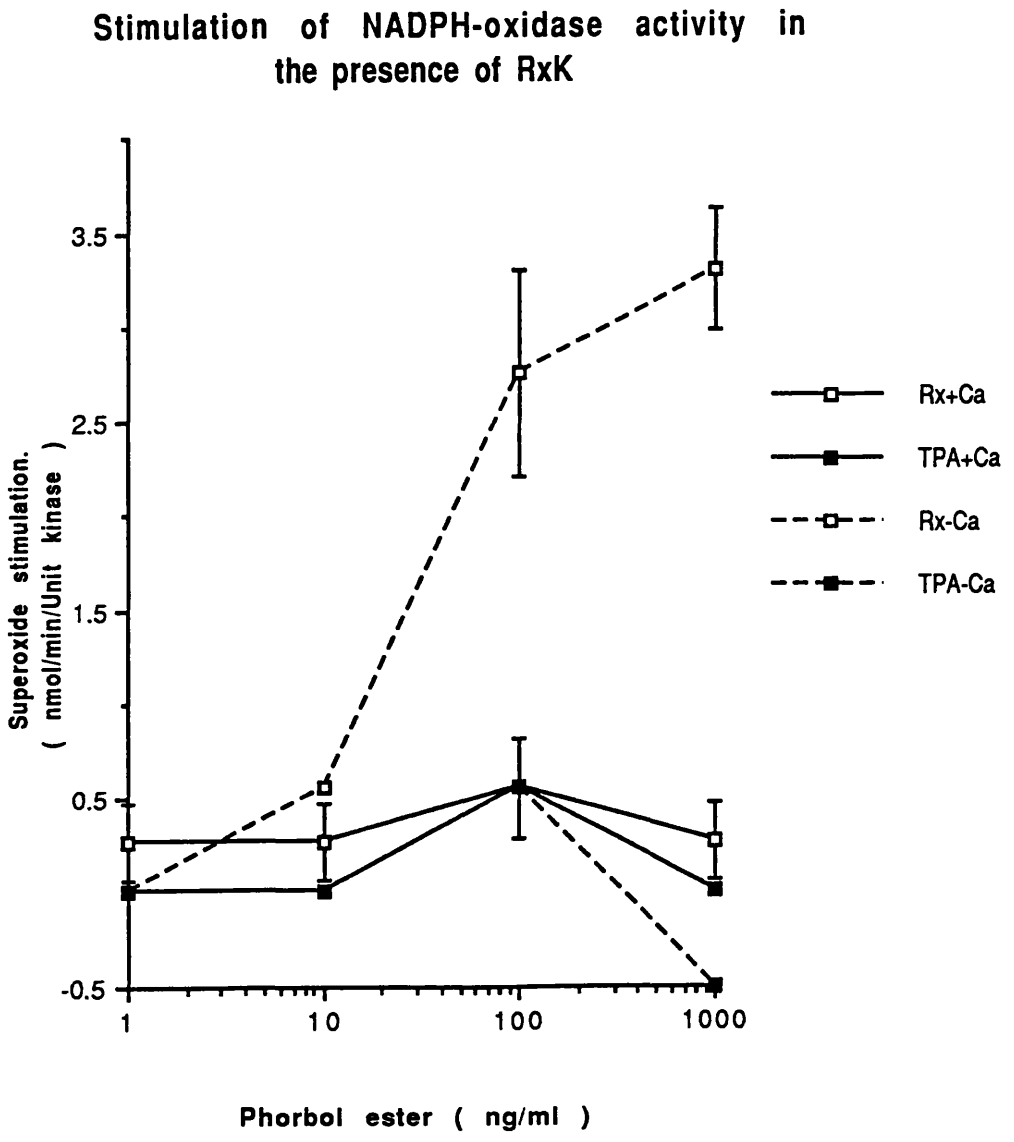
5.3.8 Comparison of activation of NADPH-oxidase by kinase stimulated with phorbol esters.

In view of earlier results when Rx stimulated O^{-}_{2} production from macrophages (see Chapter 3), it was therefore of interest to study the effect of stimulated Rx-kinase (see Chapter 4) on NADPH-oxidase activity. Since Rx-kinase was purified from macrophages, it was therefore decided that activity of NADPH-oxidase of macrophages should initially be compared to neutrophils.

Cells were lysed by sonication and membrane and cytosolic fractions were isolated (see section 2.3.2). NADPH-oxidase activity in membranes and cytosolic fractions alone or mixed (enzyme) was low from both human neutrophils and starch-elicited mouse peritoneal macrophages (SEPM) (Table 5.6). The presence of anionic amphiphile, SDS (100 uM) significantly increased the enzyme activity (19.87 ± 2.14 nmol/min). TPA (100ngml^{-1}) failed to activate the reconstituted oxidase complex but presence of SDS augmented this activity (26.74 ± 2.88 nmol/min). Rat brain pooled PKC (10 U) activated by TPA was able to activate the reconstituted oxidase in both neutrophil and SEPM and interestingly this activity did not require the presence of SDS. Like neutrophil the macrophage oxidase activity was proportional



5.9 Stimulation of NADPH-oxidase activity in the presence of pooled rat brain PKC. Superoxide anion was determined by a continious kinetic assay of SOD-inhibitable reduction of cytochrome C in the presence and absence of 100μM Ca^{2+}. Rx and TPA were both used at 100ngml^{-1} *in-vitro*. Results are expressed by calculating the initial rate of O^{-}_{2} production ±SD (n=2).



5.10 Stimulation of NADPH-oxidase activity in the presence of RxK. Superoxide anion was determined by a continious kinetic assay of SOD-inhibitable reduction of cytochrome C in the presence and absence of 100μM Ca^{2+}. Rx and TPA were both used at 100ngml^{-1} *in-vitro*. Rx-kinase was isolated from starch-elicited murine macrophages. Results are expressed by calculating the initial rate of O^{-}_{2} production ±SD (n=2).

to cell numbers (data not shown) and thus was utilised for subsequent experiments studying the effects of kinases.

Assay of NADPH-oxidase by diterpene esters in the presence of pooled rat brain PKC and SEPM Rx-kinase was determined by continuous kinetic assay and the results are expressed as stimulation above basal levels of enzymatic activity (activity of the phorbol with membranes and cytosolic fractions) per unit of kinase activity. In the presence of PKC TPA induced a strong Ca^{2+} dependent response (Fig 5.9). Rx produced only weak activation. In the presence of Rx-kinase, TPA was capable of only weak activation whereas Rx was capable of inducing a selective dose dependent activation of oxidase in the absence of added Ca^{2+} ions (Fig 5.10). This activity corresponds to the *in-vitro* histone kinase assay requirement of Rx-kinase. Under optimal conditions staurosporine (1 uM) inhibited both stimulated PKC and Rx-kinase activation of NADPH-oxidase (Table 5.7) suggesting the kinase itself caused the activation.

5.4 Discussion.

Initially plasmamembranes of TPA activated neutrophils were purified by sucrose density centrifugation followed by solubilisation with a mixture of deoxycholate and Lubrol PX, a 'mild' treatment which preserves the NADPH-dependent superoxide

NADPH-oxidase activity, nmol/min/U.		
	Rx-kinase	PKC
Rx	2.83±0.45	-
Rx+Staurosporine	0.26±0.26	-
TPA	-	2.36±0.21
TPA+Staurosporine	-	0.26±0.10

Table 5.7 Effect of staurosporine on kinase stimulation of macrophage NADPH-oxidase. 50µl cytosolic (118µg protein) and 50µl membrane (211µg) were isolated by discontinious sucrose density centrifugation from starch-elicited murine peritoneal macrophages. Stimulation by Rx (100ngml^{-1}) of macrophages Rx-kinase was in the absence of added Ca^{2+} (5mM EGTA) and, TPA of rat brain PKC was in the presence of added Ca^{2+} (100µM). Kinase activation of NADPH-oxidase was assayed with or without staurosporine (1µM) and results are expressed per Unit of kinase present and the range of values are shown from a duplicate set.

forming activity (Bellavite *et al*, 1984). The solubilised extract was chromatographed through Sephacryl S-300 column to yield a single peak of activity eluting off with void volume. UV protein trace of the profile (data not shown) showed a peak of protein at void volume followed by a broad 'tailing off'. Since not all proteins eluted at void volume, the results seem to suggest that oxidase was aggregated material with molecular weight greater than 205 kDa. Previous studies using gel filtration (Ultrogel AcA34) have also indicated that the oxidase activities were eluted with void volume and contained aggregated material which included phospholipids (Bellavite *et al*, 1985), cytochrome b_{558} and FAD (Parkinson and Gabig, 1988). Attempts were made to improve the purification of the activity, but the results were disappointing. Initially the sephacryl S-300 elution fractions were immediately subjected to superose 12 (commercial column, Pharmacia) chromatography. Distinctive peaks of protein bands were eluted between 50 - 200 kDa (calibrated region) but there was no activity observed, even at void volume. The purification of cytochrome b_{-245} (initially thought of as putative component of oxidase) has been successful through agarose resin (Harper *et al*, 1984) but when the resin was used instead of sephacryl S-300, NADPH-oxidase was not detected when eluted by a NaCl gradient even up to 0.5M NaCl. Finally an attempt was made to remove protein bound to aggregates containing the activity by a mild treatment of 0.4M NaCl (Seera *et al*, 1984) and re-subjecting it to gel filtration through sephacryl S-300 column. This treatment also induced a total loss of activity. These negative results led to the conclusion that the physico-chemicals state of the enzyme complex and contaminating proteins, possibly consisting of

proteolipid and election transport proteins, hampered a further purification by conventional methods. Attempts to purify the active oxidase subjecting it to sephacryl chromatography treated with various disaggregating detergents failed, since those treatments also caused total inactivation of the oxidase. The agents used for this purpose were stronger concentration of deoxycholate and Lubrol Px (>0.5%), Triton X-100 (0.1%), 1M KCl and 1M NaCl. All these results indicate that the aggregated state of the extract is a pre-requisite for maintaining the enzyme in its native state. For this reason it was decided to continue the pharmacological evaluation of oxidase with the aggregated material.

It is now well established that unprimed or unstimulated phagocytes express a dormant state NADPH-oxidase and thus initial experiments were investigated using TPA as the priming agent. Kinetic analysis of the activated oxidase resulted in K_m and V_{max} values which seemed to be within the range obtained by other authors for both untreated or activated NADPH-oxidase (Smith *et al*, 1989; Umeki, 1990). Interestingly the inclusion of phorbol ester, TPA or SAP-A in the assay increased the affinity for NADPH of the NADPH-oxidase and V_{max} was greatly increased. The full explanation for this result remains unclarified and requires further investigations but one suggestion could be that phorbol esters interact with a component(s) of NADPH-oxidase thus inducing conformational change producing a high affinity form of the oxidase, a more active form of oxidase associated with the membrane. For example Sasada *et al* (1983) and Tsunawaki and Nathan (1984) working with mouse

peritoneal macrophages compared the kinetics of oxidase in cell free preparations from resident and TPA activated macrophages and discovered that K_m values for NADPH decrease in activated macrophages. However interpretations of *in-vivo* actions are difficult because other factors such as TPA stimulated PKC phosphorylations cannot be ruled out. Rx did not increase the affinity for NADPH nor did the V_{max} alter. Rx has been shown to have limited biological actions which may account for this (Evans and Edward, 1987).

Initial experiments with TPA-activated cells produced a large peak of activity with gel filtration. This prompted a closer investigation using various probes of phorbol esters to stimulate neutrophils prior to homogenisation (Fig 5.5). NADPH-oxidase activity profile obtained from phorbol ester pretreatment were very similar to *in-vitro* activity correlations by phorbols esters of PKC (Ellis *et al*, 1987). TPA was slightly more potent than SAP-A and Rx produced 30% of oxidase activity compared to TPA. Interestingly NADPH-oxidase activity profile with SAP-A pretreatment resulted with efficacy in response being 85% of that achieved by TPA and, Rx generated a significantly lowered rate of about 30%. *In-vitro* analysis of PKC isotype activation by phorbol ester has shown that TPA potently activates all isotypes tested, SAP-A fails to activate δ-PKC and Rx only activates β-isotype at high doses (Ryves *et al*, 1991). Significantly in neutrophils the major isotype discovered has been β-isotype followed by minor α-PKC, ζ- and PKC (Stasia, *et al*, 1990). Additionally Ca^{2+}-inhibited Rx-kinase was isolated from neutrophils but only in cell populations from

more than one donor (ie. 'primed' (see chapter 4), but in these experiments neutrophils were obtained in large quantities using a cell separator from a single donor). There are at least two cytosolic components, p47-phox and p67-phox, which are phosphorylated and hence associated with membrane upon activation of neutrophil by TPA (Okamura *et al* 1990; Ohtsuka *et al* 1990). P47-phox is a substrate for PKC and phosphorylation occurs at multiple sites upon activation in intact cells (Hayakawa *et al*, 1986; Retrosen and Leto 1990). The most probable explanation for the observed result was that phorbol esters activate PKC subsets (most probably β-isotype) and phosphorylate cytosolic components which translocate to the membranes and oxidase becomes activated. However some studies have revealed that phosphorylation does not seem to be obligatory for activation (Badwey, 1989) in intact cells. Further investigations studying the phosphorylation in intact cells with correlation of NADPH-oxidase activity would provide a better interpretation of the results. Interestingly recent evidence has suggested that p47-phox forms the initial complex with the cytochrome and is required for the assembly of other components (Tyagi *et al*, 1992).

The study of effects of phorbol esters *in-vitro* on NADPH-oxidase partially purified from TPA and SAP-A pre-stimulated neutrophils revealed that TPA activation was only partially Ca^{2+}-dependent whereas SAP-A was Ca^{2+}-dependent. This additional *in-vitro* activation of active oxidase shows that at least *in-vitro* oxidase was a target for these ubiquitous phorbol esters and thus increasing the affinity of oxidase for

NADPH as earlier reported from kinetic studies. Interestingly TPA (and most likely SAP-A and Rx, though not tested) did not induce activation of reconstituted oxidase *in-vitro* of unstimulated neutrophils or macrophages (Table 5.6). This suggests that active oxidase with its multicomponent complex system consisting of cytosolic factor/electron transport proteins has a binding region for phorbol esters but once that is broken (or in dormant state) this region is lost or inactive. This was further backed up by the fact that SDS activated reconstituted oxidase was augmented by TPA. Whether this is a true reversible binding action or an activation process requires further investigations.

Rx pre-stimulation of neutrophils produced a partially active oxidase and this was represented by partial stimulation of active oxidase *in-vitro* by TPA and SAP-A. This may reflect weak stimulation of PKC-β by Rx or some other mechanism such as Rx-kinase. Rx-kinase seems to require a 'priming' agent for its induction (see chapter 4). Therefore it seems likely that if an initial priming agent (eg plumbagin) was used followed by Rx, then this may cause a substantial increase in purified oxidase activity. However, since this was not examined the results are difficult to postulate and hence a further investigation is recommended.

TPA and SAP-A were strong stimulants for 'priming' NADPH-oxidase. When phorbol esters were compared to variety of putative agents it was discovered that a particulate stimulus zymosan, was as equipotent as Rx whereas fMLP and DAG analogue, $DiC_{18:1}$

were stronger agents. Indeed fMLP activated neutrophils produced a slightly higher basal activity compared to TPA (see Table 5.3). Zymosan induced O^{-}_{2} stimulation has been controversial, both PKC mediated and PKC-independent pathways have been proposed (Duyster *et al*, 1992; Andre *et al*,1988). Zymosan co-treatment of murine macrophages synergise with Rx in O^{-}_{2} production, suggesting involvement of Rx-kinase (see Chapter 3). Since Rx-kinase of neutrophils used were not previously primed zymosan action was found to be diminished. Alternatively zymosan could be a poor stimulator of NADPH-oxidase of intact neutrophils in the conditions used. $DiC_{18:1}$ produced significant stimulation of the oxidase activity. This cell permeant DAG analogue is most likely acting through PKC activation. fMLP exerts its action via a receptor which is a 50-70 kDa glycoprotein with intracellular and surface localisation (for review see, Jesiatis and Allen, 1988). It has been well established that fMLP-activated neutrophils, PI hydrolysis by phopholipase C generates DAG and IP_3 (Kramer *et al*, 1989; Bass *et al*, 1989). However some studies have revealed that fMLP-activated cells generates DAG by phospholipase D which produce phosphatidic acid that are subsequently hydrolysed to DAG (Olsen *et al*, 1990; Billah, *et al*, 1989). Thus it seems that fMLP strongly generates NADPH-oxidase activation via accessory mediators.

Direct activation of 'active' NADPH-oxidase by PKC with *in-vitro* co-factor requirements was observed (see Table 5.4). The activation was further stimulated (3 - 7 fold) by phorbol ester stimulation of PKC suggesting that activation of PKC

could lead to subsequent activation of the oxidase. Since direct activation of the oxidase by phorbol esters alone and/or by unstimulated PKC was possible, the marked response with the presence of both may represent synergism as well as increased PKC activity. Interestingly PKC activation was absolutely dependent upon ATP. Direct activation by PKC *in-vitro* suggests that PKC is an important mechanism by which oxidase is activated but whether this is the major activation pathway remains to be fully clarified.

Since Rx stimulated $O^{\cdot}_2$ production was synergised by zymosan co-treatment it was of interest to test if purified Rx-kinase could activate NADPH-oxidase. As Rx-kinase was isolated from SEPM (see Chapter 4) it was decided to isolate the reconstituted system from SEPM (sufficient cell numbers were not obtainable for sephacryl S-300 chromatography) and was compared to human neutrophils. Both systems were comparable as SDS but not TPA was able to activate both the oxidase systems. However TPA was able to augment SDS activation. SDS stimulation was consistent with the established results and this activation may be due to the amphiphile mimicking a regulatory pathway such as phosphatidic acid (Tyagi *et al*, 1992). Since comparison of macrophage reconstituted system to neutrophils yielded similar results it was decided to carry on and use the macrophage system. Interestingly activation by TPA with PKC did not require the presence of SDS suggesting that PKC activation was sufficient for re-stimulation of oxidase.

Direct activation of oxidase by PKC stimulated with TPA in a dose dependent manner was observed. This activation was Ca^{2+}-dependent (ie the *in-vitro* requirement for pooled PKC activation by TPA (Ryves, 1991)). Direct activation of oxidase was also observed by Rx stimulated Rx kinase in the absence of calcium. This Ca^{2+}-inhibited activity corresponded to the *in-vitro* assay requirements for Rx-kinase. This important finding may help to explain the observed $O^{\cdot}_2$ production by Rx on primed macrophages. The suggestion is that 'priming' (although mechanism of action is unknown) results in Rx-kinase expression which upon further stimulation by Rx activates NADPH-oxidase. This is the first reported physiological substrate for Rx-kinase. However there is insufficient data to suggest whether Rx-kinase activates directly on membrane bound oxidase component(s) or phosphorylates on cytosolic regulatory protein(s). Initial *in-vitro* phosphorylation experiments proved unsuccessful mainly due to technical problems but further investigation with Rx-kinase phosphorylation studies would provide useful results. PKC (dependent on Ca^{2+}) and Rx-kinase (inhibited by Ca^{2+}) may represent separate signalling pathways involved at different states of activation.

These studies have provided a basis by which part elucidation of activation pathway can be postulated. Initial purification of pre-stimulated neutrophil oxidase were partially successful. Phorbol esters (TPA and SAP-A) were demonstrated to directly activate NADPH-oxidase from stimulated neutrophil but not the reconstituted system. PKC and importantly Rx-kinase activation provide a further mechanism of already

complex signal transduction mechanism by which NADPH-oxidase can be activated. Examples of this complexity are shown by the fact that several putative pathways some already discussed above, and others such as Guanine nucleotide induction of tyrosine phosphorylation leading to activation of respiratory burst in neutrophil (Nasmith *et al*, 1989) have been proposed. However, in essence oxidase activation is probably by several pathways co-operating possibly at different stages and further tightly regulated.

CHAPTER 6 Phorbol Ester and PKC Modulation of Phagocyte Functions

6.1 Introduction.

Stimulation of phagocytes with protein kinase C (PKC) activators such as phorbol esters results in superoxide (O^-_2) production (Kramer *et al*, 1988) and inhibits agonist stimulated bivalent cation influx (McCarthy *et al*, 1989). Stimulation of phagocytes with receptor agonists such as fMet-Leu-Phe (fMLP), results in activation of phospholipase C with inositol phosphate hydrolysis producing IP_3 and diacylglycerol (Jesaitis and Allen 1988; Smith *et al*, 1986: Rider and Niedel 1987), and diacylglycerol is a known activator of PKC (Nishizuka, 1988). Thus it seems activation of PKC plays a major role in activation of phagocytes, including neutrophils and macrophages.

The effect of a series of phorbol esters, TPA, SAP-A, DOPPA and Rx were initially studied on crude preparations of brain PKC (Ellis *et al*, 1987). However multiple subtypes of PKC have now been described (Parker *et al*, 1986; Osada *et al*, 1992). As a result of this, a series of phorbol esters were examined in detail with regard to activation of different isotypes of PKC (Ryves *et al*, 1991; Ryves, 1991). The isotypes studied were PKC α-, βi-, γ-, δ- and ε-, and it was shown that broad spectrum TPA activates all isotypes with activation of α-, βi-, γ- being slightly Ca^{2+}- dependent while δ- and ε- Ca^{2+}- independent. SAP-A activated PKC-α, -βi-, -γ in a positively Ca^{2+} dependent manner while -ε was slightly Ca^{2+}- independent. Interestingly PKC-δ was not activated by SAP-A . DOPPA activated only β- and this was absolutely dependent on Ca^{2+}. Rx caused Ca^{2+}- dependent activation of β-, at high concentration but, Rx also activates Ca^{2+} inhibited Rx - kinase (Ryves *et al*; see Chapter 4).

Since TPA, SAP-A, DOPPA and Rx show selective activities *in-vitro* with PKC isotypes, the aim of the present study was to examine their effects on two PKC-mediated responses in phagocytes. The responses studied were inhibition of fMLP-stimulated bivalent (Mn^{2+}) cation influx and stimulation of O^-_2 release in both intact cells and activation of its enzyme in cell-free system. Stimulation of neutrophils with

chemotactic peptide results in an increase in free cytosolic calcium ($[Ca^{2+}]_i$) as measured with fluorescent Ca^{2+} - indicator dye, quin-2, Indo-1 or fura-2. (Von Tscharner *et al*, 1986, Nasmith and Grinstein, 1987; Merrit *et al*, 1989). The rise in [Ca^{2+}]$_i$ appears to be the release of internal stores and fMLP- stimulated Ca^{2+} entry. These measurements of [Ca^{2+}]$_i$ responses provide only indirect evidence for agonist stimulated Ca^{2+} entry and alternative approach to measure directly against stimulated bivalent cation influx has been demonstrated (Merrit *et al*, 1989). When Mn^{2+} binds to fura -2, its fluorescence is quenched and thus cells loaded with fura-2 Merrit *et al*, (1989) were able to show fMLP stimulates Mn^{2+} influx into neutrophils. TPA was subsequently shown to inhibit fMLP stimulated bivalent cations thereby implicating the involvement of PKC in this function.

One of the major function of phagocytes upon activation is to undergo respiratory burst which utilises the enzyme NADPH-oxidase and ultimately promotes the release of O^-_2. TPA induces release of O^-_2 from phagocytes (Kramer *et al*, 1988) and PKC has been linked to phosphorylation of NADPH-oxidase of phagocytes (Cox *et al*, 1985; Tauber, 1987). Therefore initially the effect of phorbols on intact cells by manipulating [Ca^{2+}]$_i$ were studied since the phorbol esters have different Ca^{2+} requirements for activation of PKC isotypes, and the isotype differ in their requirement of Ca^{2+}. Since the biochemical sequence of activation pathways of NADPH-oxidase is likely to involve several different post-receptor steps, depending upon the agonist used (McPhail, *et al*, 1985; Grzeskowiak *et al*, 1986) activation by PKC might occur via direct phosphorylation of the components of NADPH-oxidase or via phosphorylation of 'activation proteins' (Kramer *et al*, 1988). Therefore a cell free system was devised where activation of NADPH-oxidase by PKC isotypes was examined.

6.2 Methods

6.2.1 Isolation of Human Neutrophils and Mouse Macrophages.

Human neutrophils were prepared by J. Merrit (Merrit *et al* 1993). Briefly, blood (27ml) was collected into anticoagulant (4.5ml of acid citrate dextrose; 1.5% citric acid, 2.5% trisodium citrate, 2% glucose). Dextran T500 (400mg in 12 ml 0.9% NaCl) was added, and the erythrocytes were allowed to sediment at unit gravity for 45 min. The resultant leukocyte rich plasma was removed, centrifuged (300 x g x 5min), and the cell pellet was resuspended in 8 ml of the supernatant. The concentrated cell suspension was layered over a discontinuous density gradient isosmotic of Percoll. The gradient was prepared by layering 2ml of the Percoll mixture (with 0.9% NaCl) at a density 1.110 g/ml below a 2ml layer at 1.088 g/ml. After centrifugation (600 x g x 20 min), the neutrophils (at the interface between the two layers of Percoll) were carefully removed and washed by dilution in buffer and centrifugation (300 x g x 5min). This method results in a preparation containing >95% neutrophils with a viability of >99% neutrophils (as assessed by trypan blue exclusion).

Starch - elicited murine peritoneal macrophages were obtained by aseptic lavage of the peritoneal cavity of male CD-1 mice by the method described in Chapter 3(see Section 3.2.1).

6.2.2 Loading cells with fura -2AM dye.

The bivalent cation influx and luminol chemiluminescence studies were carried out by J. Merrit (Merrit *et al*, 1993) and has given kind permission for use of her results. The neutrophils were washed and suspended at 6 x10^6 cells/ml in Hepes buffered saline (145mM NaCl, 5mM KCl, $MgCl_2$, 10mM glucose, 10mM Hepes), PH 7.4 at 37^oC supplemented with 1mM $CaCl_2$ and 1% BSA. Cells were loaded with fura-2 acetoxymethyl (fura 2-AM) ester at 0.3 uM (for all experiments except heavily buffered cells) or 2uM (for experiments with heavily buffered cells). The resultant

cytosolic fura - 2 AM concentration for incubation with 0.3uM fura -2AM was 50-100 uM ('low fura -2'). After loading with the dye, cells were centrifuged (300 x g x5 min) and resuspended in supplemented Hepes buffer as above at a density of 2 $x10^6$/ml and, maintained at RT until required.

6.2.3 Measurement of fura-2AM fluorescence.

For fura-2 fluorescence measurements, aliquots of cell suspension were centrifuged (microfuged, 6500 rev/min for 30s) and resuspended at 2 x 10^6 ml in Hepes - buffered medium containing no BSA. For fura -2 fluorescence studies lmM $CaCl_2$ or 100uM $MnCl_2$ was added as required. Fluorescence emission (500nm) was measured at 37°C in a dual - excitation wavelength fluorimeter with additions as indicated in the Figures. For manipulation of $[Ca^{2+}]_i$, the $[Ca^{2+}]_i$ concentration was calculated from the ratio of fura - 2 fluorescence at two excitation wavelength, 340nm and 380nm, as described by Grynkiewicz *et al*, (1985). For bivalent cation (Mn^{2+}) influx studies fluorescence was monitored at excitation wavelengths of 340 and 360 nm. At 340nm the fluorescence increases with $[Ca^{2+}]_i$ but at 360nm fura-2 fluorescence is insensitive to $[Ca^{2+}]_i$ and Mn^{2+} quenches both fluorescence wavelengths. Therefore quenching at 360 nm provides a direct measurement of bivalent cation (Mn^{2+}) influx.

6.2.4 Superoxide generation of neutrophils.

Superoxide generation was monitored by enhancement of luminol chemiluminescence. Luminol was solubilised in DMSO and added to aliquots of neutrophil suspension at a final concentration of 15 ug/ml, which were then pre-incubated at 37°C for 5 mins before the additions of compounds and measurement chemiluminescence. Chemiluminescence was measured by a lumiaggregometer.

6.2.5 Preparation and activation of oxidase.

10^8 -10^9 starch elicited mouse peritoneal macrophages were lysed in preparation of membrane and cytosolic fractions. Membrane and cytosol fractions were prepared as described in Chapter 5 (see section 5.2.2). For studying the effect of kinase isotypes on reconstituted complex employed the method described in Chapter 2. The assay was prepared by mixing the constituents (see section 2.3.3.2) and the mixture was incubated at room temperature for 5 mins. The reaction was started by adding NADPH-co factor (100uM) and continuously monitored on a spectrophotometer with change in absorbance at 550nm.

6.2.6 *In-vitro* phosphorylation of NADPH-oxidase.

The assay was prepared by mixing the assay mix described in Chapter 2 (see section 2.3.3.2). ATP mix buffer contained ^{32}P-ATP (0.5mCi) and the concentration was reduced from 100uM to 50uM to increase the ratio of ^{32}P-ATP/ATP. The assay mix contained 50ul membrane and cytosol fractions, 50ul PKC subtype, 50ul buffer-2 (see section 2.3.2), 10 ul TPA (10ngml^{-1}), 40ul NADPH co-factor (100uM) and, the reaction was started by adding ATP-mix buffer. Note that cytochrome C was omitted from this experiment as we were not measuring superoxide release. The reaction was terminated by pipetting 25ul of reaction mixture to 25ul double strength laemmli buffer (see section 2.3.4) at 30s intervals. After SDS-PAGE the gels were exposed to Kodak Extrom film for a maximum of 1 week at -70^{o}C.

6.3 Results

6.3.1 Effect of phorbols on fMLP-stimulated bivalent cation influx.

fMLP is known to induce bivalent cation influx in phagocytic cells such as neutrophils and initial experiments were carried out to study the effects of phorbols on this system. At 340nm excitation, fMLP (10nM) evoked a rapid and transient increase in

Legends for Fig 6.1-6.3

Figure 6.1 Effects of Sap-A, DOPPA and Rx on fMLP-stimulated Mn^{2+} influx in neutrophils. Furs-2 fluorescence (500nm emission) was monitored simultaneously at excitation wavelengths of 340nm (solid lines) and 360nm (broken lines). Additions were made as indicated: Mn^{2+} (100μM), fMLP (10nM), IONO (2μM ionomycin) and phorbols as shown. The phorbols were solubilised in DMSO, so an equivalent amount (0.1%) was included in the control experiment.

Figure 6.2 Dose response curves for inhibition of fMLP-stimulated influx by the phorbols. Mn^{2+} influx was measured as shown in Fig 6.1, with the experiments with TPA, Sap-A, DOPPA and Rx.

Figure 6.3 Effects of maximal concentration of the phorbols alone on O_2^- release from neutrophils. O_2^- was measured as enhancement of luminol chemiluminescence following a 5 min pre-incubation with luminol. Additions were made at the times shown. fMLP (1μM) is included for comparison.

Fig 6.1

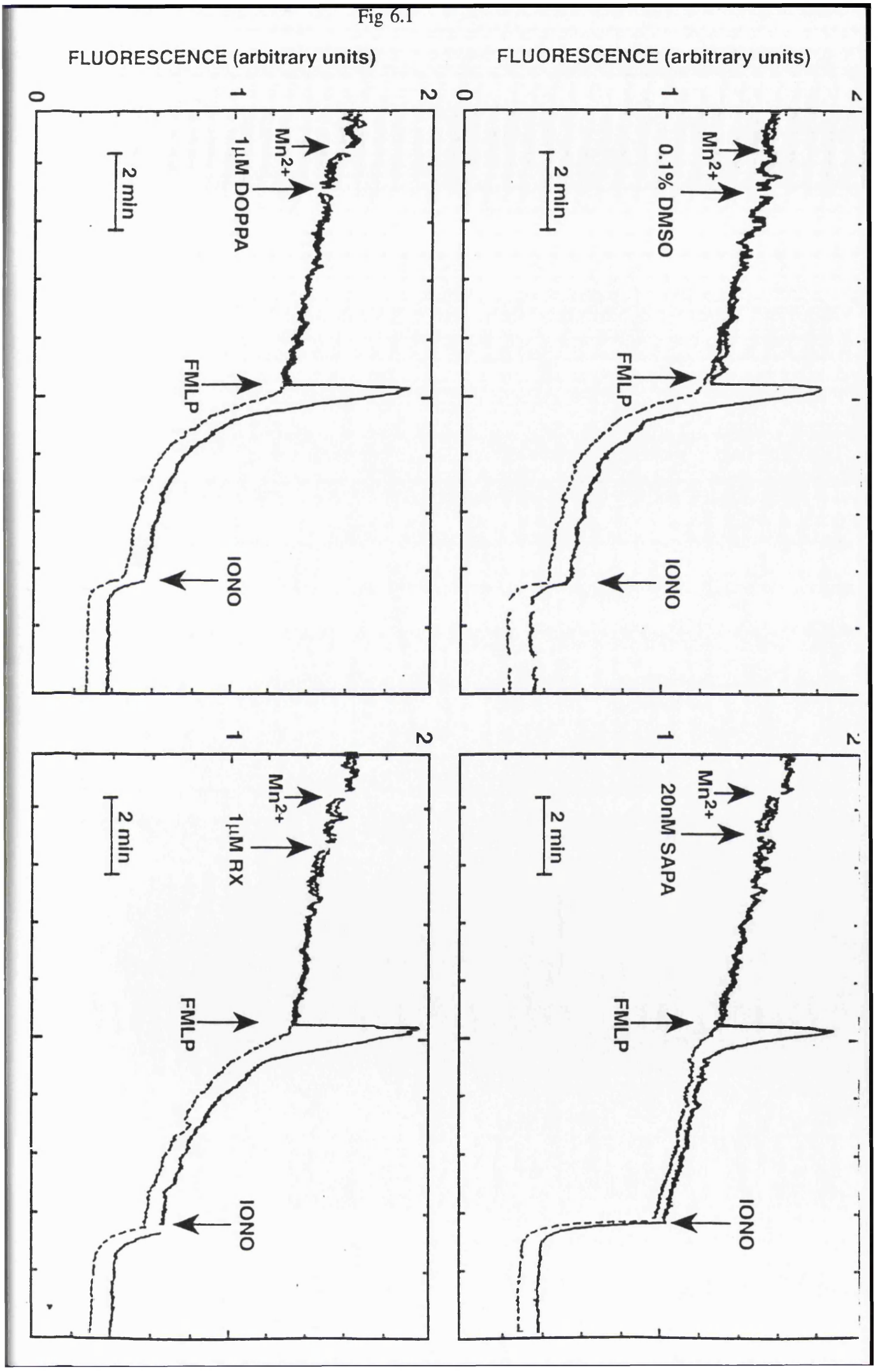
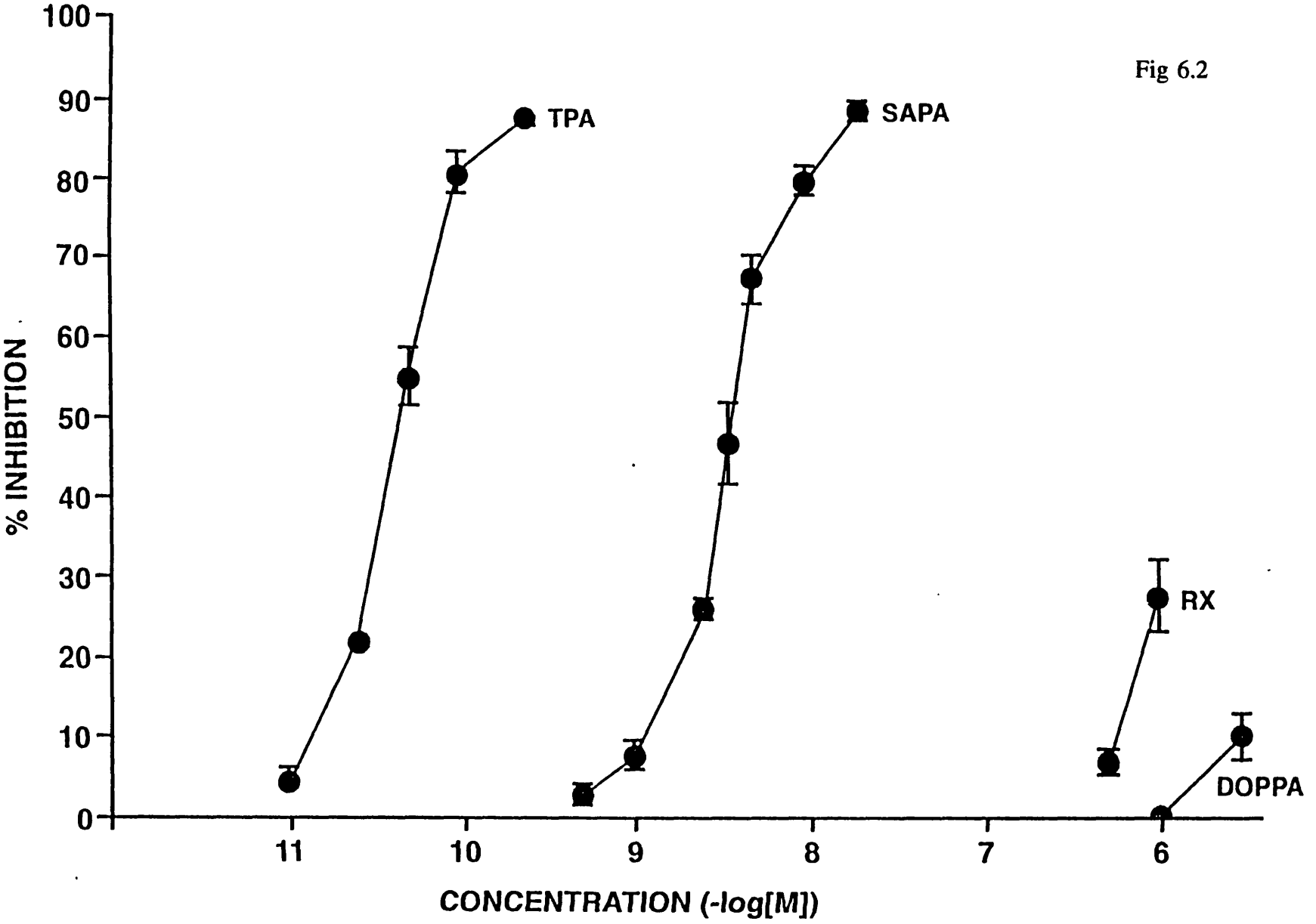

FLUORESCENCE (arbitrary units)
0
1
2
Mn^{2+}
0.1% DMSO
2 min
FMLP
IONO
FLUORESCENCE (arbitrary units)
Mn^{2+}
1μM DOPPA
2 min
FMLP
IONO
Mn^{2+}
20nM SAPA
2 min
FMLP
IONO
Mn^{2+}
1μM RX
2 min
FMLP
IONO

Fig 6.2


100
90
80
70
60
50
40
30
20
10
0
% INHIBITION
TPA
SAPA
RX
DOPPA
11
10
9
8
7
6
CONCENTRATION (-log[M])

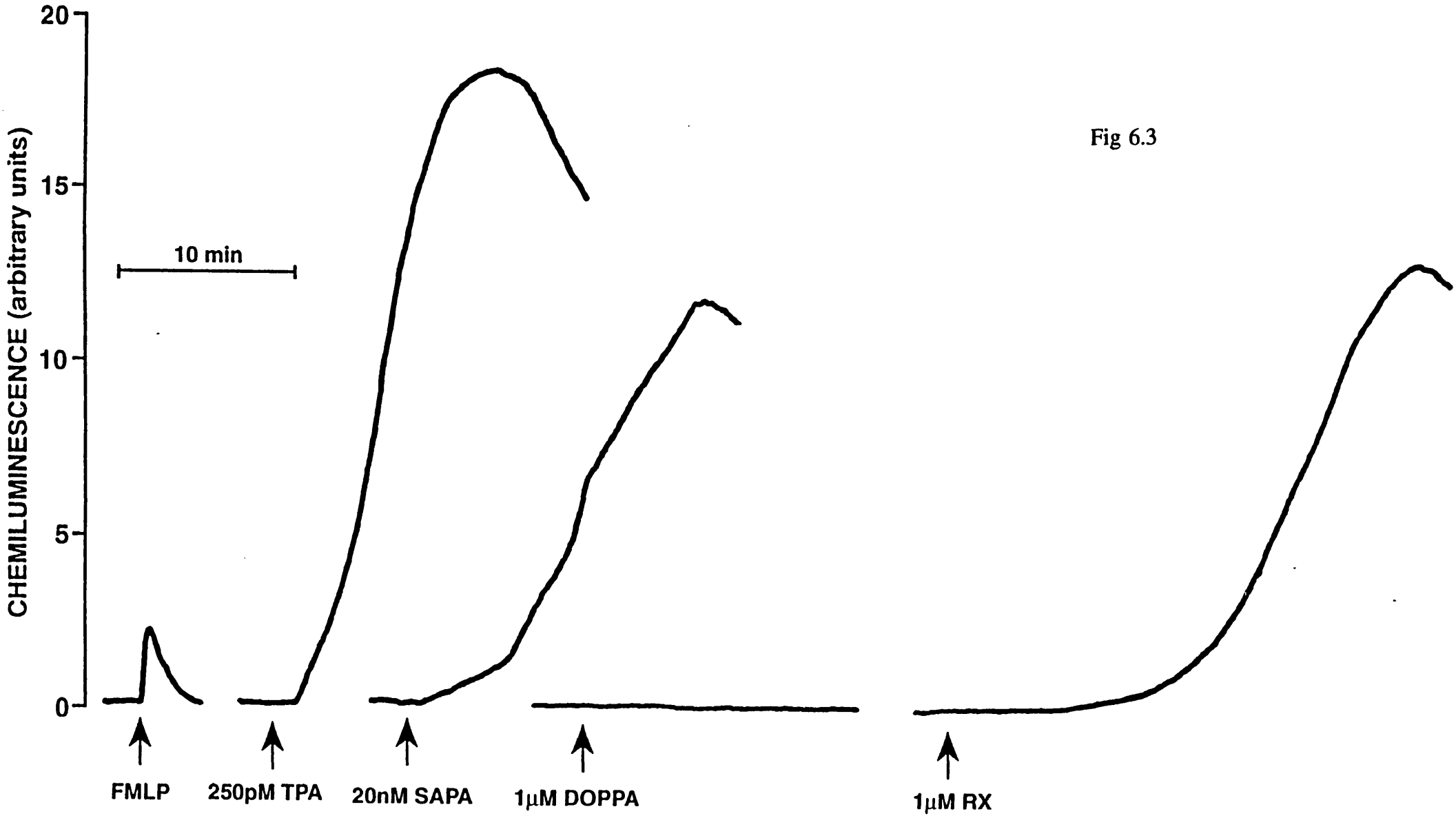

CHEMILUMINESCENCE (arbitrary units)
20
15
10
5
0
10 min
FMLP
250pM TPA
20nM SAPA
1μM DOPPA
1μM RX

Fig 6.3

fluorescence, reflecting an increase $[Ca^{2+}]_i$ due to release of Ca^{2+} from intracellular stores (as fluorescence at 360nm excitation is unaffected by $[Ca^{2+}]_i$). This rapid rise in fluorescence was followed by decline to well below pre-stimulated levels as Mn^{2+} entered the cells and quenched the intracellular dye. At 360nm excitation, only the stimulated quench in fluorescence was seen (due to stimulated Mn^{2+} entry). DOPPA (1uM) had no inhibitory effect on fMLP stimulated Mn^{2+}- influx (although some 30% inhibition was noted after 45 mins) (see Fig 6.1). Rx (luM) inhibited fMLP-stimulated Mn^{2+} influx by 30% after 5 min pre-incubation, although inhibition was time dependent with 80% inhibition occurring by 60min (Fig 6.1). When SAP-A (20nM) was pre-incubated (5 min) prior to additions of fMLP, the initial fMLP stimulated response was unaffected but the fMLP stimulated quench at both wavelength was inhibited which suggests that SAP-A totally inhibits fMLP stimulated bivalent cation influx. Dose response curves for inhibition of fMLP-stimulated Mn^{2+} influx shows that TPA was the most potent inhibitor (IC_{50} = 48 ± 4pM) and inhibition by SAP-A had an IC_{50} = 40 ± 0.3nM (Fig 6.2). Little or no inhibition was observed with DOPPA and Rx even at high concentrations. Pre-incubations of 5min was used in this study to allow comparison of 5 min pre-incubation for O^-_2 release described below.

6.3.2 Effects of phorbols on superoxide release.

Previous studies on starch elicited murine macrophages revealed that TPA and SAP-A were strong agents at stimulating O_2^-, Rx evoked a 50% maximal response and required particulate stimulus for maximum effectiveness but DOPPA was weak (see Chapter 3). Using a different method utilising enhancement of luminol chemiluminescence similar effects were observed. TPA (250 pM) and SAP-A (20 nM), at concentration that totally inhibited Mn^{2+} influx, evoked a large increase in O_2^- release after a short delay (Fig 6.3). DOPPA (1uM) did not stimulate O_2^- release even after a long incubation period. Rx (1uM) inhibited Mn^{2+} influx by 35% when pre-incubated for 10mins and

interestingly caused a large increase in O_2^- release after a delay of about 10mins.

Figure 6.4 shows the effects of fMLP- stimulated O_2^- release of neutrophils pre-incubated with phorbols 5mins before the additions of fMLP. The concentration of phorbol esters used in these experiments had no stimulatory effects. In each case fMLP response was enhanced with phorbol pre-incubation compared to fMLP alone (Fig 6.4). The largest potentiated effect was seen with TPA (100pM) followed by SAP-A (5nM) and Rx after a lag of 5min (Rx only had an effect after a lag of 10mins.). DOPPA (1uM) induced a small potentiation of fMLP response but in most experiments little or no potentiation was recorded.

6.3.3 Effects of manipulating [Ca^{2+}]$_i$ on phorbol mediated O_2^- release.

Since PKC isotypes vary in their Ca^{2+}-dependency and phorbol esters have selective Ca^{2+} requirements for PKC activation (Ryves *et al* , 1991), it was therefore decided to examine the effects of manipulating [Ca^{2+}]$_i$ on phorbol mediated O_2^- release. Two approaches to manipulating [Ca^{2+}]$_i$ were examined, firstly, a Ca^{2+}- ionophore ionomycin was used to raise [Ca^{2+}]$_i$ to known levels before examining the effects of phorbols alone or potentiation of the fMLP response. Then fMLP-stimulated rise in [Ca^{2+}]$_i$ was manipulated and the effect of phorbol esters on fMLP stimulated O_2^- release was examined under conditions where fMLP evoked either a normal or a small increase in [Ca^{2+}]$_i$.

6.3.3.1 Effect of ionomycin on stimulated O_2^- release.

Before the release of O_2^- was examined, [Ca^{2+}]$_i$ was measured in the same preparations of fura-2 loaded cells in response to range of concentration of ionomycin (2.5 -15nM). Briefly the additions of ionomycin evoked [Ca^{2+}]$_i$ rise, were as

follows: unstimulated [Ca^{2+}]$_i$ = 90nM; 2.5nM ionomycin =146nM [Ca^{2+}]$_i$; 5nM ionomycin = 233nM [Ca^{2+}]$_i$; 10nM ionomycin = 284nM [Ca^{2+}]$_i$; 15nM ionomycin = 396nM [Ca^{2+}]$_i$. Ionomycin (2.5 -15nM) alone did not evoke O_2^- release from neutrophils (Fig 6.5) and response of phorbols was measured in the presence of minimally effective ionomycin concentration. TPA (100pM) and SAP- A (5nM) induced strong release of O_2^- with the lowest dose of ionomycin (2.5nM) used (ie at the lowest elevation of [Ca^{2+}]$_i$ from 90nM to 146nM). Rx (500nM) produced a strong response when [Ca^{2+}]$_i$ was elevated from resting [90nM] to 233nM (or 5nM ionomycin). This response was rapid which contrasts to the delayed (10 min lag) response 1uM Rx at basal Ca^{2+} levels (Fig 6.3). DOPPA (1uM) only induced a response after [Ca^{2+}]$_i$ was elevated to 396nM (15nM Ionomycin).

The response to fMLP (1uM) in the presence of ionomycin (15nM) was unaffected (Fig 6.5). DOPPA (1uM) slightly potentiated fMLP response at [Ca^{2+}]$_i$ of 233nM while Rx (0.5uM) greatly enhanced the fMLP response of [Ca^{2+}]$_i$ at 146nM. Both phorbols when used alone were unable to induce O_2^- release at basal [Ca^{2+}]$_i$ but enhanced the fMLP response.

6.3.3.2 Effect of fMLP- stimulated rise in [Ca^{2+}]$_i$.

An alternative approach to studying interactions between the phorbols and [Ca^{2+}]$_i$ were done by manipulating the rise in [Ca^{2+}]$_i$ via fMLP stimulation. J Merrit *et al* (1993) describes this procedure in detail and will not be discussed here. However in order to study the effects of phorbols the results need to be addressed. Briefly, in cells with either a low or high concentrations of fura-2, the initial rise in [Ca^{2+}]$_i$ evoked by fMLP was similar; in cells with low fura-2, SKF 96365 (blocks Ca^{2+} influx but has little effect on intracellular stores) has little effect on initial rise in [Ca^{2+}]$_i$ evoked by fMLP; and in cells with high fura-2 (buffers [Ca^{2+}]$_i$) SKF 96365 considerably

Legends for Fig 6.4-6.6

Figure 6.4 Effects of adding phorbols 5 min before fMLP on stimulation of O_2^- release. O_2^- was measured as enhancement of luminol chemiluminescence. Addition were made at the times shown. The fMLP concentration was 1μM which was maximal alone or with phorbols.

Figure 6.5 Effects of ionomycin on O_2^- release stimulated by phorbols alone (a) or phorbol plus fMLP (b). Fig 6.5a shows the response at a minimally effective concentration of ionomycin for each phorbol and Fig 6.5b compares the response to fMLP alone or plus DOPPA or Rx at basal $[Ca^{2+}]_i$ or in the presence of minimally effective concentration of ionomycin.

Figure 6.6 Priming of fMLP-stimulated superoxide release by phorbols in the presence or absence of SKF&F 96365 when neutrophils contain "low" (a) or "high" (b) levels of fura-2AM. Additions were made as indicated, fMLP (1μM), SK&F 96365 (7.5μM), DMSO (vehicle 0.1%) and phorbols a shown.

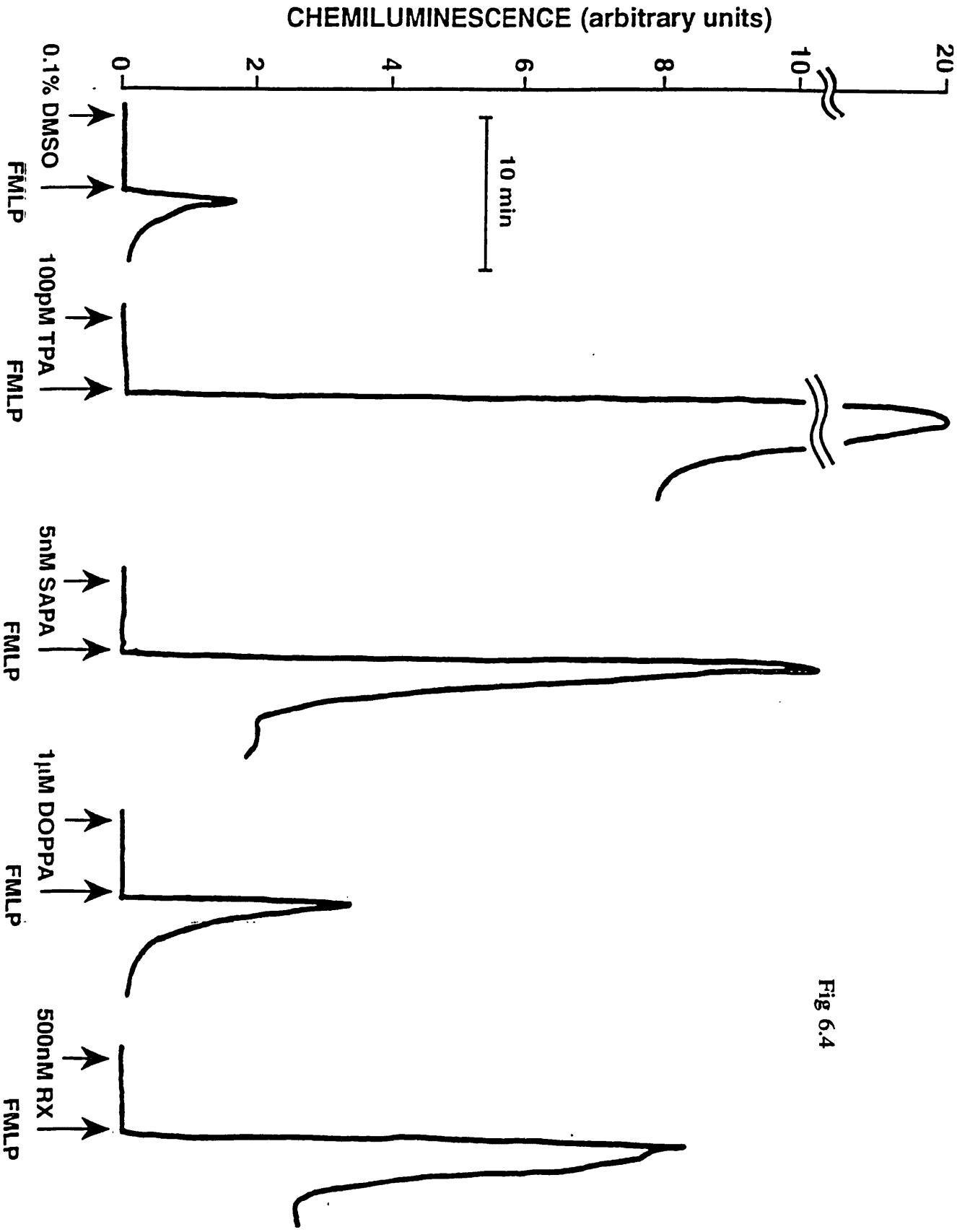
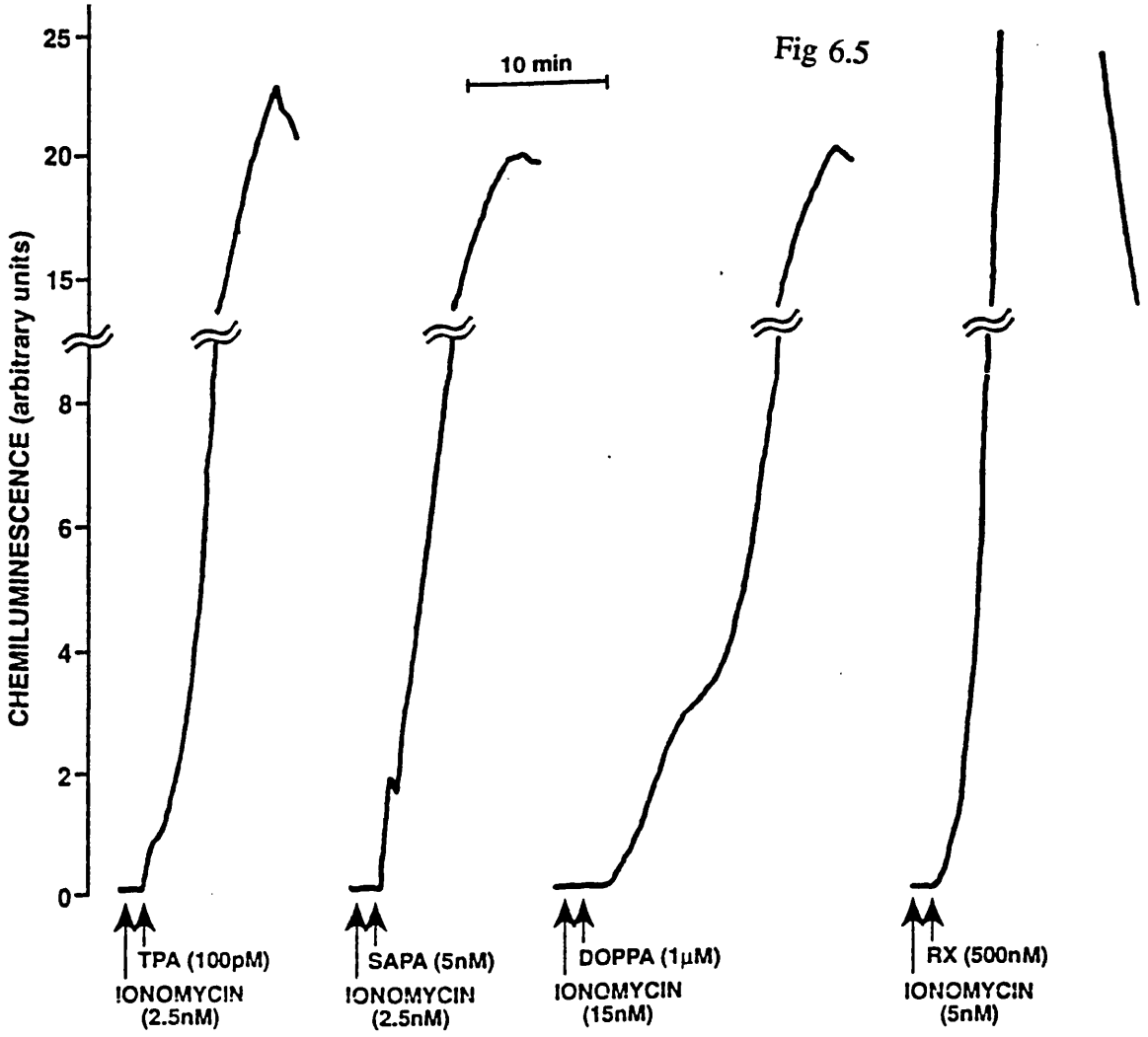

CHEMILUMINESCENCE (arbitrary units)
0
2
4
6
8
10
20
10 min
0.1% DMSO
FMLP
100pM TPA
FMLP
5nM SAPA
FMLP
1μM DOPPA
FMLP
500nM RX
FMLP

Fig 6.4

Fig 6.5


CHEMILUMINESCENCE (arbitrary units)
25
20
15
8
6
4
2
0
10 min
TPA (100pM)
IONOMYCIN
(2.5nM)
SAPA (5nM)
IONOMYCIN
(2.5nM)
DOPPA (1μM)
IONOMYCIN
(15nM)
RX (500nM)
IONOMYCIN
(5nM)

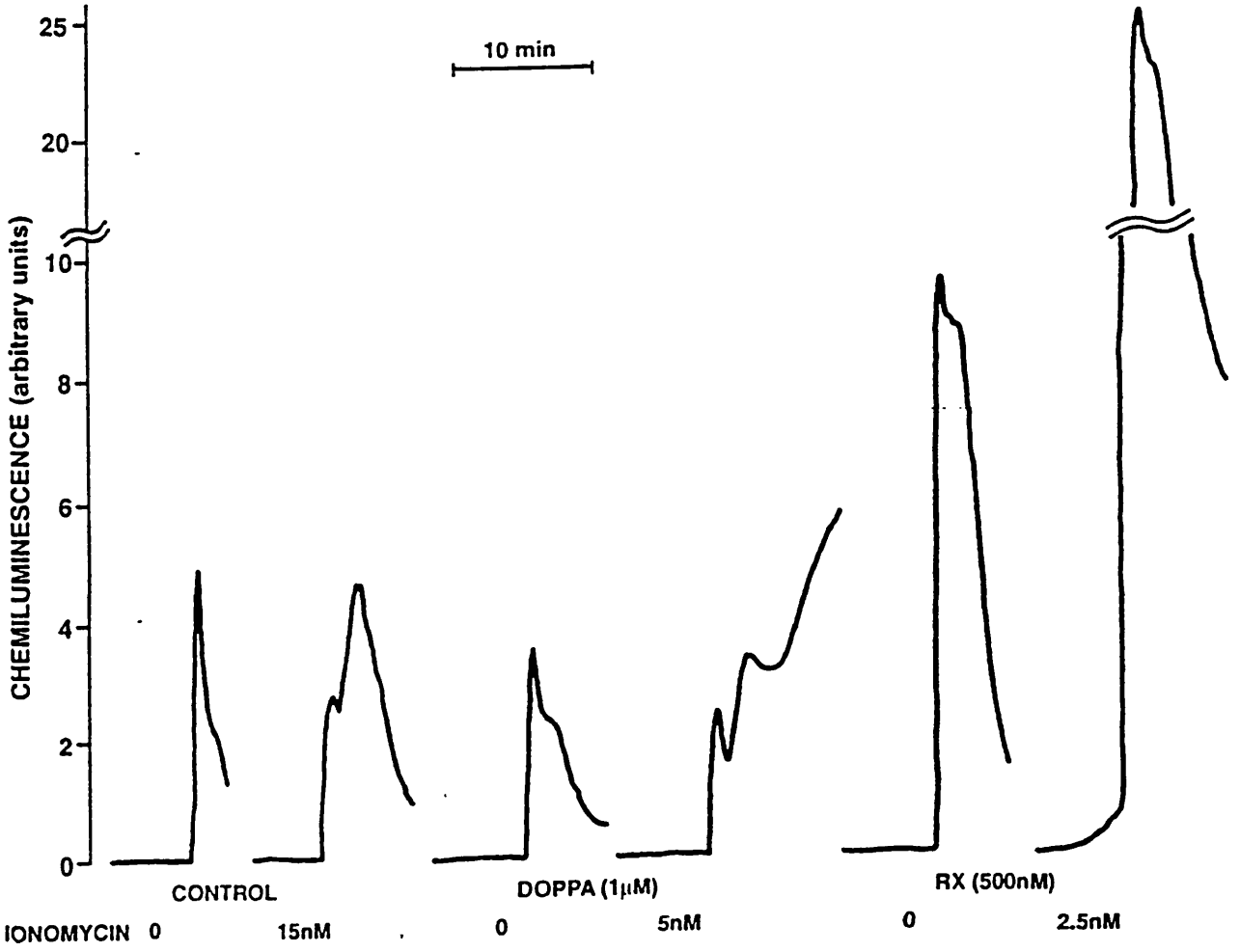
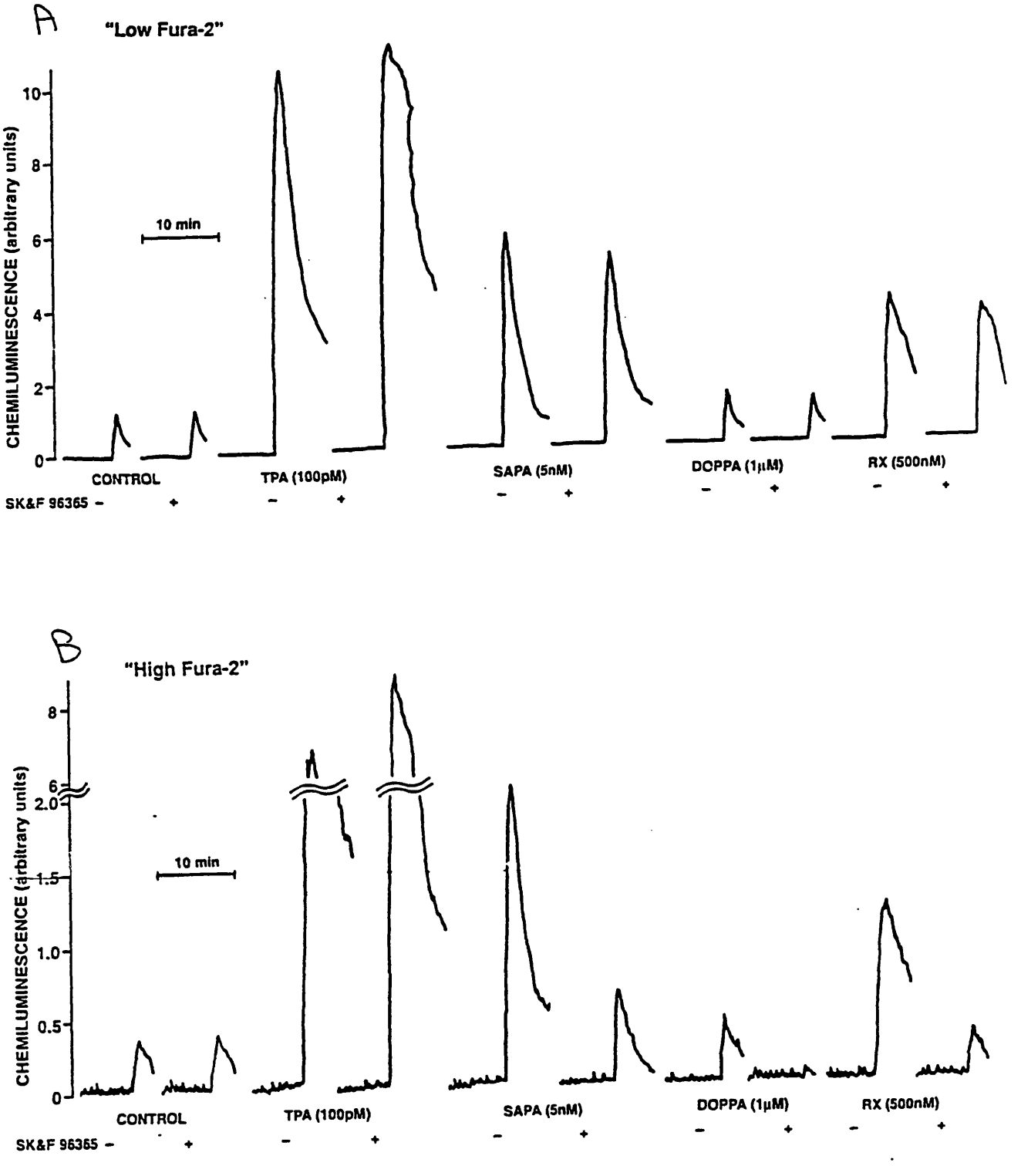

CHEMILUMINESCENCE (arbitrary units)
25
20
10
8
6
4
2
0
10 min
CONTROL
IONOMYCIN 0
15nM
DOPPA (1μM)
0
5nM
RX (500nM)
0
2.5nM

Fig 6.6


A
"Low Fura-2"
CHEMILUMINESCENCE (arbitrary units)
10
8
6
4
2
0
10 min
CONTROL
TPA (100pM)
SAPA (5nM)
DOPPA (1μM)
RX (500nM)
SK&F 96365 – + – + – + – + – +
B
"High Fura-2"
CHEMILUMINESCENCE (arbitrary units)
8
6
2.0
1.5
1.0
0.5
0
10 min
CONTROL
TPA (100pM)
SAPA (5nM)
DOPPA (1μM)
RX (500nM)
SK&F 96365 – + – + – + – + – +

reduces the initial rise in [$Ca^{2+}]_i$ evoked by fMLP.

Taking these findings into consideration studies were conducted on phorbol ester potentiation of fMLP- stimulated O_2^- in cells with low and/or high levels of fura-2 and in the presence and absence of SKF 96365. In cells with low fura-2 TPA (100pM), SAP-A (5nM), Rx (0.5 uM) but not DOPPA (1uM) potentiated fMLP O_2^- release (Fig 6.6). Under identical conditions SKF 96365 had no effect either with fMLP alone or with phorbol esters. In cells with high fura-2 similar results were obtained as TPA, SAP-A, Rx but not DOPPA potentiated fMLP response (Fig 6.7). However in these cells the additions of SKF 96365 significantly reduced the potentiated fMLP response with SAP-A and Rx but not with TPA. Thus when the fMLP stimulated rise in [$Ca^{2+}]_i$ is limited, the potentiating effects of SAP-A and Rx (but not TPA), on O_2^- release are decreased.

6.3.4 PKC- isotype activation of NADPH- oxidase.

The above results suggest that activation of PKC by phorbols in the phagocytes correlates with their Ca^{2+}- dependency for activation of PKC - *in vitro*. However the data are limited in that the involvement of PKC in the phagocyte function (O_2^-) is dependent upon exclusive activation of PKC by phorbols. Numerous targets sites of phorbols may operate ultimately leading to stimulation of O_2^-. Thus to overcome this, we decided to test the ability of PKC isotype activation of NADPH-oxidase at a biochemical level using a cell-free model. The reconstituted system was macrophage membranes and cytosol.

6.3.4.1 Dose-response curves of PKC-isotype activation of NADPH-oxidase.

Linear dose-dependent increments were observed in oxidase activity with increasing

concentration of α, β, γ- isotypes (0.01 - 1.0 u/ml) in the presence of 5ngml^{-1} TPA (Table 6.1) (δ- and ε- were not investigated due to low quantities of isotype available). Activation by these isotypes showed greater than 2- fold stimulation in the presence of Ca^{2+} as compared with the absence of Ca^{2+}- ions. The Ca^{2+}- dependency observed for the isotypes for activation of the physiological substrate, oxidase, correlates with their *in-vitro* activity in standard histone/kinase assays (Marias and Parker, 1989). From these dose response studies, it was calculated that further experiments used 0.1 u/ml of isotypes.

When direct activation of oxidase was compared with PKC isotypes it revealed that unstimulated intrinsic activity of PKC- isotypes were dependent of Ca^{2+}- for oxidase activation (Table 6.2). However the values were lower, with plus Ca^{2+} the activation by basal PKC was 5-8 fold lower and without Ca^{2+} the activation was 3-4 fold lower than when TPA (50 ngml^{-1}) was present. The basal activity of δ- (maximum response 144±36 pmol/min /U) and ε- (maximal response 162±36 pmol/min/U) were 2.0 to 4.0 fold more effective than α- (maximal response 36±18), β- (maximal response 54±18 pmol /min/U) and γ- (maximal value 72±36 pmol/min/U) at oxidase activation.

6.3.4.2. Activation of NADPH-Oxidase by TPA- stimulated PKC isotypes.

In the presence of TPA maximal responses to PKC isotypes activation of oxidase were up to 5- fold higher than compared to basal levels. In the presence of free Ca^{2+} ions (100uM), a dose-dependent response was observed with an EC_{50} value of 10-20ng/ml TPA for all the isotypes used (Fig 6.7) with the maximal response achieved by β-PKC at 0.447 nmol/min/U. However in the absence of Ca^{2+} ion (or 10mM EGTA), Ca^{2+}- independent δ- and ε-PKC showed an active dose dependent rise in NADPH-oxidase activation to increasing concentrations of TPA (Fig 6.8). The Ca^{2+} dependent isotypes α- and γ-PKC were weakly active in the absence of Ca^{2+} with the maximal

response of only 0.188 nmol/min/U by α-PKC. β-PKC was more active than α- or γ- but less potent than δ- and ε- in the absence of Ca^{2+} ions.

6.3.4.3 *In Vitro* phosphorylation with PKC -isotypes.

Although cell-free assay as above indicate NADPH-oxidase is activated by PKC isotypes the individual protein phosphorylated during activation are not. In order to clarify this, purified α-, β-, γ-PKC isotypes (δ- and ε- were not available) were added to the membrane plus cytosol fractions obtained from macrophages. Fig 6.9 shows the time course for phosphorylation with each lane representing 30s. Almost similar phosphorylation patterns were observed for all three isotypes used with the major band appearing at approximately 47k Da. This phosphorylated protein appears within 30s for both α- and β-PKC and by 1 min with γ-PKC. Minor phosphorylated bands also appear after 1 min at apparent molecular weight of 56 kDa and 92 kDa.

6.4 Discussion

Involvement of phorbol esters and PKC mediated activities in two functions of phagocytes (human neutrophil and murine macrophages) have been studied, inhibition of fMLP bivalent cation influx, and stimulation of O_2^- release (either in intact cells or in cell-free systems). Phorbol esters with selective stimulation of PKC isotypes *in-vitro* were used to activate intact neutrophils to evoke functional responses and this was linked to direct activation by PKC isotypes in a cell-free system.

Previous work reported that TPA activation of PKC inhibits agonist stimulated bivalent cation influx (McCarthy *et al*, 1989) and studies were thus probed with SAP-A, DOPPA and Rx and not TPA. SAP-A also inhibited fMLP stimulated Mn^{2+} influx and

Activation of NADPH oxidase by stimulated PKC in the presence of calcium

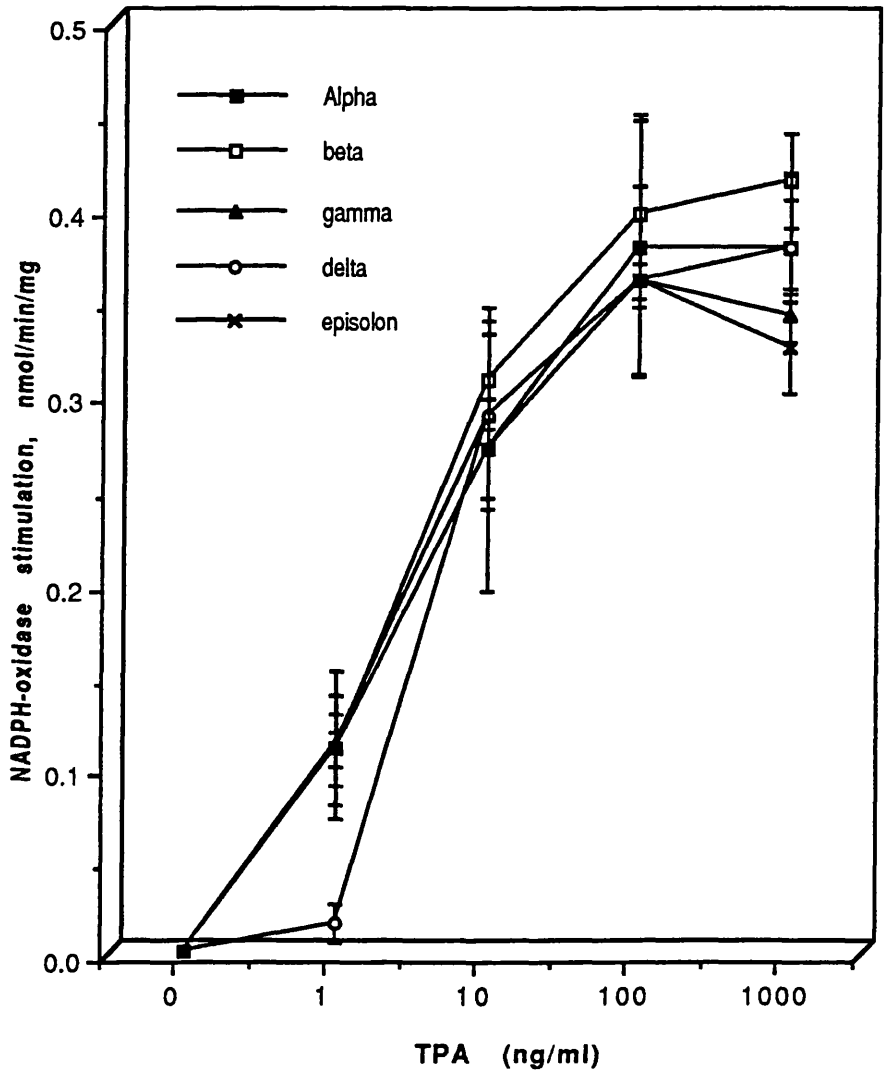


Fig 6.7 Activation of NADPH-oxidase by TPA stimulated PKC isotypes in the presence of Ca^{2+}. Superoxide anion was determined by a continious kinetic assay of SOD inhibitable reduction of cytochrome C. For PKC isotype studies the phosphate buffer detailed in Chapter 2 (section 2.3.3.2) contained additional 100μM ATP, 10mM $MgCl_2$, 5.2mM Ca^{2+}, 250μgml^{-1} PS and 100ngml^{-1} TPA in 0.02% Triton X-100. Results are calculated using the initial rate of $O^{\cdot}_2$ ±SD (n=2).

Activation of NADPH oxidase by stimulated PKC in the absence of calcium

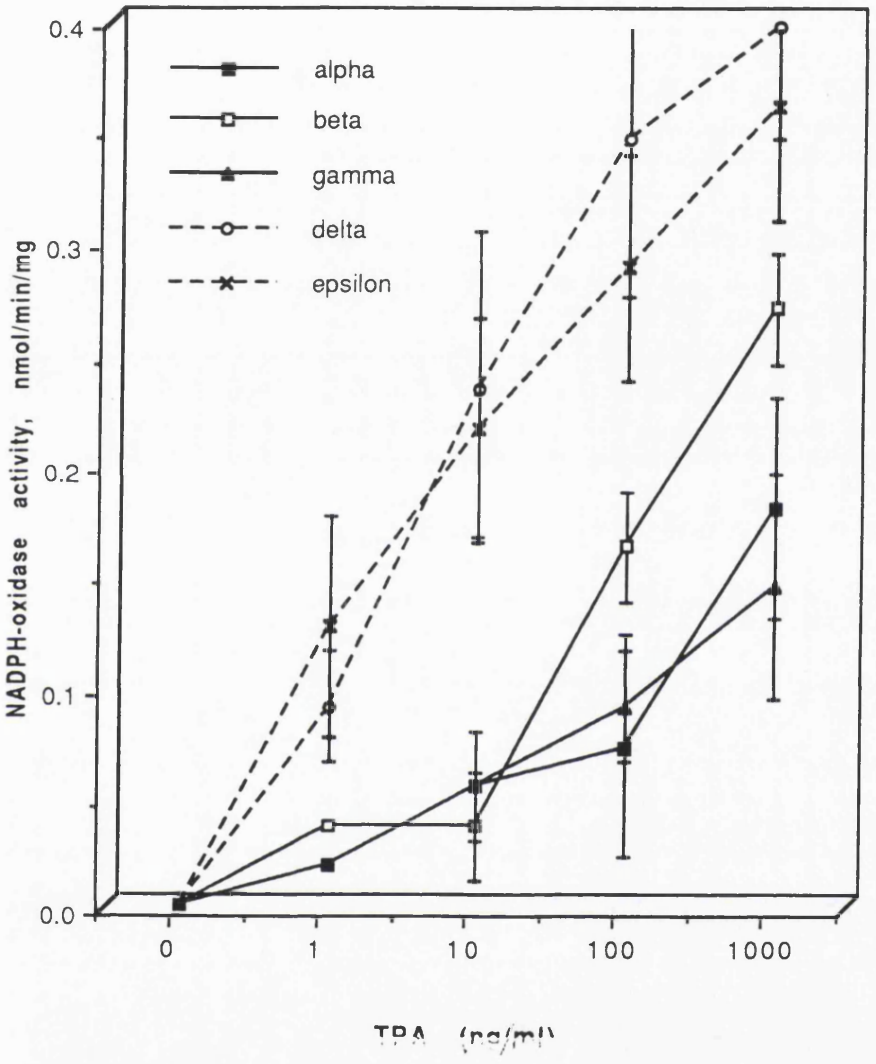


Fig 6.8 Activation of NADPH-oxidase by TPA stimulated PKC isotypes in the absence of Ca^{2+}. Superoxide anion was determined by a continious kinetic assay of SOD inhibitable reduction of cytochrome C. For PKC isotype studies the phosphate buffer detailed in Chapter 2 (section 2.3.3.2) contained additional 100μM ATP, 10mM $MgCl_2$, 10mM EGTA, 250μgml^{-1} PS and 100ngml^{-1} TPA in 0.02% Triton X-100. Results are calculated using the initial rate of O^{-}_2 ±SD (n=2).

this inhibition was also blocked by staurosporine. SAP-A is a Ca^{2+}- dependent phorbol ester which activates α-, β-, γ- and ε- PKC but not δ- isotype *in-vitro* (Ryves *et al*, 1991). Taken together these results indicate a major role for δ-PKC is ruled out on inhibition of fMLP - stimulation of Mn^{2+} influx of intact neutrophils. Rx (1uM) also inhibited fMLP stimulated Mn^{2+} influx but only 30% by 5mins (74% after 45 min pre-incubation). This smaller effect (and increasing with longer pre-incubation time) of Rx suggests involvement of Rx-kinase, in additions to PKC (effects observed with TPA and SAP-A) in modulation of bivalent cation influx. It was interesting to note that prolonged incubations caused greater inhibition by Rx since Rx-kinase is known to be 'primed' by Rx-pre-incubations (see Chapter 3, section 3.4). Another suggestion could be that Rx initially stimulate β_i-PKC isotype (Ryves *et al*, 1991) before Rx-kinase was primed and activated to modulate inhibition of agonist stimulated bivalent cation influx. DOPPA, even at high concentrations had no inhibitory effects after 5 mins and only 30% after 45 mins. DOPPA was shown to be a poor activator of PKC with only a major stimulation of β_i- isotype (Ryves, *et al*, 1991; Ryves, 1991). These results indicate that a major role for β_i-PKC seems unlikely for inhibition of agonist stimulated bivalent cation influx. However activation of β_i-PKC by DOPPA was totally dependent upon Ca^{2+} and it may be possible that $[Ca^{2+}]_i$ under these conditions may not be sufficient to sustain an activation of β_i-PKC by DOPPA.

There is mounting evidence to suggest that PKC activation promotes O_2^- production from phagocytes. Previous results have revealed that phorbol esters directly stimulate O_2^- release by probably acting through protein kinases (PKC and Rx-kinase; see Chapter 3 and Chapter 7). TPA stimulated O_2^- release after a short delay at concentrations which totally inhibited fMLP-stimulated bivalent cation influx. SAP-A also produced similar results. Both TPA and SAP-A were ineffective at lower doses (100pM TPA and 5nM SAP-A). DOPPA (1uM) at high concentrations which did not inhibit agonist stimulated Mn^{2+} influx, also did not induce O_2^- release. Rx (1 uM) on the other hand stimulated O_2^- release but after a lag of 10 mins (Rx at this concentration

Units of enzyme activity (U)			
Enzyme	0.01	0.10	1.00
$\alpha + Ca^{2+}$	0.234±0.03	0.288±0.05	0.324±0.051
$\alpha - Ca^{2+}$	0.108±0.05	0.144±0.05	0.126±0.018
$\beta + Ca^{2+}$	0.216±0.051	0.270±0.025	0.306±0.02
$\beta - Ca^{2+}$	0.126±0.025	0.144±0.036	0.144±0.051
$\gamma + Ca^{2+}$	0.216±0.051	0.270±0.025	0.288±0.06
$\gamma - Ca^{2+}$	0.108±0.06	0.162±0.02	0.162±0.025

Table 6.1 Dose dependent activation of NADPH-oxidase by PKC-isozymes in the presence of TPA. PKC isotype were kindly donated Dr P J Parker and diluted in 20mM Tris-Cl, pH 7.5 containing 2mM EDTA, 0.02% Triton X-100, 1mM DTT and 10% glycerol to give an activity in the range of 0.01 - 1.0 U (nmol/min/ml) of kinase activity. TPA was used at concentration of 5ng/ml. Results are expressed as mean ±SD (n=3).

Isozyme	Ca^{2+} (100uM)	EGTA (5.0 mM)
α	36±18	36±18
β	54±18	36±18
γ	54±36	72±36
δ	144±36	126±18
ε	126±36	162±36

Table 6.2 Unstimulated intrinsic PKC-isozymes mediated activation of NADPH-oxidase. Activity is shown as pmol superoxide anion produced per minute per unit enzyme activity ±SD. Unit of enzyme is measured by γ-^{32}P incorporated/min into histone IIIs for α, β and γ and specific peptide pseudosubstrates for δ and ε.

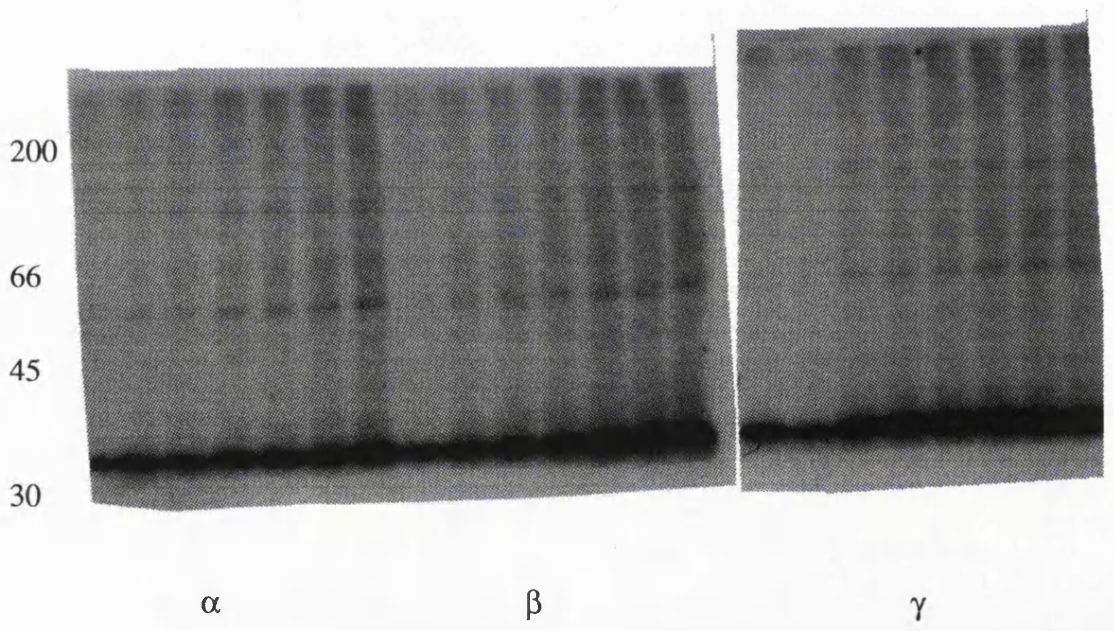


Fig 6.9 Time course for in-vitro phosphorylation of NADPH-oxidase by PKC isotypes. The assay was prepared by mixing the assay mix described in the Methods. The reaction was started by adding ATP mix buffer and terminated at every 30s by adding equal volume odf double strength lammeli buffer. The results represent gels exposed to 1 week at -70oC

inhibited Mn^{2+} influx by 35% after 10 mins incubation). This delay in response may reflect poor cell permeability but seems unlikely since lower concentrations of Rx (500nM) were effective at priming the fMLP response following only a 5 mins pre-incubation (see Fig 6.4). Rx induction of O_2^- release suggests Rx-kinase involvement particularly in view of the lag response. Stimulation of O_2^- by TPA, SAP-A and Rx was inhibited by staurosporine which confirms the involvement of a protein kinase in this response. These results are not sufficient to identify the precise mechanism of action of the protein kinase. However there is accumulating evidence to suggest that PKC mediates its action by phosphorylation of cytosolic components of the superoxide generating, NADPH-oxidase (Okamura *et al*, 1990; Ohtsuka *et al*, 1990).

fMLP (1 uM) alone evoked a small but immediate release of O_2^- compared to the short delay and large response of phorbol esters. When TPA, SAP-A and Rx were pre-incubated fMLP response was markedly enhanced but was still immediate and transient. DOPPA at concentrations which did not inhibit Mn^{2+} influx, had little or no potentiating effect of fMLP stimulated O_2^- release. All these results suggest that involvement of PKC-δ (not activated by SAP-A) and PKC-β_i (activated by DOPPA) were less likely of inhibition of fMLP-stimulated bivalent cation influx and potentiation of fMLP-stimulated O_2^- release. However the results do implicate involvement of Rx-kinase in addition to PKC. This is an important discovery in that Rx-kinase has previously been shown to stimulate O_2^- release by directly activating NADPH-oxidase (Evans *et al*, 1990; see Chapter 3 and Chapter 5). The potentiation of fMLP response and inhibition of agonist stimulated Mn^{2+} influx by Rx-kinase (and PKC) is described here for the first time (see also Merrit *et al*, 1993). The precise mechanism of action however remains to be fully identified

The phorbol esters have selective Ca^{2+} dependencies for *in-vitro* activation of PKC isotypes (Ryves *et al*, 1991). Using this rationale, the effect of manipulating $[\ Ca^{2+}\ ]_i$ on phorbol-mediated O_2^- release was examined. Two methods were employed to

manipulate [Ca2+]$_i$. Firstly different concentration of Ca^{2+} ionophore ionomycin were used to increase [Ca^{2+}]$_i$ and method employing high levels of fura-2 (buffers [Ca^{2+}]$_i$) and SKF 93635 (blocks Ca^{2+} influx) were used. TPA, SAP-A and Rx at concentration which were ineffective at basal [Ca^{2+}]$_i$ (90nM) evoked a large O_2^- generation after [Ca^{2+}]$_i$ was elevated to 230nM. At even higher levels [Ca^{2+}]$_i$ (400nM) DOPPA was able to stimulate O_2^-. This stimulation by DOPPA at high [Ca2+]$_i$ may reflect its Ca^{2+}- dependency for its *in-vitro* activation of PKC-β_i (Ryves *et al*, 1991). fMLP stimulated a large rise in [Ca^{2+}]$_i$ which potentiated fMLP-stimulated O_2^- release by TPA, SAP-A and Rx in cells with lightly or heavily loaded fura-2 and in cells with SKF 93635 in lightly loaded cells. Heavily loaded cells with SKF 93635 limits only a small increase in fMLP-stimulated [Ca^{2+}]$_i$. In these cells potentiation by TPA was unaffected, but potentiation by SAP-A and Rx was considerably diminished.

Taken together these results are consistent with *in-vitro* Ca^{2+} requirement for TPA and SAP-A, since SAP-A is markedly Ca^{2+} dependent than TPA (Ryves *et al*, 1991). The synergy between Rx and Ca^{2+} was an unexpected result since Rx-Kinase is inhibited by Ca^{2+} (See Chapter 4, Section 4.3.5). However nothing is known about the physiological regulation of Rx-kinase and its *in-vivo* mechanism of action, and these results suggest that physiological levels of [Ca^{2+}]$_i$ could synergise with Rx to activate Rx-kinase. However it is well established that Rx-kinase has to be 'primed' before its expression. Previous results have suggested that both fMLP and a Ca^{2+} ionophore (although the compound used was A23187 rather than ionomycin) were not good agents for 'priming' Rx-kinase in mouse peritoneal macrophages (See Chapter 3, section 3.4). Therefore an alternative explanation could be that Rx was activating PKC-β when [Ca^{2+}]$_i$ was elevated; PKC-β can be activated by high doses of Rx in the presence of Ca^{2+} ions *in-vitro* (Ryves *et al* 1991).

The method employed to measure O_2^- release was luminol chemiluminescence of

neutrophils and is different to the previous study which utilised SOD inhibitable reduction of cytochrome C (see Chapter 3) on mouse peritoneal macrophages. Interestingly, the results with both systems are comparable since TPA, SAP-A and Rx were active but DOPPA ineffective. The above results do not however identify the precise mechanism of action in phagocyte function. Therefore it was decided to examine the effect of PKC isotypes on O_2^- production by activating directly on O_2^- generating enzyme, NADPH-oxidase complex. Although the above results were obtained on intact neutrophils, the reconstituted NADPH-oxidase was partially purified from mouse peritoneal macrophages because of the data in Chapter 3. However mouse peritoneal macrophage NADPH-oxidase is comparable in activation profile to human neutrophil NADPH-oxidase (see Chapter 5, Section 5.3.8).

In-vitro activation of NADPH-oxidase by PKC-isotypes revealed that all isotypes can activate NADPH-oxidase to some degree. Activation of oxidase by PKC-α, -β and -γ were Ca^{2+} dependent and is consistent with the activity in the standard histone/kinase assays (Marias & Parker, 1989). The Ca^{2+}- independent isotypes -δ and -ε were 2-4 fold more active than Ca^{2+}- dependent isotypes. The relevance of this finding has to be fully characterised before any conclusions can be reached although the specific activity of PKC-α, -β and -γ were not absolutely comparable with -δ and -ε isotypes since latter were standardised using peptide substrates and not histone IIIs. Activation of PKC itself by TPA caused marked rise in NADPH-oxidase stimulation. TPA is known to stimulate 'active' NADPH-oxidase but not the reconstituted complex (see Chapter 5) therefore the increased activity noted was caused by PKC activation itself.

Although purified PKC has been implicated in the activation of NADPH-oxidase (Heinecke *et al*, 1990) and specific protein phosphorylations demonstrated (Kramer *et al*, 1988), individual PKC-isotypes mediated activation /phosphorylation has not. Therefore an attempt was made to study the *in-vitro* phosphorylation of PKC isotypes, specifically -α, -β and -γ (as -δ and -ε were unavailable). The results show a major

phosphorylated band at apparent molecular weight 47kDa appearing within 30s - 1 min. Minor bands also appear at 56kDa and 92kDa. It is likely that 47kDa protein is 47 *Phox*, a cytosolic component of NADPH-oxidase (Tyagi *et al*, 1992) and this protein has been shown to be a substrate for purified PKC (Krane *et al*, 1988). However it is not possible to distinguish between cytosolic and membrane phosphorylated proteins since the experiment used a mixture of both (same as the activation studies). 92kDa may be one unit of the cytochrome b_{-245} (alpha = 22 kDa & beta = 92 kDa). The autoradiographs were left for a maximum of 1 week for exposure and if a longer exposure time was used there may have been further phosphorylated protein bands of interest.

Functions of phagocytes were studied on intact cells and in cell-free systems. Using phorbol esters with isotype specificity it was possible to conclude that PKC-α (and PKC-β at elevated Ca^{2+} levels) and Rx-kinase may function in the regulation of the neutrophil respiratory burst. Finally using purified PKC isotypes it was possible to show that respiratory burst enzyme NADPH-oxidase was target for PKC. Taken together future experiments could utilise selective diterpene ester with restricted isotype specificity and make it possible to identify selective substrate phosphorylation by specific isotypes in intact cells.

CHAPTER 7 Comparison of Stimulation of Human Neutrophils and Monocyte by Phorbol Esters

7.1 Introduction.

Protein kinases particularly the PKC has become the focus of studies on signalling in many cell types including phagocytes (Majmunder *et al*, 1991; Pontremoli *et al*, 1990). A role for PKC as a positive signal for the activation sequence of neutrophils has been proposed since activators of PKC, such as phorbol esters and cell permeate DAG activate NADPH-oxidase to generate superoxide anion (O_2^-) (Lambeth, 1990; Rider and Niedel, 1987; see also Chapter 3 and Chapter 5). PKC can also down regulate ligand induced signalling (Della-Bianca *et al*, 1986). The ability of PKC to trigger positive and negative signals in neutrophils suggests the activity of multiple PKC isotypes.

PKC is now known to exist in a number of isozymic forms (Nishizuka, 1988), and phorbol esters with different biological actions can selectively activate specific isotypes of PKC (Ryves *et al*, 1991; Ryves, 1991). Rx, a daphnane orthoester has been shown to selectively stimulate a novel calcium-inhibited kinase termed Rx-kinase from phagocytes (see Chapter 4; Evans *et al*, 1990). Additionally phorbol esters, TPA and TxA also stimulate kinases which are as yet unidentified in rat brain homogenates (Evans *et al*, 1991).

It is well known that phorbol esters stimulate O_2^- production of potentiated and resting phagocytes and this has been linked amongst other pathways, to PKC and other protein kinases. It was therefore decided to examine phorbol ester stimulated kinase activity profile of neutrophils and monocytes.

7.2 Methods.

7.2.1 Isolation of human neutrophils and monocytes.

Whole blood was collected from healthy donors by venous puncture and mixed in a ratio 9:1 with citrate buffer (3.6% trisodium citrate). Human monocytes and neutrophils were isolated by density centrifugation as previously described in Chapter 4, section 4.2.1.

7.2.2 Assay of superoxide anion production.

O_2^- production was monitored spectrophotometrically by measuring the SOD inhibitable reduction of cytochrome C and by adapting the method described in Chapter 2 (see Chapter 2, section 2.3.1). The assay contained the following :

100µl cells
50µl cyt C (80µM)
50µl SOD (300U)
10µl Phorbol
40µl Buffer or
10µl Plumbagin/ 30µl buffer

The procedure was then followed according to the methods described in Chapter 2. Phorbol esters were diluted in 50% ethanol and plumbagin was dissolved in PBS. The protein content of cultures were determined by using a Sigma protein assay kit.

7.2.3 Protein kinase purification.

Cells were suspended in ice-cold homogenisation buffer (see Chapter 2, section 2.3.6 a) and were lysed by probe sonication (3 x 10s) at 4°C to prepare homogenates. Homogenates were centrifuged at 25000 x g x 15mins at 4°C. Supernatant was loaded into a 10ml superloop and pumped onto hydroxyapatite (HPT) column with protein kinase separation buffer A (see section 2.3.6 b). The column was washed with buffer

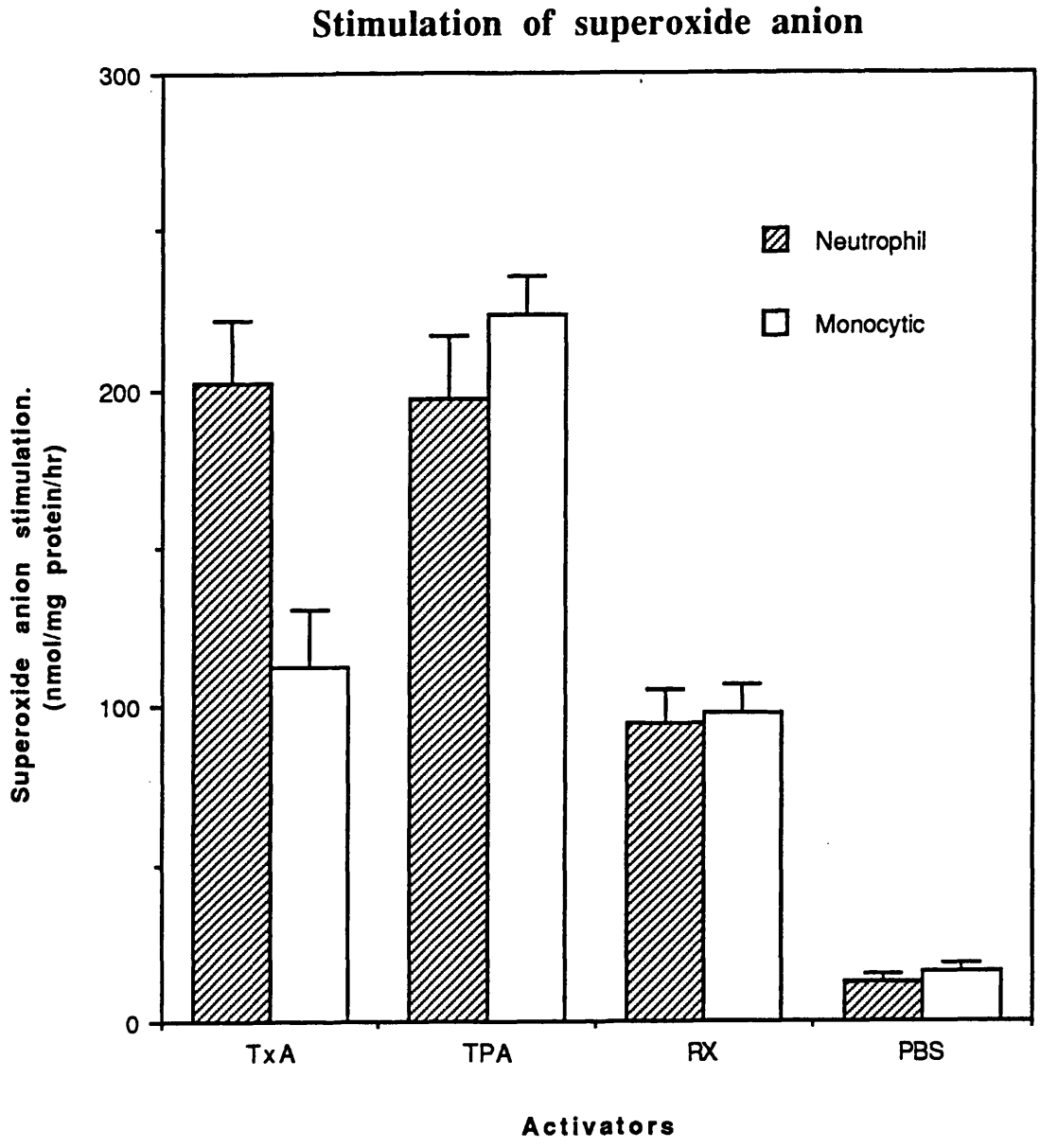


Figure 7.1 Stimulation of superoxide anion from human neutrophils and monocytes. 1 x 10^5 cells were incubated with TxA (100ng/ml), TPA (100ng/ml) or Rx (100ng/ml) for 2h. The superoxide anion was measured by SOD (300U) inhibitable reduction of cytochrome c (80μM) at 550nm. Results are expressed as mean ±SEM (n=4).

Stimulation of superoxide anion in the presence of plumbagin.

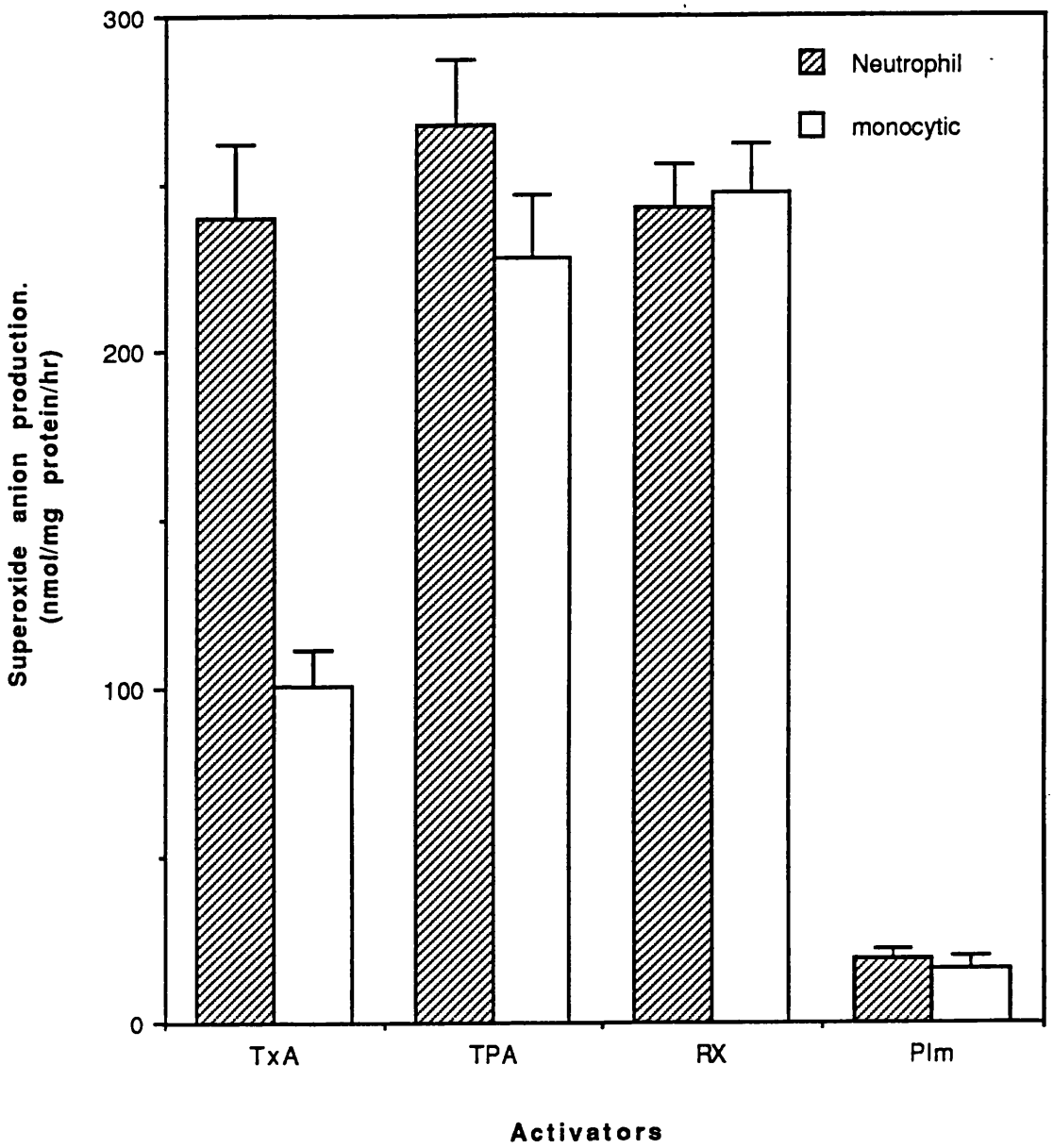


Figure 7.2 Stimulation of superoxide anion from human neutrophils and monocytes in the presence of plumbagin. 1 x 10^5 cells were incubated with TxA (100ng/ml), TPA (100ng/ml) or Rx (100ng/ml) in the presence plumbagin (0.1μg/ml) for 2h. The superoxide anion was measured by SOD (300U) inhibitable reduction of cytochrome c (80μM) at 550nm. Results are expressed as mean ±SEM (n=4).

A to remove unbound proteins and then gradients of 20-300 mM phosphate buffer was applied at a flow rate of 1ml/min over 60mins. Fractions of 1ml were collected on ice and immediately vortexed with 25% storage buffer (see section 2.3.6 d). The fractions were stored at -70^{o}C until analysis.

7.2.4 Assay of kinase activity.

Kinase activity was determined on all fractions generated from HPT by measuring the transfer of ^{32}P from [^{32}P]-ATP to histone IIIs as described in Chapter 2 (section 2.3.7.1).

7.3 Results.

7.3.1 Stimulation of O_2^- anion.

Cultures of human neutrophils and monocytes (both at 1 x 10^6 viable cells/ml) were stimulated by phorbol esters TPA, TxA and Rx over a 2h period and the results are expressed as nmoles O_2^- / h / mg of total protein. TPA (100 ngm^{-1}), the broad spectrum phorbol ester was a strong stimulator of O_2^- production of both monocyte and neutrophil cell populations (see Fig 7.1). A naphthoquinone, plumbagin (0.1 $ugml^{-1}$) augmented the TPA response by approximately 20-40% (Fig 7.2). TxA (100$ngml^{-1}$), daphnane orthoester and a second stage tumour promoter induced potent O_2^- production of neutrophil with a maximum rate of 220 nmol/hr/mg protein (Fig 7.1). However TxA was relatively weak at stimulating human monocytes with a maximal rate of just 130 nmol/hr/mg. Plumbagin augmented the TxA response of neutrophils by approximately 20% but had little effect on monocytes. Rx (100 $ngml^{-1}$), a potent daphnane irritant, weakly stimulated O_2^- generation of neutrophils and monocytes

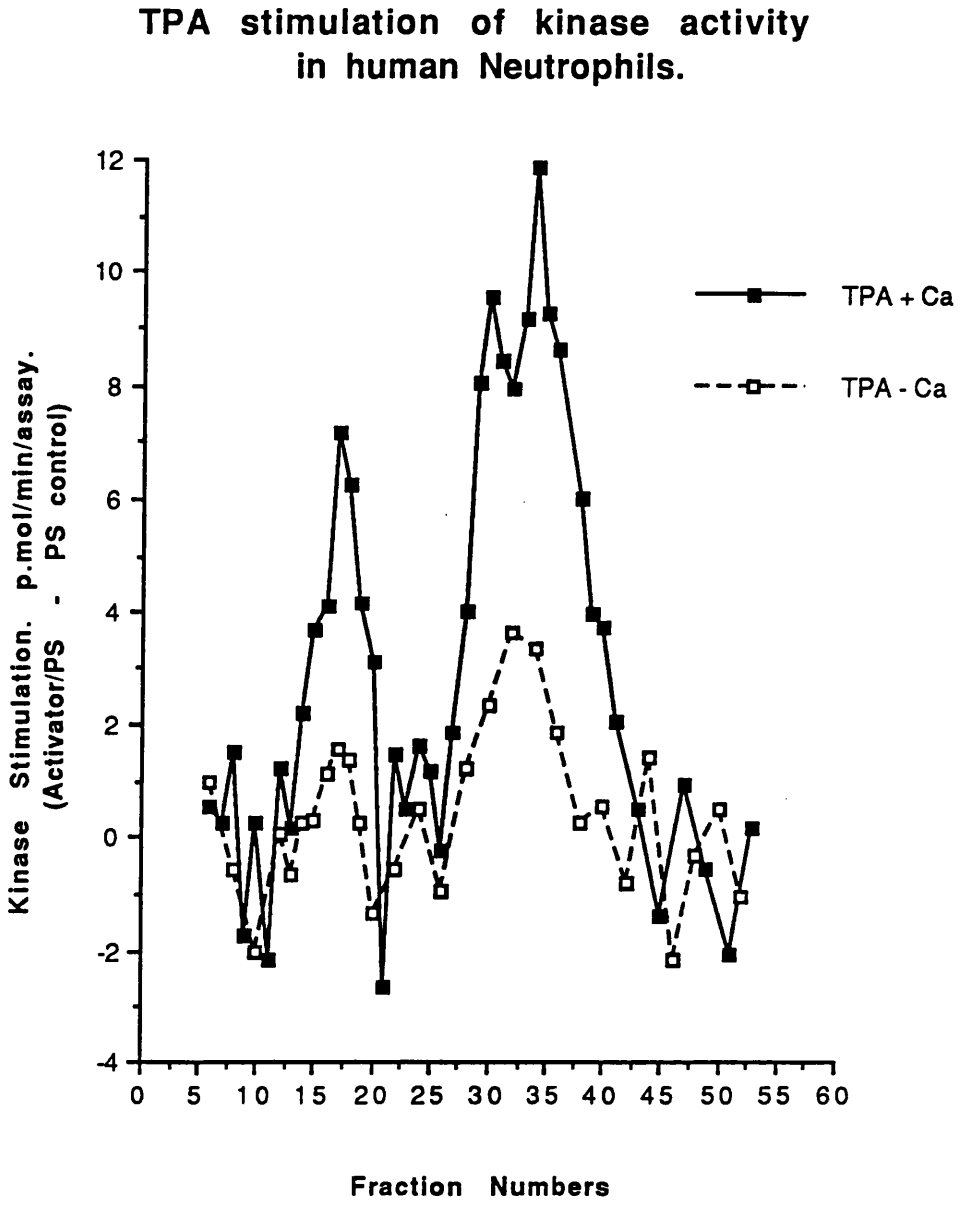


Figure 7.3 TPA stimulation of kinase activity in human neutrophils. 1 x 10^8 cell homogenates was subjected to FPLC HPT chromatography. Fractions were generated from phosphate gradient (20-300mM) over 1h at 1ml/min. Fraction were assayed using the standard micellar assay which contained TPA (100ng/ml) in a molar ratio 0.003% to 20% triton micelles in the presence of and absence of added Ca^{2+} (100μM). Results are expressed as a typical profile of a representative experiment.

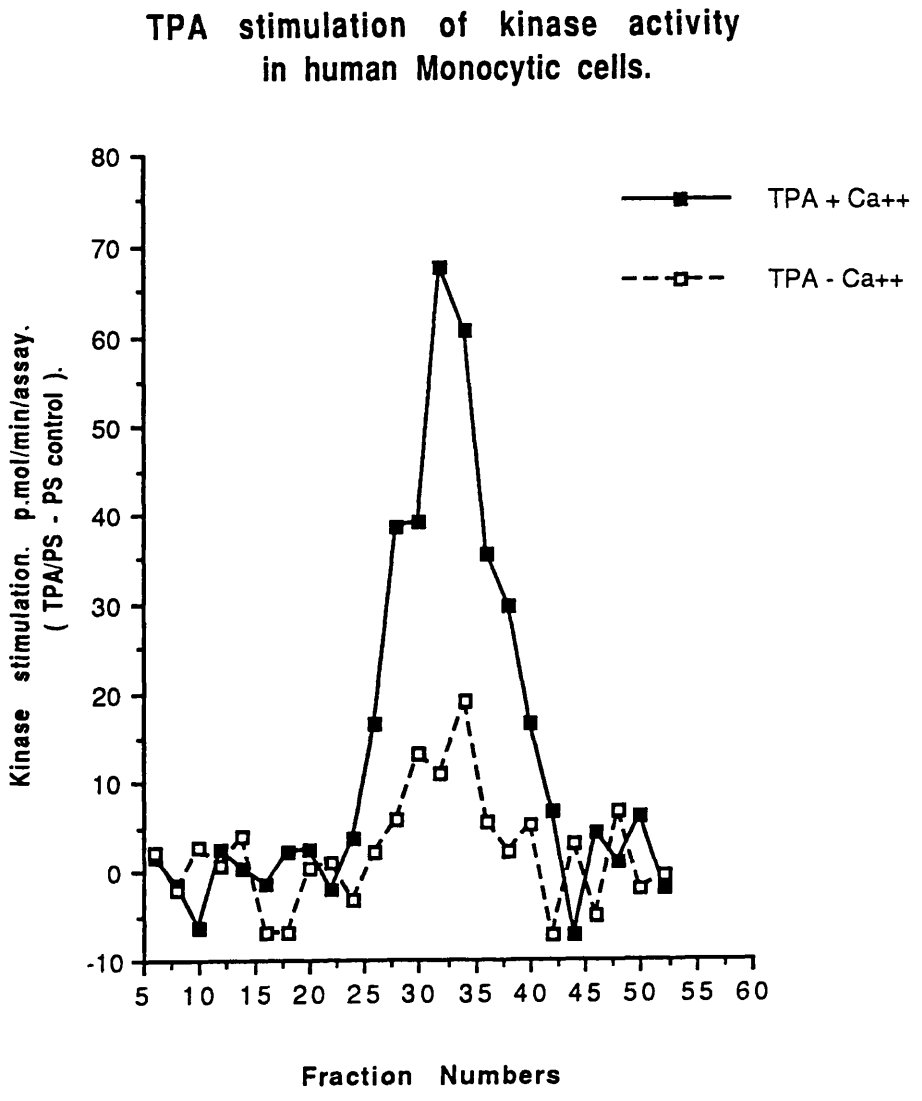


Figure 7.4 TPA stimulation of kinase activity in human monocytes. 1 x 10^8 cell homogenates was subjected to FPLC HPT chromatography. Fractions were generated from phosphate gradient (20-300mM) over 1h at 1ml/min. Fraction were assayed using the standard micellar assay which contained TPA (100ng/ml) in a molar ratio 0.003% to 20% triton micelles in the presence of and absence of added Ca^{2+} (100μM). Results are expressed as a typical profile of a representative experiment.

compared to TPA. This response was markedly enhanced by the presence of plumbagin and was similar to response observed with TPA. Interestingly plumbagin on its own was ineffective at stimulating O_2^- generation of both neutrophils and monocytes.

7.3.2 Protein kinase profiles of human neutrophils and monocytes.

7.3.2.1 TPA stimulation profile.

1 x 10^8 human neutrophil and monocyte homogenates were subjected to HPT chromatography through FPLC. The results are reported here as stimulated kinase activity with basal (PS alone) activity subtracted and expressed as pmol / min/ assay (assay was equivalent to 25 ul enzyme used). All the kinase profiles were generated from a phosphate gradient (20-300 mM) over 1h at a flow rate of $1ml/min^{-1}$.

TPA ($100ngml^{-1}$) stimulation of kinase activity in human neutrophils produced two distinct range of activities (Fig 7.3). The first range was within 85 - 110 mM phosphate gradient with peak of activity, termed pre-peak I at 105 mM phosphate buffer. The second range of activity was between 160 - 215 mM phosphate gradient and within it contain two distinct peaks of activities, peak II (eluting at 170 mM) and peak III (190 mM). All these were observed with the presence of calcium (100 uM) however, absence of calcium (10 mM EGTA) the peak I was abolished whereas peak II and peak III merged to create a minor peak (only 30% maximal; 180 mM).

Kinase profile of monocytes by TPA produced a single range of activity (150 - 210 mM; Fig 7.4). This range had two peaks, peak I (160 mM) and peak II (180 mM) in the presence of Ca^{2+} . Without Ca^{2+} , only a minor peak was observed again only 30% maximal.

7.3.2.2 TxA stimulation profile.

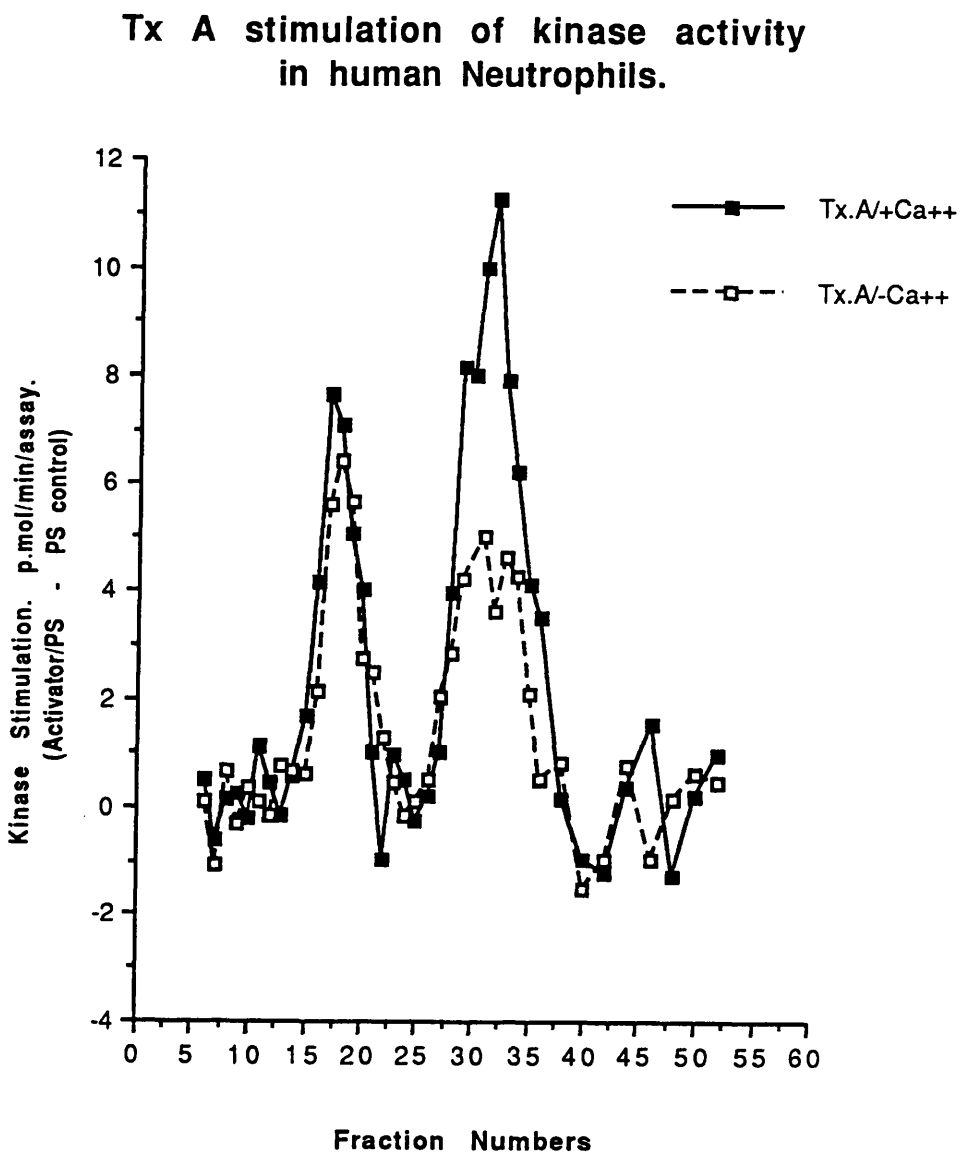


Figure 7.5 TxA stimulation of kinase activity in human neutrophils. 1 x 10^8 cell homogenates was subjected to FPLC HPT chromatography. Fractions were generated from phosphate gradient (20-300mM) over 1h at 1ml/min. Fraction were assayed using the standard micellar assay which contained TxA (100ng/ml) in a molar ratio 0.003% to 20% triton micelles in the presence of and absence of added Ca^{2+} (100μM). Results are expressed as a typical profile of a representative experiment.

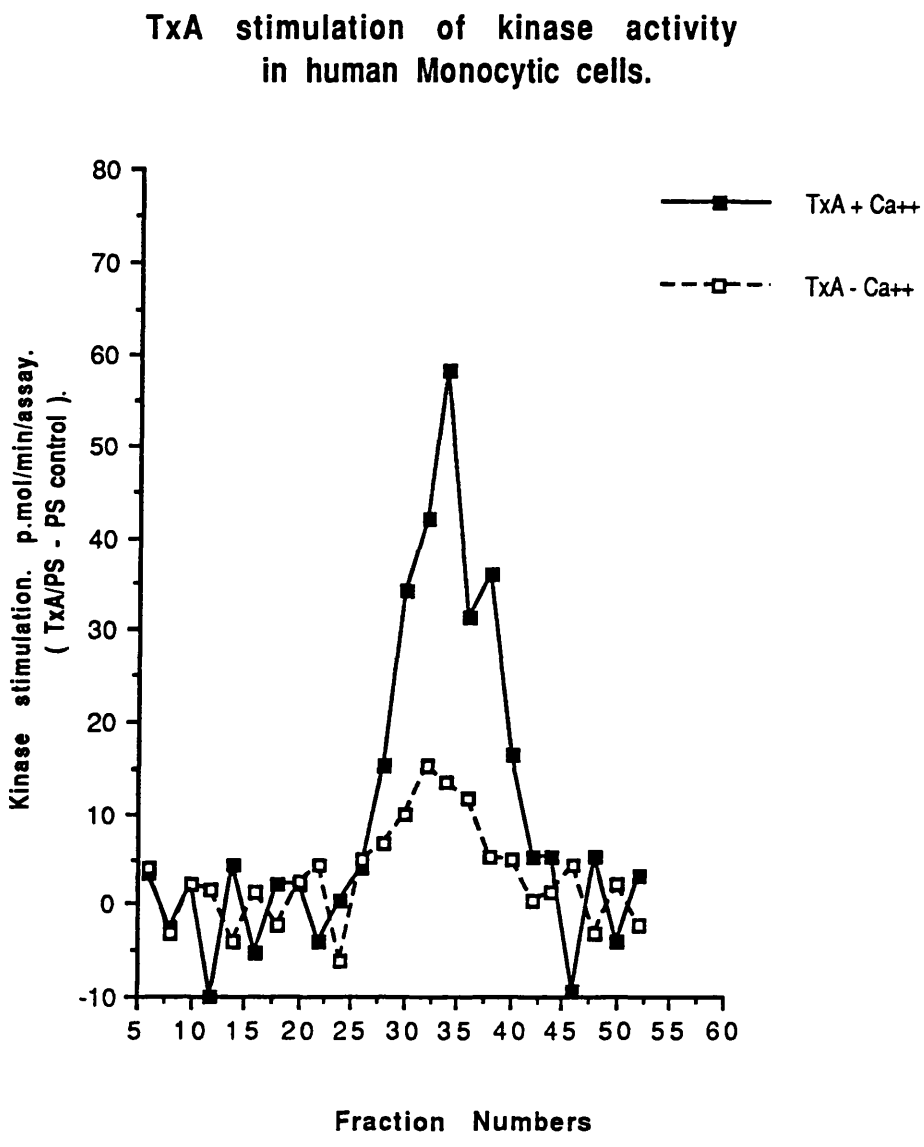


Figure 7.6 TxA stimulation of kinase activity in human monocytes. 1 x 10^8 cell homogenates was subjected to FPLC HPT chromatography. Fractions were generated from phosphate gradient (20-300mM) over 1h at 1ml/min. Fraction were assayed using the standard micellar assay which contained TxA (100ng/ml) in a molar ratio 0.003% to 20% triton micelles in the presence of and absence of added Ca^{2+} (100μM). Results are expressed as a typical profile of a representative experiment.

The kinase profile of neutrophils stimulated by TxA (100 $ngml^{-1}$) was similar to results obtained with TPA (Fig 7.5). Again two major range of activities were detected. With plus Ca^{2+} (100 uM) the first range (90 - 110 mM phosphate gradient) had a pre-peak I at 105 mM phosphate buffer and, the second range (160 - 200 mM) had two peaks of activities, peak II (165 mM) and peak III (180 mM). However with minus Ca^{2+} peak I was not abolished like the TPA response and had peak activity of nearly 85% of that achieved with plus Ca^{2+} . Peak II and peak III were also observed with minus Ca^{2+} but again as with TPA response the activity was low (approaching 40 % of maximal).

The kinase profile of monocytes stimulated by TxA was similar to TPA response (Fig 7.6). TxA stimulated a single range (160 - 230 mM) which had two peaks of activities, peak I (190 mM) and peak II (200 mM) both with the presence of Ca^{2+} . Without Ca^{2+} only a minor peak was detected at just 30% maximal.

7.3.2.3 Rx stimulation profile.

Rx (100 $ngml^{-1}$) stimulation of kinase activity profiles of neutrophils and monocytes are shown in Fig 7.7 and Fig 7.8 respectively. Rx failed to stimulate any kinase activity upto 300 mM phosphate gradient of both sets of cell homogenates. However Rx created a greater 'noise' between the range where kinase activity was stimulated by TPA and TxA (ie 160 - 210 mM). At 300 mM phosphate gradient FPLC was programmed to increase the concentration upto 500 mM over 1min and to further collect several fractions in order to explore for any Rx-kinase expressed in neutrophils and monocytes (see also Chapter 4, section 4.3.1). Peak Rx-kinase activity of both types of phagocytes are shown in Table 7.1. TPA (100 $ngml^{-1}$) and TxA (100 $ngml^{-1}$) were unable to stimulate Rx-kinase whereas Rx (100 $ngml^{-1}$) was and only in the absence of Ca^{2+}. The results were standardised to Units/ cell equivalents (10^8) and the values

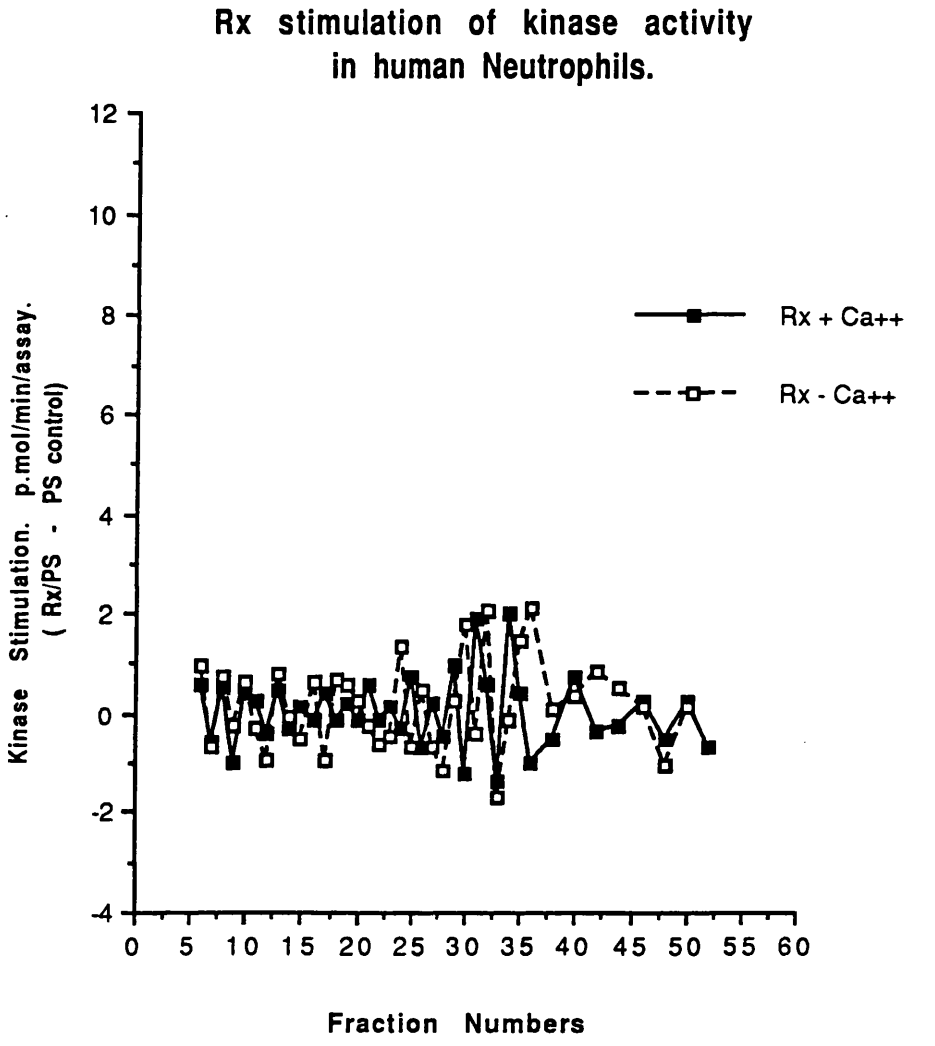


Figure 7.7 Rx stimulation of kinase activity in human neutrophils. 1 x 10^8 cell homogenates was subjected to FPLC HPT chromatography. Fractions were generated from phosphate gradient (20-300mM) over 1h at 1ml/min. Fraction were assayed using the standard micellar assay which contained Rx (100ng/ml) in a molar ratio 0.003% to 20% triton micelles in the presence of and absence of added Ca^{2+} (100μM). Results are expressed as a typical profile of a representative experiment.

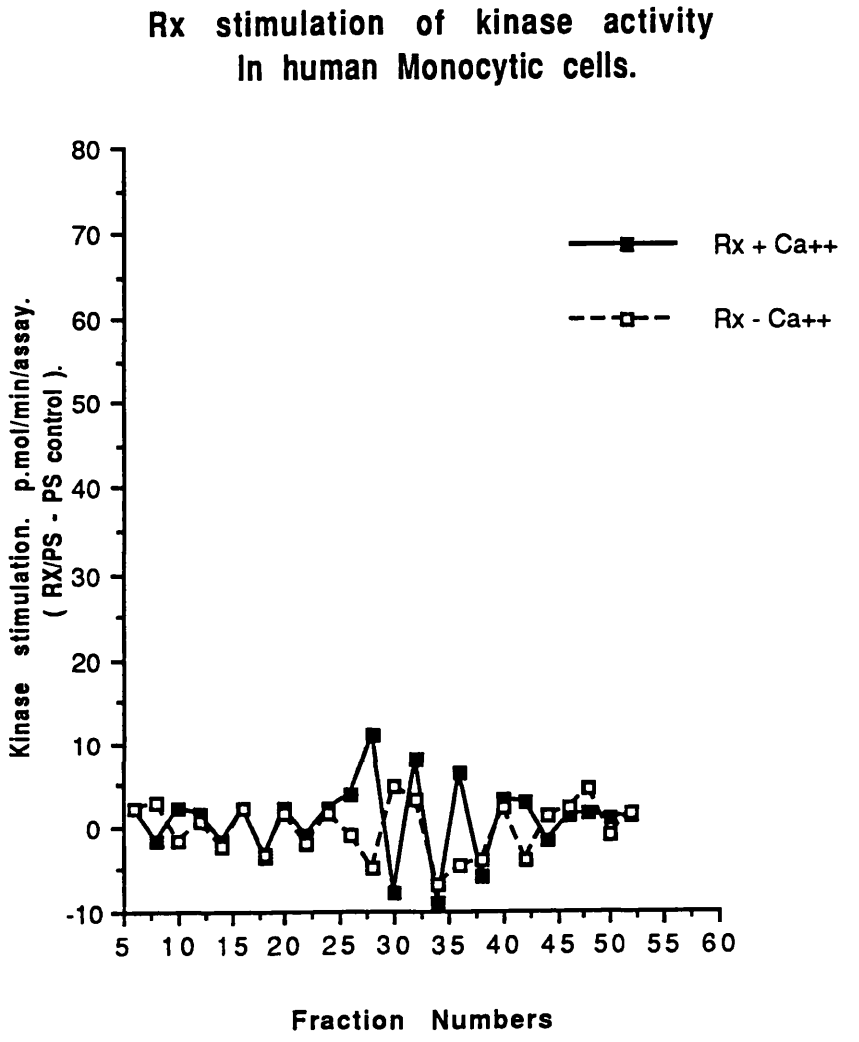


Figure 7.8 Rx stimulation of kinase activity in human monocytes. 1 x 10^8 cell homogenates was subjected to FPLC HPT chromatography. Fractions were generated from phosphate gradient (20-300mM) over 1h at 1ml/min. Fraction were assayed using the standard micellar assay which contained Rx (100ng/ml) in a molar ratio 0.003% to 20% triton micelles in the presence of and absence of added Ca^{2+} (100μM). Results are expressed as a typical profile of a representative experiment.

of Rx-kinase were comparable in both neutrophils and monocytes.

7.3.3 Dose response studies of pre-peak I kinase of human neutrophils.

TPA and TxA stimulatable pre-peak I kinase of neutrophils was further studied for dose response curves to establish Ca^{2+} -dependencies and phorbol ester potencies. TPA was able to activate the enzyme with or without Ca^{2+} (Fig 7.9). However, with plus Ca^{2+} TPA shifts the curve dramatically to the left increasing the potency by greater than 50-fold. EC_{50} for TPA plus Ca^{2+} was calculated as 22.4 $ngml^{-1}$ and, EC_{50} for TPA minus Ca^{2+} as 1.12 $ugml^{-1}$. The Ca^{2+} dependent response of TPA was not seen with TxA but was instead Ca^{2+} independent (Fig 7.10). EC_{50} for TxA and plus Ca^{2+} 45.7 $ngml^{-1}$, and EC_{50} for minus Ca^{2+} 36.3 $ngml^{-1}$. These results suggest partial selectivity of phorbol ester to stimulate this peak of kinase activity *in-vitro* .

7.4 Discussion.

Human neutrophils and monocytes were initially subjected to phorbol ester/ plumbagin to investigate the generation of O_2^-. The results conclusively indicate that O_2^- was generated by both neutrophils and monocytes in response to phorbol esters TPA and TxA and, to a lesser extent Rx. Moreover addition of plumbagin substantially increased the rate of O_2^- production and this was particularly true for Rx response. These results are consistent with the previous observation that TPA and TxA stimulate O_2^- of mouse macrophages and that plumbagin induces Rx sensitivity of mouse peritoneal macrophages thereby increasing the rate of O_2^- generation (see Chapter 3). Plumbagin is the active constituent of *Plumbago spp.*, a group of medicinal plants widely used in the tropics as traditional antimicrobial agents and for treatment of skin

Stimulated kinase activity, pmol/min/assay (Rx/PS - PS control)		
Monocytes		
Compound	+ Ca^{2+}	- Ca^{2+}
TxA	1.27±0.61	0.90±0.5
TPA	0.56±0.14	1.37±0.49
Rx	0.98±0.65	14.11±2.16
Neutrophils		
Compound	+ Ca^{2+}	- Ca^{2+}
TxA	0.79±0.43	0.44±0.23
TPA	0.89±0.57	1.88±0.46
Rx	1.37±0.45	15.35±3.67

Table 7.1 Peak Rx-kinase activities observed in neutrophils and monocytes. Cells (1 x 10^8) homogenates were subjected to HPT chromatography and PKC containing fractions were washed off by 0 - 60% phosphate gradient. Rx-kinase fractions were eluted at 100% phosphate (500mM) gradient and assayed using micellar system. Results are expressed as mean of triplicates ± SEM (n=3).

diseases (Evans, 1986). These compounds appear to work by generating free radicals which interfere with biological electron transport chains (Docampo and Moreno, 1984). Plumbagin as stated above potentiated the response of all phorbols but particularly Rx. Previously I have reported that plumbagin induces Rx-kinase (Ca^{2+} inhibited / phospholipid dependent kinase) activity (see Chapter 3 and Chapter 4). This may account for the potentiated Rx-response by plumbagin. It is not yet known whether plumbagin induces increased PKC activity although the TPA and TxA results seem to suggest a partial action. However increased activities may also represent plumbagin directly acting on O_2^- -generating NADPH-oxidase (although plumbagin on its own did not stimulate superoxide production) or may be due to some cellular damage caused by the naphthoquinone triggering a defunct response.

O_2^- generation of monocytes by TxA was relatively weak compared to TPA even with plumbagin pre-treatment. This result remains to be explained. However further clarification could be acquired if kinase profiles were obtained from these cells on the basis that phorbol esters target protein kinase cascades (Ryves, 1991; Evans *et al*, 1991). Accordingly neutrophil and monocyte homogenates were then individually fractionated by FPLC on HPT chromatography. Eluted fractions were assayed for the presence of phorbol ester-stimulated kinase activity using a PS/ micellar assay (see Chapter 2, section 2.3.7.1).

Working on the assumption that routine preparations of PKC can successfully be resolved by its high affinity for HPT, a slow phosphate gradient (20 - 300 mM over 60min at 1ml/ ml) was applied resulting in distinct peaks of activity stimulated above basal level. Kinase profile of neutrophils stimulated by TPA was Ca^{2+} -dependent in nature. Since TPA is known to activate all PKC isotypes *in-vitro* (Ryves *et al*, 1991), then these results suggest presence of Ca^{2+} dependent protein kinases in human neutrophils, possibly a mixture but predominantly β-PKC and minor α-PKC as has previously been reported (Pontremoli *et al*, 1990; Smallwood and Malawista, 1992;

Tx A stimulation of pre-peak (I) kinase activity in human neutrophils.

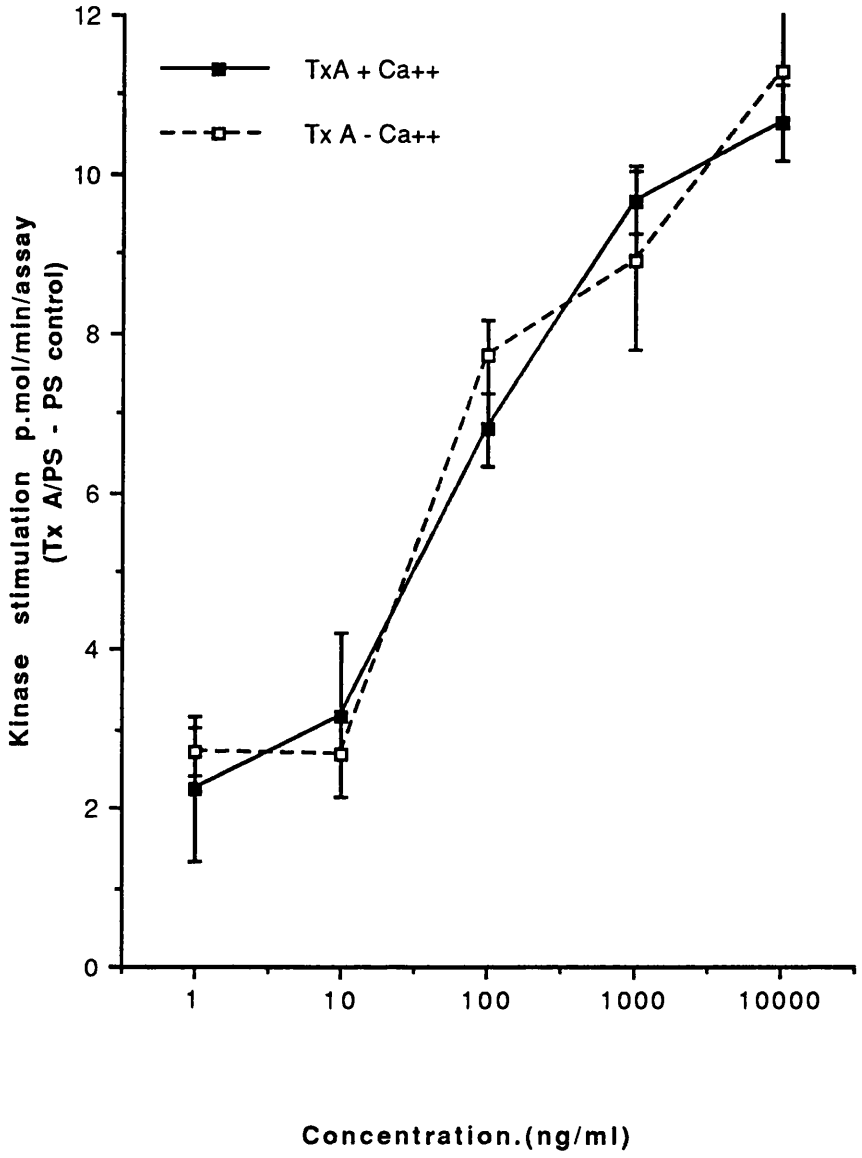


Fig 7.9 Dose response studies of pre-peak I with TxA. Resits are expresses as mean of triplicates +/- SEM (n=3)

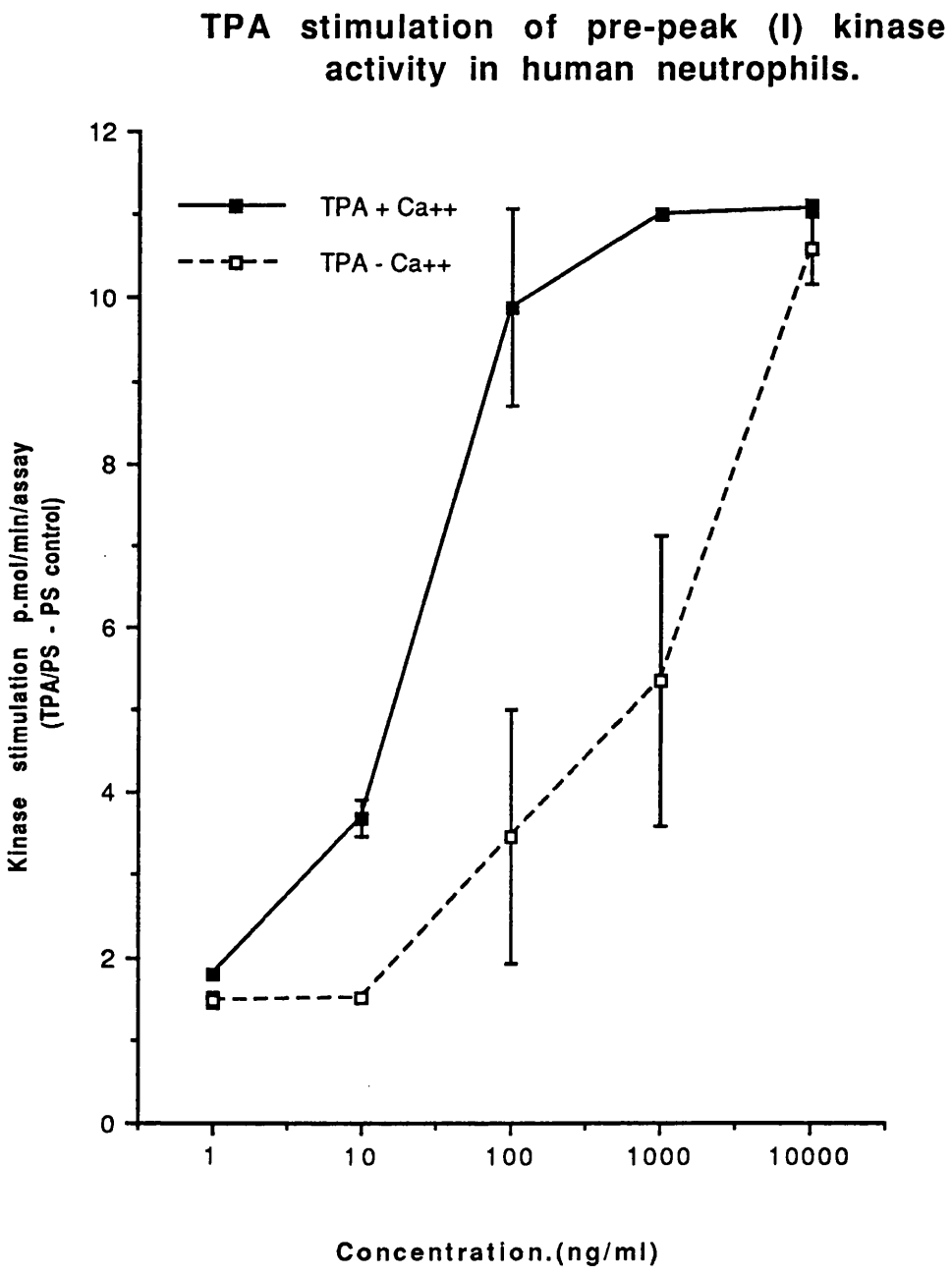


Fig 7.10 Dose response studies of pre-peak I with TPA. Results are expressed as mean of triplicates +/- SEM (n=3)

Balazovich *et al*, 1992). Although individual isotypes could not be confirmed since Western blots were not examined, the elution profile of rat brain PKC seems to coincide with the elution obtained from neutrophils (rat brain PKC eluted at 150 - 220 mM (Ryves, 1991), and neutrophil PKC eluted at 150 - 210 mM phosphate gradient). It was interesting to note that TxA stimulated the early pre-peak I in a Ca^{2+} -independent manner although stimulation of other peaks appeared to be much more Ca^{2+} dependent. TxA has been shown to activate PKC isotypes *in-vitro* , specifically α-, β-, and γ-isotypes in a Ca^{2+} dependent manner but considerably less potent at activating the Ca^{2+} -independent isotypes δ- and ε- PKC (Ryves *et al*, 1991). It therefore appears that TxA was stimulating PKC (β and α) during the second range (150 - 210 mM) of activity (see Fig 7.). However the identity of pre-peak I remains to be fully characterised.

Pre-peak I which elutes early on HPT chromatography seems unlikely to be a known PKC-isotype since Western blot analysis, have shown that all isotypes are eluted between 150 - 220 mM phosphate buffer (Ryves, 1991). Some authors have reported existence of Ca^{2+} - independent protein kinases from human neutrophils termed nPKC (Majmunder *et al*, 1991; 1993) and ζ- PKC (Stasia *et al*, 1990). Pre-peak I and nPKC are probably not related since nPKC elutes between PKC-α and PKC-β, and not early (Majmunder *et al*, 1991). ζ-PKC and pre-peak I also appear to be unrelated as ζ-PKC elutes (although early) with bulk PKC-β and ζ-PKC has not yet been discovered in human neutrophil (ζ- was discovered in bovine neutrophils (Stasia *et al*, 1990)). Additionally ζ-PKC is a atypical PKC since it is not activated by phorbol derivatives (Ways *et al*, 1992) .

Pre-peak I kinase activity has also been previously observed in rat brain homogenates (Ryves , 1991; Evans *et al*, 1991). TPA and TxA both stimulate the rat brain pre-peak which we resolved prior to the immunologically detected isotypes of PKC on the phosphate gradient. And interestingly TxA also activated the rat brain pre-peak Ca^{2+}-

independently whereas TPA activation was Ca^{2+}- dependent. Additionally the position of elution of this similar activity in rat brain coincides with neutrophil pre-peak I (105mM phosphate concentration in neutrophil and 106.6mM rat brain).

Further *in vitro* studies revealed that TxA activation of pre-peak I was totally Ca^{2+} independent whereas TPA activation was distinctly Ca^{2+}- dependent. These calcium dependencies may merely reflect co-factor requirement for activation of this particular enzyme by different phorbol derivatives. However these findings suggest an existence of a novel protein kinase or as a co-elution of protein kinases in human neutrophils and activated by selective phorbol esters. Human monocytes on the other hand did not elute a pre-peak I and since TxA was significantly more potent at stimulating O_2^- of neutrophils, the results suggest that pre-peak I may be involved in activating NADPH-oxidase thereby ultimately resulting in O_2^- generation. Further studies using cell-free assays of NADPH-oxidase activated by the kinase and *in-vitro* phosphorylation of NADPH-oxidase could elaborate the function of pre-peak I on superoxide generation. Interestingly nPKC described by Majmunder *et al* (1993) (see above text) does not seem to be involved in activation of NADPH-oxidase.

Elution profile of human monocytes was more typical of the profile of rat brain PKC containing one broad range of activity containing PKC - isotypes with distinct lack of pre-peak. Again using Western blots with anti-PKC antibody would confirm the presence of PKC-isozymes however, TPA and TxA stimulation patterns suggest Ca^{2+}-dependent isotypes being dominant. Monocytic U937 cells have been shown to contain PKC -α, PKC-β and PKC -ζ (Ways, 1992). TPA but not TxA was a stronger stimulator of O_2^- generation of monocytes. This observation cannot be explained only on the basis of PKC activation by these phorbol derivatives since both are potent PKC activators *in-vitro* (Ryves, 1991). However TxA is relatively weak activator of PKC-δ and PKC-ε and this may be one of the reasons why TxA was weak stimulator of O_2^- , particularly since human monocytic cell lines contain PKC-ε. Additionally the lack of

Neutrophils				
	Pre-pk I	Pk II	Pk III	Rx-kinase
TPA	14.42±2.1	16.8±3.4	21.3±2.6	-
TxA	18.8±1.9	14.7±2.9	19.4±4.8	-
Rx	-	-	-	71.6±6.74
Monocytes				
	Pre-pk I	Peak I	Peak II	Rx-kinase
TPA	-	10.9±1.2	13.8±1.7	-
TxA	-	16.1±2.8	12.1±3.9	-
Rx	-	-	-	68.6±4.5

Table 7.2 Effects of plumbagin pre-treatment on kinase activities of neutrophils and monocytes. Cells (1 x 10^8) were subjected to plumbagin ($0.1\mu gml^{-1}$) prior to homogenisation. The homogenates were chromatographed through HPT and fraction collected were assayed using micellar system. Results are expressed as percent increase in peak kinase activities ± SEM (n=3).

pre-peak I in monocytes may also account for poor stimulation of O_2^- by TxA.

As stated above stimulation of O_2^- was markedly increased by the presence of plumbagin. To see whether plumbagin induces changes in kinase profiles further experiments were carried out by pre-stimulating the cells with plumbagin (0.1 ugml-1) prior to homogenisation. The kinase profiles remained unchanged but increased in activity (see Table 7.2). The percentage increase in kinase correlated with increase in O_2^- generation by these phorbols in the presence of plumbagin. However as expected a marked increase in Rx-kinase activity was observed with plumbagin pre-treated phagocytes and this was consistent with the results observed in earlier chapters (see Chapter 3 and Chapter 4).

Stimulation within the PKC-range by Rx was not observed. This was expected since Rx was shown to be non-activator of all the PKC isotypes tested although it was a slight activator of PKC-β at very high doses (Ryves *et al*, 1991). Rx was a weak stimulator of O_2^- of neutrophils and monocytes but the response was clearly amplified with plumbagin. Rx is known to stimulate Rx-kinase of phagocytic cells (Ryves *et al*, 1989; Evans *et al*, 1990). Rx-kinase was obtained from human neutrophils and monocytes (both of these preparations were from mixed donors since with a single donor sufficient quantity of cells was unobtainable). The rates of Rx-kinase activity were similar in both neutrophil and monocyte homogenates and this activity was enhanced by greater than 65% with plumbagin pre-treatment. These results are consistent with the hypothesis that O_2^- generation of 'primed' phagocytes are activated by PKC-mediated process but significantly are also activated by PKC-independent Ca^{2+}-inhibited phospholipid dependent Rx-kinase (which itself is 'primed').

Chapter 8 Final Discussion and Conclusions

8.1 Concluding remarks.

Protein phosphorylations play significant roles in modulation of cell actions and particularly in transducing intracellular signals to ultimately produce a physiological response. A vast array of protein kinases have been discovered in the last ten years (for review see Blackshear *et al*, 1988). The functions of a relatively few enzymes have been well characterised but the rest are placed into groups or 'families'. One such group, the PKC-isotypes was initially isolated as monomeric protein (Inoue *et al*, 1977) but was discovered to be a family of related isotypes α, β and γ PKC (Coussens *et al*, 1984). Subsequently by using molecular biological techniques several 'novel' PKC have been discovered (δ, ε, ζ, η, θ, ι, λ, μ; for review see Dekker and Parker 1994). Frequently the novel PKC are found to be more abundant than the classical isoforms and these were often overlooked in earlier investigations. Further studies will be required, once the PKC system in general is better defined, to clarify the many interesting results of signal transduction experiments that have implicated PKC.

Such diversity of protein kinases and the wide range of biological activities of phorbol ester would inevitably result in discoveries of novel kinases, possibly newer forms of PKC isotypes or clearly distinct enzymes activated by phorbol esters. Ryves *et al* (1989) first described a Ca^{2+} independent kinase eluting at a higher phosphate gradient than the conventional PKC, and activated by pro-inflammatory daphnane ester Rx (termed Rx-kinase). The problem with Rx-kinase was that it was highly unstable and thus difficult to characterise. However this was

overcome by the present studies which describes that Rx-kinase can be 'primed' for expression. Ryves (1991) also describes several phorbol ester stimulatable kinase activities in rat brain which cannot be immunologically identified with antisera presently available (also see Evans *et al*, 1991). The present study demonstrated that neutrophils have distinct kinase profiles and comparing elution patterns and TxA activation, pre-peak I of neutrophils seems to be similar to peak I of rat brain. However to characterise this peak of activity further experiments probing specific anti-PKC antibody against pre-peak I to confirm its novel identity has to be investigated. Once this is established the kinase could be tested to see whether other phorbol esters (DOPP, DOPPA, Sap-A) can fully activate the enzyme. Since TxA was better at stimulating superoxide anion of neutrophils compared to monocytes and the pre-peak I kinase was only detected in neutrophils it would useful to test this enzyme on partially purified NADPH-oxidase. Initial parameters of activation patterns of NADPH-oxidase by pre-peak I kinase can be established followed by *in-vitro* phosphorylation studies. If any specific substrate phosphorylation are observed then this would provide a vital breakthrough in understanding the mechanism of action of this kinase. However a caution has to be applied as the results of activation and phosphorylation patterns have to be correlated since some studies have found that PKC phosphorylation and activation have not correlated (Badwey *et al*, 1989b; Uhlingher *et al*, 1990).

Phorbol esters in particular TPA has been extensively used to study the phagocyte function of $O^{\cdot}_2$ release. However whole range of phorbol ester differing in biological activities have not been screened. Our results on $O_2^{\cdot}$ release by phorbol

esters correlate in both murine macrophages and human neutrophils and in by using two separate experimental approaches such as the spectrophotometrically and chemiluminescence studies. Rx on both occasions induced the release of O_2^- but Rx does not activate PKC isotypes at low doses (although it does activate β-PKC at high doses; Ryves *et al*, 1991). Rx was clearly targeting a separate signalling pathway or receptor. Therefore we hypothesise that Rx was acting through the then recently described Rx-kinase to release O_2^-.

The effects of Rx and zymosan appears to be primed and Rx-kinase is primed. Additionally Rx-kinase directly activates NADPH-oxidase. However *in-vitro* phosphorylation studies with Rx-kinase on NADPH-oxidase would provide a direct evidence of its involvement in O^-_2 release. A further study recommended would be to load whole cells with ^{32}Pi and activate the cells with Rx and correlate with the pharmacological response. Since Rx has limited biological actions the observed phosphorylation patterns would provide valuable clues as to its mode of action.

The irritant potency of Rx has been linked to a neurogenic nature (Szallasi and Blumberg, 1990a; 1990b) by acting as an ultrapotent analogue of capsaicin. A detailed study has shown that Rx induced inflammatory response is of a mixed aetiology (Gordge, 1992; Evans *et al*, 1992). Their results suggest that biochemical mechanism of action of Rx may involve Rx-kinase, although how this enzyme activation interacts with biochemical mechanism of nociceptive stimuli is unclear. Rx-kinase is the receptor for Rx since ^3H-Rx binds to Rx-kinase, which is reported here for the first time. However studies could be undertaken to assess whether Rx-

kinase is a 'daphnane receptor', that is if other daphnanes would bind to Rx-kinase in preference to PKC. Chemical studies could produce compounds with different residues attached to homovallinic acid to further investigate the process of neurogenic inflammation.

Rx-kinase is described in these studies as the Ca^{2+}-inhibited, phospholipid dependent histone kinase with at least one physiological substrate, NADPH-oxidase. Some Ca^{2+}-inhibited kinases have been reported, for example rat brain DEAE-cellulose chromatography contaminating PKC fractions was found to have a greater activity with EGTA than Ca^{2+} in the absence of phospholipid (LePauch *et al*, 1983). In another study using HPT fractionated rabbit and rat brain extracts, an apparent Ca^{2+}-inhibited kinase was co-eluting with PKC activity profile (Buday *et al*, 1989; Farago *et al*, 1989). In our studies Rx-kinase was fully characterised with assay parameters identified. However evaluation of the co-localisation of Rx-kinase could be carried out to determine whether the enzymes can be translocated to the membrane from the cytosol or whether its actions are localised. The Rx-kinase may itself exist as series of isoforms, all of which could be distinct from recognised forms of PKC. Our results of the alveolar macrophages and lungs with two distinct peaks of Rx-kinase may be the preliminary results to identifying further isoforms.

Our results provide the basis from which to scale up the preparation to produce bulk and pure Rx-kinase. Use of pig lungs as available from local slaughter houses would provide a readily available source of Rx-kinase. Pig lungs would provide large quantities of Rx-kinase from which to concentrate the enzyme either by

ammonium sulphate precipitation or ultracentrifugation. The concentrated enzyme could be further chromatographed through gel filtration to make it homogenous or sequenced for amino acid. Nucleotide sequence can then be deduced resulting in the potential to clone the enzyme. Another possibility with the concentrated sample is to produce specific antibodies or antibodies to a recognised peptide sequence of the kinase. This would provide a powerful tool for investigating mechanisms of action of Rx-kinase and its distribution. Antibodies would provide the probe from which to study the dynamic distribution and subcellular localisation of Rx-kinase by techniques such as microinjection.

The work described here suggests novel classes of kinases which are target for the ubiquitous phorbol esters. Where for example Rx-kinase or pre-peak I kinase act in the complex signal transduction pathways is a challenging and intriguing problem for a future research. As a Ca^{2+}-independent and Ca^{2+}-inhibited kinase they have major implications for a significant role in Ca^{2+} homeostasis and signalling pathways.

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