MECHANISMS CONTROLLING THE ACTIVITY AND SUBCELLULAR LOCALISATION OF PKD/PKCμ

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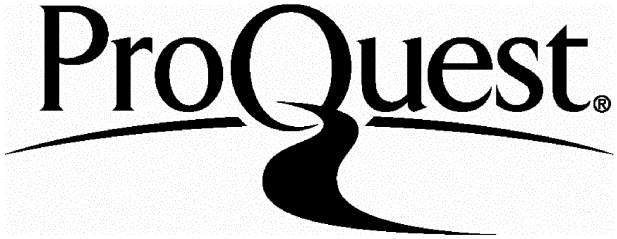

ABSTRACT

Protein kinase C (PKC) enzymes have been implicated in a wide range of biological responses *in vivo*, including cell morphology, differentiation and proliferation. A related kinase is Protein kinase D (PKD), also known as PKCμ. The catalytic domain of PKD is distinct from that of PKC enzymes, although it contains a conserved C1 domain that binds phorbol esters and diacylglycerol (DAG) in a manner similar to classical and novel PKCs.

The experiments described here demonstrate that the pharmacological agent bryostatin 1, like phorbol esters, stimulates PKD activity through a novel PKC-dependent signalling pathway in fibroblasts. Importantly, physiological antigen receptor triggering also induces PKC-dependent activation of PKD, in T and B cell lines, in mast cells and in peripheral blood-derived T lymphocytes. Significantly, PKD activity is dynamically regulated by both positive and negative signals from antigen receptors in B lymphocytes.

The second cysteine-rich motif within the C1 domain of PKD is the major binding site for phorbol esters, both *in vitro* and *in vivo.* However, direct phorbol ester/DAG binding to PKD is dispensable for its activation, as shown by analysis of a PKD C1 domain mutant, consistent with the proposed role for classical and novel PKC enzymes in the regulation of PKD activity. A functional C1 domain is however required for the translocation of PKD from the cytosol to the plasma membrane of intact cells in response to antigen receptor ligation or phorbol ester treatment. Strikingly, PKD only briefly translocates to the plasma membrane of B lymphocytes and mast cells following receptor stimulation, before returning to the cytosol within minutes of the initiation of antigen receptor signalling. In contrast, a sustained phase of PKD activity is observed in antigen receptor-activated B lymphocytes which is maintained over a period of hours.

These data suggests that both direct and indirect DAG signalling pathways contribute to the regulation of PKD *in vivo* and indicate that PKD may disseminate DAG signals away from the plasma membrane into the cell interior during sustained responses to antigen receptor engagement.

PUBLICATIONS ARISING FROM THIS THESIS

1. **Matthews, S. A.**, G. R. Pettit, and E. Rozengurt. 1997. Bryostatin 1 induces biphasic activation of protein kinase D in intact cells. *Journal of Biological Chemistry*. 272:20245-50.

2. Iglesias, T., **S. Matthews**, and E. Rozengurt. 1998. Dissimilar phorbol ester binding properties of the individual cysteine-rich motifs of protein kinase D. *FEBS Letters*. 437:19-23.

3. **Matthews, S.A.**, T. Iglesias, D. Cantrell, and E. Rozengurt. 1999. Dynamic redistribution of Protein Kinase D (PKD) as revealed by a GFP-PKD fusion protein: dissociation from PKD activation. *FEBS Letters*. 457:515-521.

4. **Matthews, S. A.**, E. Rozengurt, and D. Cantrell. 1999. Characterisation of S916 as an *in vivo* autophosphorylation site for PKD/PKCμ. *Journal of Biological Chemistry*. 274:23543-26549.

5. **Matthews, S.A.**, T. Iglesias, D. Cantrell, and E. Rozengurt. 1999. Spatial and temporal regulation of Protein Kinase D by antigen receptors. *Submitted*.

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For V.E.M.

(1946-1995)

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ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BCR	B cell antigen receptor
Br-1	Bryostatin 1
CD	cluster of differentiation
c.p.m.	counts per minute
cDNA	complementary DNA
C-terminal	carboxy-terminal
DAG	*sn*-1,2-diacylglycerol
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGTA	[ethylene-bis(oxyethylenenitrilo)] tetra-acetic acid
ELISA	enzyme-linked immunoabsorbent assay
ERK	extracellular signal-regulated kinase
FcεR1	high affinity receptor for IgE
g	unit of gravity
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
HRP	horse raddish peroxidase
Ig	immunoglobulin
IL	Interleukin
IP_3	Inositol (1,4,5) trisphosphate
ITAM	immuno-receptor tyrosine based activation motif
ITIM	immuno-receptor tyrosine based inhibitory motif
JNK	c-jun N-terminal kinase
kb	kilobase
K_d	dissociation constant
kDa	kilodalton
KLH	keyhole limpet hemocyanin
K_m	Michaelis constant
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated erk kinase
MHC	major histocompatibility complex
min	minute
NFAT	nuclear factor of activated T cells

N-terminal	amino-terminal
°C	temperature, degrees celsius
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDBu	phorbol 12,13-dibutyrate
PDGF	platelet derived growth factor
PDK1	3'-phosphoinositide dependent kinase 1
PH	pleckstrin homology
$PI(3,4)P_2$	phosphatidylinositol 3,4-bisphosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
$PI(3,4,5)P_3$	phosphatidylinositol 3,4,5-trisphosphate
PI3-Kinase	phosphatidylinositol 3-kinase
PIPES	piperazine-N,N-bis(ethanesulfonic acid)
PKB/Akt	protein kinase B (or Akt)
PKC	protein kinase C
PKD	protein kinase D
PLC	phosphoinositide-dependent phospholipase C
PLD	phospholipase D
rIL-2	recombinant IL-2
rpm	revolutions per minute
RPMI-1640	Rosewell Park Memorial Hospital Medium 1640
RT	room temperature
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulphate
SH	src homology domain
SHP-1/2	SH_2 domain containing tyrosine phosphatases-1/2
SHIP	SH_2 domain containing 5' inositol phosphatase
TCR	T cell antigen receptor
TPA	12-O-tetradecanoylphorbol 13-acetate
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	octyl-phenoxy-polyethoxyethanol
Tween-20	polyoxyethylenesorbitan monoluarate

CHAPTER 1 : General Introduction

1.1. INOSITOL LIPID METABOLISM.

The regulated metabolism of inositol phospholipids has been recognised for many decades as a major mechanism by which cell surface receptors transmit signals from the plasma membrane into the interior of cells to regulate effector responses. In the context of signal transduction pathways two major routes of inositol lipid metabolism have been extensively studied, controlled by PLC enzymes and PI3-kinases respectively. Hydrolysis of membrane-localised lipid $PI(4,5)P_2$ by PLC enzymes generates the inositol polyphosphate IP_3 and diacylglycerol (DAG) which control the release of calcium from intracellular stores and activate members of the PKC family of serine/threonine kinases, respectively (Berridge and Irvine, 1989; Berry and Nishizuka, 1990; Nishizuka, 1988). In contrast, PI3-Kinases phosphorylate $PI(4,5)P_2$ at the 3' position of the inositol ring to generate $PI(3,4,5)P_3$ (and its metabolite $PI(3,4)P_2$) which function to target PH domain-containing signalling proteins to the plasma membrane for activation (Leevers *et al.*, 1999).

1.1.1 Phospholipase C.

Stimulation of $PI(4,5)P_2$ hydrolysis by phosphoinositide-specific PLC is one of the earliest signalling events induced by a wide variety of extracellular stimuli, including hormones, neurotransmitters, antigens and growth factors. Three distinct classes of PLC enzymes (PLCβ, δ, γ) exist which share common catalytic properties, although each class is regulated by different signalling pathways (Figure 1.1a), (Exton, 1997; Katan, 1998; Nishizuka, 1995; Rhee and Bae, 1997).

Thus, PLCγ is activated by receptors with intrinsic tyrosine kinase activity: receptor stimulation induces the autophosphorylation of specific tyrosine residues within the cytoplasmic tail of these receptors which creates docking sites for the two SH_2 domains of PLCγ. Once bound, PLCγ is itself phosphorylated on tyrosine residues by the receptor kinase domain and activated (Katan, 1998; Rhee and Bae, 1997). In addition, non-receptor tyrosine kinases (for example antigen receptors) regulate PLCγ through activation of cytosolic Src- and Tec-family tyrosine kinases, which recruit specific adaptor molecules through phosphotyrosine/SH_2 domain interactions, leading to PLCγ activation (as discussed more fully in section 1.3.2). In contrast, PLCβ enzymes do not contain SH_2 domains and are instead activated by binding to the GTP-bound α-subunits of Gq proteins or to the βγ dimers of Gi/o proteins released upon the activation of G protein-coupled

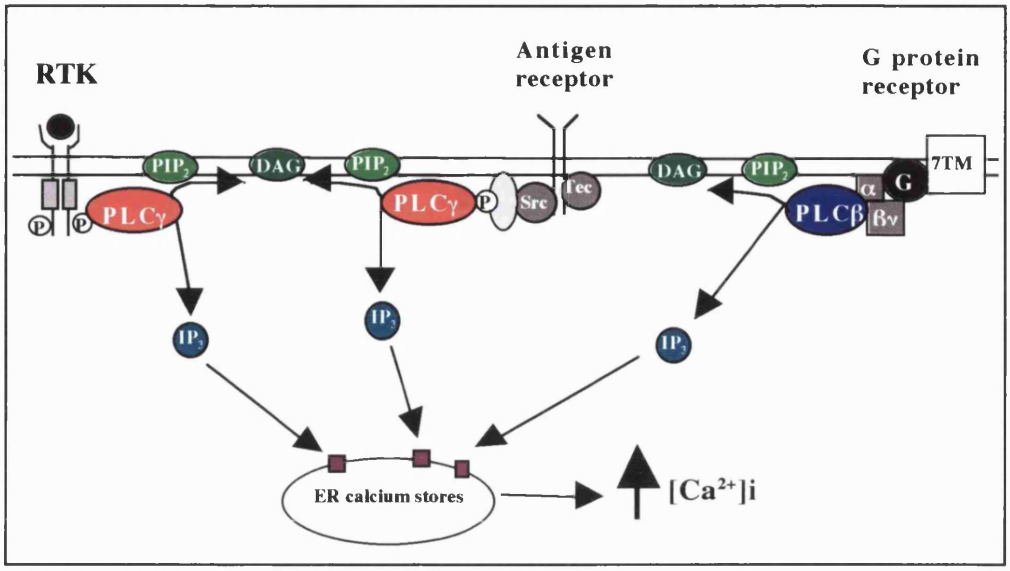


Fig. 1.1a Regulation of PLC enzymes by cell surface receptors.

PLCγ and PLCβ isoforms are regulated by distinct mechanisms: The SH_2 domain of PLCγ binds to phosphotyrosine motifs on receptor tyrosine kinases or specific adaptor molecules that target PLCγ to the plasma membrane for phosphorylation and activation by the receptor kinase domain or cytosolic tyrosine kinases. PLCβ is regulated through binding to G protein α and βγ subunits. See text for full details.

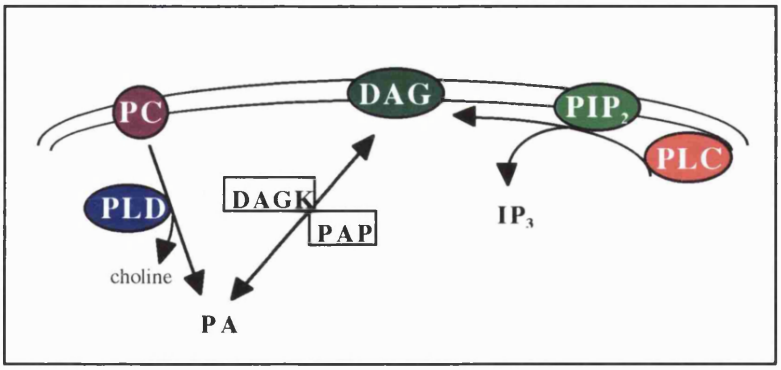


Fig. 1.1b Pathways leading to DAG sunthesis.

Receptor stimulation induces biphasic DAG generation: an initial rapid increase in $PI(4,5)P_2$ derived DAG generated by PLC activity is followed by a sustained phase of phosphatidylcholine derived DAG production via PLD.

Key: PA, phosphatidic acid; PAP, PA phosphohydrolases; DAGK, DAG kinase

receptors (Exton, 1997; Katan, 1998). Regulation of the PLCδ enzymes is less well characterised, although physiological calcium levels are sufficient to stimulate $PLC\delta_1$ (but not $PLC\beta_1$ or $PLC\gamma_1$) activity in an *in vitro* permabilised cell system (Allen *et al.*, 1997). Recently it has been demonstrated that DAG/IP_3 generation in $PLC\delta_1$-overexpressing PC12 cells is enhanced upon stimulation of the PLCβ-linked bradykinin receptor, a response that is dependent on the influx of extracellular calcium (Kim *et al.*, 1999a). Thus, $PLC\delta_1$ may be regulated by PLCβ-induced calcium signals *in vivo.* There is also evidence supporting the regulation of $PLC\delta_1$ by a novel GTP-binding protein (Gh), distinct from the heterotrimeric G proteins. Complexes between $PLC\delta_1$ and Ghα subunits have been observed in cells stimulated via adrenergic receptors and Ghα subunits can bind to and activate purified $PLC\delta_1$ *in vitro* (Feng *et al.*, 1996; Hwang *et al.*, 1995; Im *et al.*, 1992; Katan, 1998).

The activity of PLC enzymes is only one mechanism by which DAG can be generated. Mammalian cells contain ~50 distinct species of DAG some of which are the by-products of *de novo* glycerolipid synthesis but most are generated in response to activation of cell surface receptors (Pessin and Raben, 1989; Pettitt and Wakelam, 1993). Receptor activation leads to a biphasic accumulation of DAG: an initial rapid rise in DAG, which consists mainly of polyunsaturated species, is due to the hydrolysis of $PI(4,5)P_2$ by PLC enzymes, as described above. This response is quickly terminated by the action of DAG kinases (which phosphorylate DAG to phosphatidic acid) and also more slowly by DAG lipase (which catalyses the deacylation of DAG). The elevated level of DAG within stimulated cells often presists due to a second slower, sustained phase of DAG (consisting mostly of monounsaturated or saturated species) that arises from phosphatidylcholine, via the dephosphorylation of phosphatidic acid generated by PLD (Figure 1.1b), (Nishizuka, 1995; Wakelam, 1998). A putative phosphatidylcholine-specific PLC has also been implicated as an alternative pathway for DAG production, although the significance of this in mammalian cells is unclear.

The two lipid products of PLC enzymes, DAG and IP_3, play critical roles in the regulation of many biological responses, including cell proliferation and differentiation, contraction, secretion, gene transcription and synaptic transmission (Clapham, 1995; Clemens *et al.*, 1992; Dekker and Parker, 1994; Dolmetsch *et al.*, 1997; Kikkawa *et al.*, 1989; Li *et al.*, 1998; Nishizuka, 1992; Nishizuka, 1995; Timmerman *et al.*, 1996; Toker, 1998) as is clearly illustrated by the early embryonic lethal phenotype of $PLC\gamma_1$ or $PLC\beta_3$ deficient mice (Ji *et al.*, 1997; Wang *et al.*, 1998).

1.1.2. PI3-kinase signalling.

A second major pathway that regulates signal transduction cascades via inositol lipid metabolism is controlled by Phosphoinositide 3-Kinases, which phosphorylate inositol lipids at the 3' position of the inositol ring to generate PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3, as illustrated in Figure 1.2.

The PI3-Kinases constitute a large family of heterodimeric dual specificity lipid and protein kinases that comprise a regulatory p85 and a catalytic p110 subunit (Vanhaesebroeck *et al.*, 1997). Targeting of PI3-Kinase to the plasma membrane upon stimulation of receptor tyrosine kinases, non-receptor tyrosine kinases and G protein-coupled receptors (through phosphotyrosine/SH_2 and Gβγ interactions, respectively) brings PI3-Kinase into close proximity to its lipid substrates and is sufficient to fully activate PI3-Kinase, as demonstrated by fusion of N-terminal myristoylation or C-terminal isoprenylation sequences to PI3-Kinase (Klippel *et al.*, 1996) or by attaching the catalytic subunit of PI3-Kinase to the transmembrane CD2 protein (Reif *et al.*, 1996).

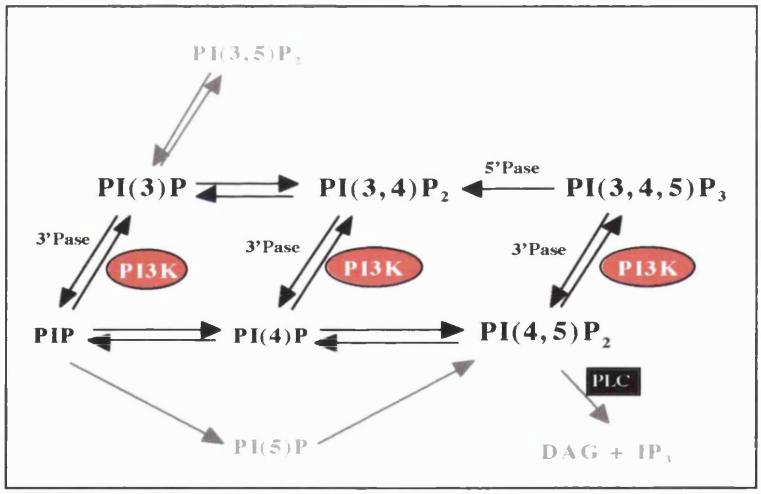


Fig. 1.2. Phosphoinositide metabolism and PI3-Kinase signalling.
Cellular levels of both PI(3,4)P_2 and PI(3,4,5)P_3 levels are low in unstimulated cells and are rapidly increased upon agonist stimulation by the phosphorylation of PI(4,5)P_2 at the D3 position by PI3-Kinase. A potential direct route for PI(3,4)P_2 synthesis maybe occur through phosphorylation of PI(3)P by PI3-Kinase, however the delayed accumulation of PI(3,4)P_2 following receptor activation would suggest its production is predominantly via the action of 5'phosphatases (e.g. SHIP) on newly generated PI(3,4,5)P_2 (Hawkins *et al.*, 1992; Stephens *et al.*, 1991; Stephens *et al.*, 1993). The recently identified PTEN tumour suppressor antagonises the actions of PI3-Kinase by functioning as a 3'phosphoinositide phosphatase (reviewed in (Leevers *et al.*, 1999)).

Numerous studies using inhibitors of PI3-Kinase or expression of PI3-Kinase mutants, and more recently mice deficient for the p85α regulatory and p110α catalytic

subunits of PI3-Kinase, have demonstrated the importance of PI3-kinase for the regulation of cell proliferation and survival, metabolism, cytoskeletal reorganisation and membrane trafficking. An exhaustive description of the biological roles of PI3-Kinase is beyond the scope of this thesis but is the subject of several excellent recent reviews (Bi *et al.*, 1999; Fruman *et al.*, 1999; Leevers *et al.*, 1999; Rameh and Cantley, 1999; Wymann *et al.*, 1999).

Briefly, two major types of signals emanate from PI3-Kinase. Firstly, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (generated by active PI3-Kinase) bind to the pleckstrin homology (PH) domains of a number of intracellular signalling molecules with high affinity and either allosterically modify their activity or induce relocalisation of the protein to defined areas of the plasma membrane where activation can occur (Leevers *et al.*, 1999; Lemmon, 1999; Rameh and Cantley, 1999). PH-domain containing proteins regulated by the 3'phosphoinositide products of PI3-Kinase include PKB/Akt; tyrosine kinases of the Tec family (such as Btk) and several guanine nuc leotide exchange factors for small GTPases (e.g. Vav). Similarly, the PI3-Kinase lipid product PI3P appears to regulate a number of proteins (including EE1A, early endosome antigen 1) involved in intracellular membrane trafficking through binding to recently identified FYVE domains (Leevers *et al.*, 1999).

The second major function of PI3-Kinase is as a protein serine/threonine kinase and substrates include PI3-Kinase itself (which appears to negatively regulate the lipid kinase activity of PI3-Kinase) and the insulin receptor substrate 1 (Wymann *et al.*, 1999). Recently, Bondeva *et al* have demonstrated that PI3-Kinase γ hybrid proteins (with no detectable lipid kinase activity) retain their protein kinase activity and are able to activate ERK2 in response to cell stimulation (Bondeva *et al.*, 1998).

1.1.3. Crosstalk between PLCγ and PI3-kinase signalling pathways.

PLC- and PI3-Kinase-regulated signalling pathways do not function independently, rather they are co-ordinated into an integrated network. For example, the $PI(3,4,5)P_3$ lipid product of PI3-Kinase bind to the PH domains of certain Tec family tyrosine kinases (including Btk, Itk and Tec) localising them to the plasma membrane where they play a central role in the regulation of PLCγ and calcium signalling by antigen receptors (August *et al.*, 1997; Bolland *et al.*, 1998; Fluckiger *et al.*, 1998; Li *et al.*, 1997b; Scharenberg *et al.*, 1998a).

Several studies have also suggested that a second direct link between PI3-Kinase and PLCγ may exist. Specific high affinity binding of $PI(3,4,5)P_3$ to the PH and/or SH2 domains of PLCγ has been detected *in vitro* (Bae *et al.*, 1998; Falasca *et al.*, 1998; Rameh

et al., 1998) and an isolated PH domain from PLCγ translocates to the plasma membrane of serum-starved COS1 cells in a $PI(3,4,5)P_3$-dependent manner in response to cell stimulation (Falasca *et al.*, 1998). Moreover, inhibition of PI3-kinase signalling (using PI3-Kinase inhibitors or expression of dominant negative PI3-Kinase mutants or PDGF receptor mutants that do not bind PI3-Kinase) partially reduces IP_3 generation and calcium mobilisation in PDGF-treated cells (Bae *et al.*, 1998; Falasca *et al.*, 1998; Rameh *et al.*, 1998). Thus, several signals may stabilise PLCγ at the plasma membrane for efficient substrate hydrolysis during receptor stimulation: SH_2-mediated binding to the phosphotyrosine motifs of receptors and SH_2/PH domain-mediated interactions with plasma membrane $PI(3,4,5)P_3$. It should be noted however that the relative importance of PI3-Kinase in the regulation of PLCγ signalling appears to be cell type-dependent. For example, PI3-Kinase inhibitors markedly reduce calcium mobilisation in COS1 cells expressing a wild-type PDGF-receptor and in leukaemia cells, but no inhibition is detected in A31 cells (Rameh *et al.*, 1998). Similarly, DAG production by a wild-type PDGF-receptor and a mutant receptor that lacks the PI3-Kinase binding site are equivalent (Van Lint *et al.*, 1998).

1.2. THE PKC SUPERFAMILY.

1.2.1. PKC introduction.

Receptor-mediated phospholipid metabolism has been recognised for many years and several early studies postulated a linked to biological functions such as calcium mobilisation (Michell, 1975). PKC was initially defined as a histone kinase activity in brain that was activated by limited proteolysis (Inoue *et al.*, 1977) which was rapidly followed by the discovery that this kinase activity could be stimulated by phospholipid metabolism. Thus acidic phospholipids and DAG (a product of $PI(4,5)P_2$ hydrolysis) were found to stimulate PKC activity in a calcium-dependent manner (Nishizuka, 1984a). At the same time Berridge and co-workers demonstrated that IP_3 (the other product of $PI(4,5)P_2$ turnover) was linked to calcium mobilisation from internal stores (Berridge and Irvine, 1984).

A series of reports in the early 1980's identified PKC as cellular target for the tumour promoting phorbol esters (reviewed in (Nishizuka, 1984b)). Thus, the phorbol ester receptor could be co-purified with a calcium and phospholipid-dependent kinase activity (PKC) and phorbol esters could mimic the actions of DAG in binding to and activating this kinase in the presence of calcium and phospholipids (Castagna *et al.*, 1982; Kikkawa *et al.*, 1983; Niedel *et al.*, 1983; Yamanishi *et al.*, 1983). Moreover, chronic

treatment of cells with phorbol esters downregulated PKC, rendering the cells insensitive to a second addition of phorbol esters or DAG (Collins and Rozengurt, 1982; Collins and Rozengurt, 1984; Jaken *et al.*, 1981; Rodriguez-Pena and Rozengurt, 1984; Rozengurt *et al.*, 1984). Hence, phorbol esters have provided a useful tool with which to study PKC functions *in vivo.*

A biological role for DAG/calcium-regulated-PKC was demonstrated during platelet degranulation (Kaibuchi *et al.*, 1983; Yamanishi *et al.*, 1983) establishing for the first time the important link between lipid metabolism and protein phosphorylation in the regulation of intracellular signal transduction pathways. Purification of this kinase activity later indicated that it was composed of at least three distinct PKC species, designated α, β and γ (Huang *et al.*, 1986). Now more than 20 years after its initial characterisation, genetic screening has identified ten mammalian PKC isoforms, as well as a number of related kinases, which comprise the PKC superfamily.

1.2.2. The PKC family.

PKC is now known to constitutute a large family of serine/threonine kinases each with distinct features. The first PKCs to be identified and cloned from brain cDNA libraries were the classical α, β and γ PKC isoforms (Coussens *et al.*, 1986; Parker *et al.*, 1986). PKCβ exists in two alternate splice forms, β_I and β_{II}, which differ only at their extreme C-terminal regions (Coussens *et al.*, 1987). Subsequent lower stringency screening of cDNA libraries from brain and other tissues led to the identification of six additional PKC isoforms: δ, ε and ζ (Ono *et al.*, 1987); η (Osada *et al.*, 1990); θ (Baier *et al.*, 1993; Osada *et al.*, 1992); λ/ι (Akimoto *et al.*, 1994; Selbie *et al.*, 1993).

Although sharing a high degree of homology within their catalytic domains, these kinases have been classified into distinct groups based on amino-acid sequence and distinct regulatory elements (Fig. 1.3). Of these the classical PKCs (α, β_I, β_{II} and γ) have been most extensively studied and are activated by phosphatidylserine in a calcium-dependent manner. In addition, classical PKCs are also regulated by DAG which increases the specificity of the enzyme for phosphatidylserine and shifts the affinity for calcium into the physiological range (Nishizuka, 1984a; Takai *et al.*, 1979). The novel PKCs (δ, ε, η and θ) are also regulated by DAG in the presence of phosphatidylserine but are insensitive to calcium (e.g. (Ono *et al.*, 1988)). In addition, tumour-promoting phorbol esters bind to classical and novel PKCs with high affinity, eliminating the requirement for DAG and decreasing the calcium concentration needed for activation of classical PKCs

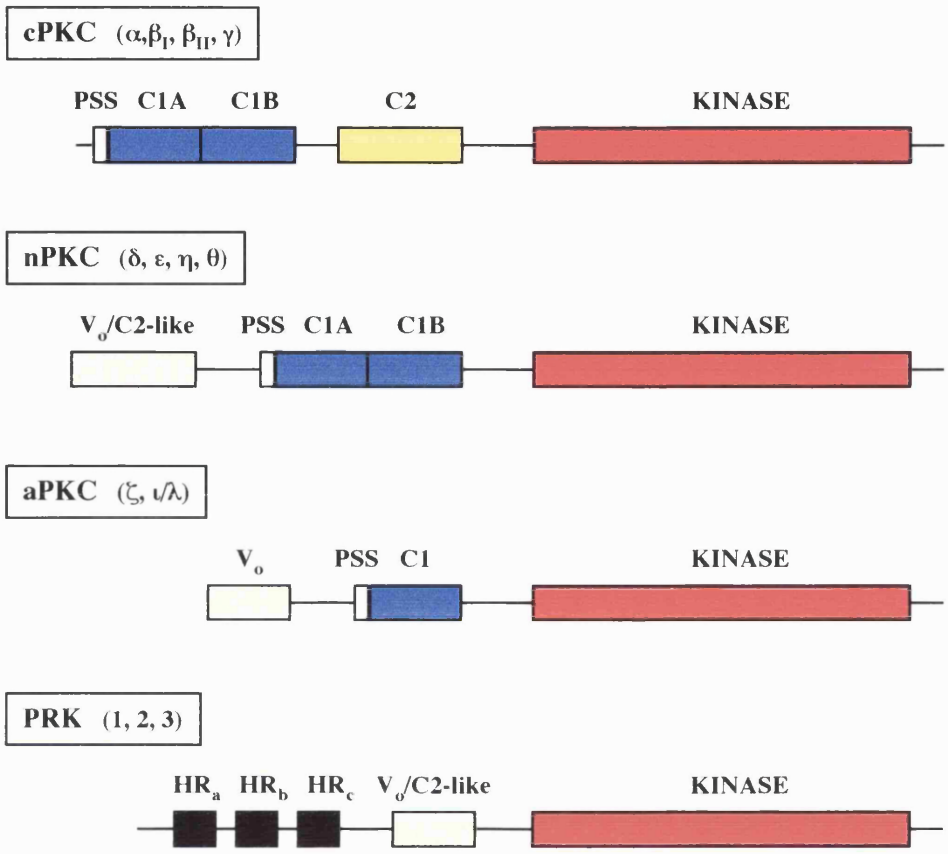


Fig. 1.3. The PKC superfamily.

(Castagna *et al.*, 1982). In contrast, the atypical PKCs (ξ and λ/ι) are not regulated by either calcium, DAG or phorbol esters but are regulated by $PI(3,4)P_2$ and $PI(3,4,5)P_2$ both *in vitro* and *in vivo* (Nakanishi *et al.*, 1993; Ono *et al.*, 1989a). In addition ceramide, which is produced upon stimulation of the TNFα receptor, binds to and activates atypical PKCζ *in vivo* (Muller *et al.*, 1995).

More recently a family of PKC-related kinases, the PRK kinases, have been identified (Mukai *et al.*, 1994; Palmer *et al.*, 1995a). The PRKs show similarities to atypical PKCs in that they are not regulated by either calcium or DAG. Activation of the PRKs by acidic phospholipids, including $PI(4,5)P_2$ and $PI(3,4,5)P_3$, has been documented *in vitro* (Palmer *et al.*, 1995b). The PRKs also interact with GTP-loaded Rho through a unique HR motif (Flynn *et al.*, 1998). Interactions between PRK1 and Rho^{GTP} *in vitro* stimulate PRK1 autophosphorylation (Amano *et al.*, 1996; Watanabe *et al.*, 1996) and LPA stimulates PRK1 autophosphorylation in a Rho-dependent manner *in vivo* (Amano *et al.*, 1996). PRK2 also interacts with Rho^{GTP}, although the role played by Rho in the activation of PRK2 is unclear (Quilliam *et al.*, 1996).

Activation of classical and novel PKCs by lipid cofactors is accompanied by their translocation to specific cellular membranes, in particular to the plasma membrane (Mellor and Parker, 1998; Newton, 1997; Nishizuka, 1995; Toker, 1998) and is mediated by the regulatory domains described below.

1.2.3. The C1 domain.

The identification of the C1 domain of PKC enzymes as the site of phorbol ester/DAG binding arose from mutational studies of PKCα in which the C1 domain was either completely deleted or where several apparently conserved cysteine residues were mutated (Ono *et al.*, 1989b). A subsequent more refined mutational study defined a minimal highly conserved ~50 amino acid motif as the DAG/phorbol ester binding site (Quest *et al.*, 1994a). Classical and novel PKC enzymes contain two tandemly repeated copies of this motif (named the C1A and C1B domains, respectively). In contrast, atypical PKCs contain only a single C1 domain which cannot bind DAG or phorbol esters. Additional C1 domain-containing proteins have now been described, as discussed later in this chapter.

C1 domains contain a highly conserved cysteine-rich $H\text{-}X_{12}\text{-}C\text{-}X_2\text{-}C\text{-}X_{13/14}\text{-}C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_2\text{-}C\text{-}X_7\text{-}C$ motif, which co-ordinates two zinc atoms (Hurley *et al.*, 1997). Several groups have now reported the three-dimensional structure of C1 domains from PKCα and

PKCδ (Hommel *et al.*, 1994; Zhang *et al.*, 1995). From these studies, and from subsequent computer modelling, it is apparent that the C1 domain folds into an elongated globular structure in which the top part of the domain is made up of two parallel β-sheets which form a water filled cavity. A number of highly conserved residues, including Pro11, Gly23 and Gln27, play a critical role in maintaining the precise "unzipped" nature of this DAG/phorbol ester binding groove. In addition, other residues at positions 8, 13, 20, 22 and 24 (usually Met, Val, Leu, Ile, Phe, Tyr or Trp) form a hydrophobic wall around this groove. Mutation of these conserved residues in PKCδ has been demonstrated to abolish or greatly diminish the sensitivity of this kinase to DAG and phorbol esters (Kazanietz *et al.*, 1995). C1 domains have also been described in proteins that do not bind DAG or phorbol esters, here polar residues are often found in place of the conserved hydrophobic residues of DAG-binding C1 domains. For example, structural analysis of the non-DAG binding C1 domain of Raf has shown that the DAG-binding site is compromised by the absence of hydrophobic residues, such that one entire face of the binding groove is missing (Mott *et al.*, 1996). In addition, atypical PKCs, which are unresponsive to DAG or phorbol esters, contain a single C1 domain which deviates from the consensus at two positions: Pro11 and a hydrophobic residue at position 20. However, mutation of the residue at position 11 back to proline does not confer phorbol ester binding (Kazanietz *et al.*, 1994b). Whether mutation of the other site would be sufficient to restore phorbol ester sensitivity is not known.

Hurley and co-workers have elucidated the crystal structure of an isolated C1 domain, from PKCδ, complexed to phorbol ester (Zhang *et al.*, 1995). The C1 domain does not appear to undergo significant conformational changes upon phorbol ester binding. Rather the ligand caps the binding site groove forming a continuous hydrophobic surface over the top third of the domain. Thus, membrane targeting of classical and novel PKCs is achieved through hydrophobic interactions induced by DAG/phorbol ester binding to the C1 domains.

The stochiometry of phorbol ester binding to classical or novel PKCs is 1:1 (Hannun *et al.*, 1985; Konig *et al.*, 1985; Mosior and Newton, 1995) despite the presence of two C1 domains that are both capable of binding phorbol ester with similar affinities when expressed *in vitro* as isolated fusion proteins (Burns and Bell, 1991; Kazanietz *et al.*, 1995; Ono *et al.*, 1989b; Quest and Bell, 1994b). Interestingly, an emerging theme in the regulation of classical and novel PKCs is that individual C1 domains within PKC enzymes play distinct roles in mediating phorbol ester/DAG binding and membrane association *in*

vivo. Thus one study using a novel fluorescent phorbol ester analogue has proposed that the C1A and C1B motifs of PKCα bind phorbol esters and DAG with distinct and opposing affinities (Slater *et al.*, 1996). Moreover, mutation of a highly conserved proline residue within the C1A motif of PKCδ has little effect on its response to phorbol ester, whereas mutation of the equivalent site in C1B caused a 125-fold decrease in phorbol ester binding affinity and membrane translocation (Szallasi *et al.*, 1996b). This concept has recently been visualised by following the membrane targeting of GFP-tagged C1A and C1B domains (from PKCγ) in response to phorbol ester stimulation, where these two domains show significant differences in their affinity for cellular membranes (Oancea *et al.*, 1998a).

1.2.4. The C2 domain.

Immediately C-terminal to the C1 domain of classical PKCs is a calcium binding C2 domain similar to that present in a number of other proteins including the synaptotagmins, phospholipase C and GAPs (Ponting and Parker, 1996; Shao *et al.*, 1996). In a manner analogous to the C1 domain, calcium-induced association of the C2 domain with acidic phospholipids is implicated in the membrane targeting of classical PKCs (Mellor & Parker, 1998; Newton, 1997; Newton & Johnson, 1998; Nishizuka, 1995; Toker, 1998). Thus, treatment of cells with calcium ionophore is sufficient to induce repetitive transient associations of GFP-tagged classical PKC enzymes or isolated C2 domains with cellular membranes, in a manner that exactly correlates with calcium spikes (Almholt *et al.*, 1999; Oancea and Meyer, 1998b).

The regulatory region of calcium-independent novel PKCs do not contain a classical C2 domain. However a motif termed the V_0 region at the extreme N-terminus of these kinases shows sequence homology to the C2 domain of classical PKCs but lacks several conserved aspartate residues that are important for calcium binding (Ponting and Parker, 1996; Sossin and Schwartz, 1993). Whether this V_0 motif is able to bind phospholipids is unclear although novel PKCs are regulated by phospholipids *in vitro* and it has recently been demonstrated that the V_0 region of PKCδ is structurally similar to that of a calcium-bound C2 domain (Pappa *et al.*, 1998).

A second function of the C2 domain of classical PKC enzymes may be to mediate protein-protein interactions. A class of proteins, termed receptors for activated PKCs (RACKs), have been shown to interact with the C2 domain of PKCs (reviewed in (Mochly-Rosen, 1995; Ron and Kazanietz, 1999a)). RACKs are not PKC substrates but appear to

target activated PKCs to specific membrane compartments, as discussed further in section 1.2.7.

1.2.5. Regulation of PKC by phosphorylation.

PKCs are synthesised as precursor proteins that undergo post-translational modification by sequential phosphorylation of conserved serine/threonine residues, resulting in reduced mobility on SDS-PAGE, to form the mature kinase (Borner *et al.*, 1989; Flint *et al.*, 1990). Three of these phosphorylation sites, referred to as 'priming' phosphorylation sites are located within the activation loop (T500 in PKCβ_{II}) and at the C-terminus of these enzymes (at residues T641 and S660 for PKCβ_{II}) (Karanen *et al.*, 1995; Tsutakawa *et al.*, 1995). Phosphorylation at these priming sites is required for the correct processing and maturation of PKC to ensure maximal catalytic competency upon subsequent lipid cofactor binding. Cazaubon and co-workers first identified the T497 activation loop phosphorylation site of PKCα as critical for the permissive activation of this kinase when an alanine substitution at this site resulted in a non-activatable kinase (Cazaubon *et al.*, 1994; Cazaubon *et al.*, 1993). The equivalent phosphorylation site in the activation loop of PKCβ_{II} is also essential for lipid-induced catalytic activity (Orr and Newton, 1994). Modelling studies have since indicated that a negative charge at this activation loop sites is important for the correct alignment of residues for catalysis following lipid binding.

Mutation of the middle priming phosphorylation site of PKCβ_{II} (T641) (or dephosphorylation of this site in the mature fully phosphorylated enzyme) results in a non-activatable kinase (Karanen *et al.*, 1995). In contrast, mutation of the equivalent site (S643) in PKCδ does not prevent lipid-induced activation (Li *et al.*, 1997a). Moreover, Parker and co-workers have demonstrated that mutation of the two C-terminal phosphorylation sites in PKCα (T638 and S657) does not prevent PKC activation as such rather these mutants are unstable, exhibiting increased sensitivity to protein phosphatases, heat and oxidation (Bornancin and Parker, 1996; Bornancin and Parker, 1997). The discrepancy between these studies may be explained by the possibility of compensatory phosphorylation events within the C-terminal region of PKC enzymes, as proposed by Newton and co-workers. For example, mutation of several putative compensatory phosphorylation sites around the T641 residue of PKCβ_{II} has demonstrated that C-terminal phosphorylation events are essential in the correct processing of this enzyme for catalytic competence (Edwards *et al.*, 1999).

Recently, the molecular mechanisms underlying these priming phosphorylation events have been described. Sequence alignment studies revealed a highly conserved TFCGTX(E/D)YXAPE motif surrounding the activation loop phosphorylation site of all PKC enzymes that shows homology to similar phosphorylation motifs within the kinase domains of other serine/threonine kinases, including PKB/Akt and $p70^{S6}$ kinase. The identification of the PKB/Akt activation loop kinase as PDK1 (phosphoinositide-dependent kinase 1) followed by the subsequent demonstration that PDK1 could also phosphorylate the comparable site of $p70^{S6}$ kinase (Alessi *et al.*, 1997b; Alessi *et al.*, 1997a; Pullen *et al.*, 1998; Stokoe *et al.*, 1997) led to the hypothesis that PDK1 might also transphosphorylate the activation loop of PKC enzymes. Indeed, complexes between PDK1 and different PKC isoforms have been detected (Le Good *et al.*, 1998) and several groups have now shown that PDK1 phosphorylates the activation loop priming site of classical, novel and atypical PKC enzymes in a PI3-Kinase-regulated manner both *in vitro* and *in vivo* (Chou *et al.*, 1998; Dutil *et al.*, 1998; Le Good *et al.*, 1998).

In a situation analogous to the activation loop priming phosphorylation site, the extreme C-terminal priming phosphorylation site of classical and novel PKCs lies within a conserved hydrophobic phosphorylation motif, FXXF(**S/T**)(Y/F), that is also found at the C-terminus of other serine kinases notably PKB/Akt, $p70^{S6}$ kinase and PRK1/2. In contrast, atypical PKCs contain a glutamic acid residue in place of the serine in this motif, mimicking a constitutive phosphorylation event. The Newton laboratory have reported that this C-terminal phosphorylation site in $PKC\beta_{II}$ (S660) is regulated through an autophosphorylation mechanism since purified $PKC\beta_{II}$ (which has been dephosphorylated *in vitro* by phosphatases) can re-autophosphorylate on S660 *in vitro* (Dutil *et al.*, 1994; Karanen *et al.*, 1995). In addition a kinase-dead $PKC\beta_{II}$ mutant is defective for S660 phosphorylation (Benn-Krappa and Newton, 1999). This may not be the case for all PKC enzymes however. A recent study by Zeigler and co-workers has implicated atypical $PKC\zeta$ (in complex with a rapamycin-sensitive component) in the transphosphorylation of the analogous hydrophobic C-terminal site in $PKC\delta$ and $PKC\alpha$ (Ziegler *et al.*, 1999). Interestingly, the corresponding hydrophobic phosphorylation motif in the C-terminus of $p70^{S6}$ kinase is also the primary target for the inhibitory action of rapamycin against this kinase (Pearson *et al.*, 1995) and recent studies have described the regulation of $p70^{S6}$ kinase by atypical PKC enzymes (Akimoto *et al.*, 1998; Romanelli *et al.*, 1999). Thus it is possible that a conserved mechanism for the phosphorylation of hydrophobic FXXF(**S/T**)(Y/F) motifs might involve atypical PKC enzymes.

Not all PKC phosphorylation sites are priming events however, for example autophosphorylation of PKCα on T250 following TPA-stimulation has been described (Ng *et al.*, 1999a). In addition to serine/threonine phosphorylation, PKCδ undergoes tyrosine phosphorylation in response to various stimuli, including hydrogen peroxide (Denning *et al.*, 1996; Gschwendt *et al.*, 1994; Konishi *et al.*, 1997; Li *et al.*, 1994). The effects of tyrosine phosphorylation on activity differ depending on the experimental conditions used and the sites involved have not been mapped. The biological significance of such tyrosine phosphorylation events for PKCδ (or other PKCs) is unknown.

1.2.6. Regulation of PKC activity.

The phosphorylation sites described above are 'priming' events that ensure the correct processing of immature PKC for efficient catalytic competency. Activation of PKC, often measured by the translocation of PKCs from the cytosol to cellular membranes arises from lipid cofactor binding to C1 (DAG) and C2 (calcium and acidic phospholipids) domains. As discussed in section 1.1.1, cells contain different types of DAG that arise from distinct sources, PLC and PLD respectively. Although the majority of different DAG species can activate classical/novel PKCs in *in vitro* assays there is a marked preference for polyunsaturated DAG (Marignani *et al.*, 1996; Schachter *et al.*, 1996). Indeed, $PKC\beta_{II}$ has the highest affinity for polyunsaturated *sn*-1,2-diacylglycerol DAG (Newton and Johnson, 1998). *In vivo* it appears that classical and novel PKC enzymes are specifically activated by polyunsaturated DAGs since PKC activity appears to correlate better with the transient phase of $PI(4,5)P_2$-hydrolysis rather than with the later sustained phase of PLD-derived saturated DAG (Hodgkin *et al.*, 1998; Pettitt *et al.*, 1997; Wakelam, 1998). For example, the initial polyunsaturated-enriched DAG produced in stimulated Swiss 3T3 cells is a more potent *in vitro* activator of PKC than monounsaturated/saturated DAG species produced at later times (Pettitt and Wakelam, 1998). Stimulation of Swiss 3T3 cells with bombesin induces a transient increase in polyunsaturated DAG levels and a transient activation of PKC, whilst a combination of bombesin plus TGFβ (which results in the sustained elevation of polyunsaturated DAG) now activates PKC in a sustained manner (Olivier *et al.*, 1996). Similarly, LPA-stimulation of porcine endothelial aortic cells does not induce PKC activity and in these cells PLD, but not PLC, is coupled to the LPA receptor (Pettitt *et al.*, 1997). In addition, sustained DAG production does not correlate with the association of classical/novel PKC enzymes with cellular membranes in living cells (Feng *et al.*, 1998a; Oancea and Meyer, 1998b; Oancea *et al.*, 1998a; Ohamori *et al.*, 1998).

These observations fit with the hypothesis that PLD-derived monounsaturated/saturated PA (and not DAG) is the active signalling product of PLD (Fig 1.4). Thus, PLD activity is essential for LPA-induced actin stress-fibre formation in porcine aortic endothelial cells and inhibition of PA production blocks this response (Pettitt *et al.*, 1997). Furthermore, addition of exogenous PA to these cells stimulates stress-fibre formation. Rho has been implicated as a downstream effector of PA since the *Clostridium botulinum* C3 toxin (which ADP-ribosylates and inactivates Rho) blocks LPA- and PA-induced stress-fibre formation (Cross *et al.*, 1996).

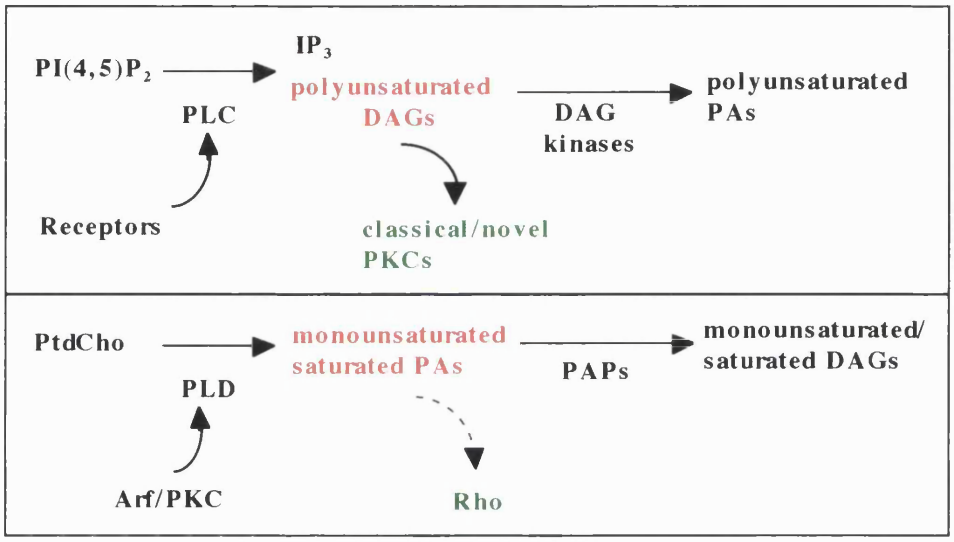


Figure 1.4. Distinct intracellular signalling molecules generated by PLC and PLD.
Top, receptor-mediated $PI(4,5)P_2$ hydrolysis at the plasma membrane. Bottom, receptor-mediated PLD-regulated phosphatidylcholine turnover. Active polyunsaturated DAGs regulate classical and novel PKC enzymes. Active monounsaturated/saturated PAs are involved in the regulation of the actin cytoskeleton, Rho is implicated as a downstream effector in this process. See main text for more details. Key: PA, phosphatidic acid; PAPs, PA phosphohydrolases. Adapted from (Hodgkin *et al.*, 1998).

PKC was originally characterised as a kinase activity that was enhanced by proteolysis indicating that PKCs are structurally regulated by an autoinhibitory domain. Sequence analysis has identified a regulatory pseudosubstrate motif upstream of the C1 domain of PKC enzymes that closely resembles the optimal substrate phosphorylation motif of individual PKC enzymes but with an alanine residue at the predicted serine/threonine phosphorylation site (House and Kemp, 1987). Mutation of this motif results in constitutive effector-independent PKC activity (Pears *et al.*, 1990). These data suggest a model whereby the pseudosubstrate motif maintains PKC enzymes in an inactive conformation by sterically blocking the catalytic domain active site. Thus, lipid cofactor binding to

classical/novel PKCs is thought to induce a conformational change that releases the pseudosubstrate motif increasing the catalytic activity of these enzymes.

1.2.7. Regulation of PKC subcellular localisation.

Once activated PKCs phosphorylate specific substrates and regulate downstream signalling cascades, as discussed later in section 1.2.9. The heterogeneity of the PKC family suggests that different PKC isoforms have specific functions within intracellular signalling cascades. However, as discussed above the lipid regulators of different PKCs are very similar. Cellular mechanisms must therefore exist to determine which PKC enzymes participate in which signalling pathways.

One potential mechanism that may regulate the specificity of PKC *in vivo* may be through the control of subcellular localisation. Membrane targeting of classical/novel PKCs via their C1 and C2/V_0 motifs has been extensively characterised. However, different PKC isoforms are frequently localised at distinct subcellular sites both before and after activation (reviewed in (Goodnight *et al.*, 1995; Jaken *et al.*, 1989)). A number of PKC interacting proteins have been identified (through overlay assays or yeast-2-hybrid screening) which appear to function as regulators of PKC localisation. These include scaffolding proteins such as the AKAPs and receptors for activated PKCs (RACKs). Interestingly a number of these proteins appear to bind to PKCs in an isoform-specific manner and have been proposed to regulate which intracellular sites PKC enzymes are targeted to and thus their association with downstream substrates (Mochly-Rosen, 1995; Newton, 1996; Pawson and Scott, 1997).

The AKAP79 anchoring protein is a multi-enzyme scaffolding protein that complexes three inactive enzymes: classical PKCs, calcineurin and cAMP-dependent kinase (Klauck *et al.*, 1996). A peptide based on the PKC-binding site of AKAP79 inhibits PKC activity *in vitro*, indicating that AKAP79 may function to keep PKC in an inactive state *in vivo* (Klauck *et al.*, 1996). Calcium/calmodulin complexes can antagonise the interaction between AKAP79 and PKC, thus releasing PKC and allowing concomitant activation by DAG following receptor-mediated stimulation of PLC (Faux and Scott, 1997). It is possible that AKAP79 concentrates these enzymes near specific substrates. Moreover, co-localisation of two kinases with a phosphatase would allow the tight regulation of downstream effector pathways.

RACK proteins, which bind to the C2 domains of activated PKC enzymes appear to do so in an isoform specific manner. Thus the RACK1 protein binds specifically and

directly to PKCβ_{II} (Ron *et al.*, 1994), whilst β'COP binds to PKCε (Csukai *et al.*, 1997). A recent report has described a role for RACK1 as a shuttling protein that localises PKCβ_{II} to appropriate sites (Ron *et al.*, 1999b). The hypothesis that interactions with RACK proteins target active PKC enzymes to specific intracellular sites/substrates is supported by the observation that peptides based on the RACK binding regions of PKCβ_{II} or ε disrupt phorbol ester-induced membrane targeting of the respective enzymes *in vivo* (Ron *et al.*, 1994; Ron *et al.*, 1995; Yedovitzky *et al.*, 1997).

1.2.8. Inactivation of PKC enzymes.

The degradation of classical and novel PKC enzymes (downregulation) upon prolonged exposure to phorbol esters has long been recognised and results from a net increase in PKC proteolysis without changes in the rate of PKC synthesis (Young *et al.*, 1987). PKC downregulation appears to be a function of the catalytic activity of PKC enzymes since PKC inhibitors can block phorbol ester-induced degradation of PKCα and expression of a catalytic active kinase domain fragment of PKCα is sufficient to induce PKC downregulation *in trans* (Hansra *et al.*, 1999). Chronic exposure to phorbol esters has been shown to induce the ubiquitination and degradation of PKCα by the proteasome (Lee *et al.*, 1996a) suggesting that this might be a mechanism by which PKC enzymes are downregulated *in vivo*. Indeed phorbol-induced degradation of PKCα is blocked by lactacystin, a proteosome inhibitor.

Downregulation of PKC enzymes has been correlated with several dephosphorylation events (Hansra *et al.*, 1996; Lee *et al.*, 1996b). Recently the Parker laboratory have demonstrated that downregulation of PKC enzymes correlates with an upregulation of vesicle trafficking events (Goode *et al.*, 1995; Goode *et al.*, 1994). Using GFP-tagged PKCα to visualise the downregulation process it was shown that PKCα is internalised into cytosolic vesicles within ~10-15 min of phorbol ester treatment and is subsequently delivered to a detergent-insoluble perinuclear region (within ~2h) before it is finally degraded (Hansra *et al.*, 1999). Dephosphorylation of the three 'priming' sites described in section 1.2.5. occurs after phorbol ester-induced internalisation of classical PKC enzymes (Hansra *et al.*, 1999). Since phosphorylation of these sites is required for optimal catalytic competency of PKC a model has been proposed in which membrane localised PKC enzymes are internalised in an activated form where they are subsequently attacked by phosphatases and inactivated prior to proteolytic degradation. Indeed, Ng *et al* have shown that active PKCα is associated with cytosolic vesicles in TPA-stimulated

cells during sustained responses and is autophosphorylated on T250 (Ng *et al.*, 1999a). The actual mechanism of PKC internalisation and how PKC enzymes couple to it are unknown at present.

The downregulation of certain classical/novel PKC enzymes under conditions of physiological stimulation has been demonstrated (Kiley *et al.*, 1991; Olivier and Parker, 1994) but this is by no means a widely reported event. The importance or significance of downregulation as a means to inactivate PKC enzymes following receptor ligation is therefore not known. Classical and novel PKC enzymes only transiently associate with the plasma membrane of a variety of cell types upon receptor-engagement and require lipid cofactor binding for catalytic activity. Thus, it is frequently assumed that the plasma membrane dissociation of classical and novel PKC enzymes is associated with their catalytic inactivation, see for example (Feng and Hannun, 1998b; Oancea and Meyer, 1998b). However, the location of (and the duration of) PKC activity under physiological conditions is not known at present. In the future phospho-specific antisera against inducible PKC autophosphorylation sites, such as the T250 site of PKCα (Ng *et al.*, 1999a) may help to address these questions.

1.2.9. Biological functions of PKC.

The diversity of the PKC family coupled with their distinct tissue and subcellular expression patterns suggests that individual PKC isotypes are likely to have specific biological roles *in vivo* and not solely redundant. Through the use of the tumour promoting phorbol esters, which mimic DAG, classical/novel PKC enzymes have been implicated in a wide range of biological actions, including changes in ion fluxes, secretion, contraction, adhesion, gene expression, apoptosis and cell proliferation & differentiation (Clemens *et al.*, 1992; Dekker and Parker, 1994; Kikkawa *et al.*, 1989; Nishizuka, 1992; Nishizuka, 1995; Toker, 1998). An exhaustive description of these events is beyond the scope of this thesis, however a few areas will be discussed briefly.

PKC and the cell proliferation.

Phorbol esters function as tumour promoting agents *in vivo* which first prompted the hypothesis of a link between PKC and cell proliferation. Over the last 20 years numerous *in vitro* and *in vivo* studies (utilising phorbol esters, PKC inhibitors and overexpression systems) have been published regarding the role played by PKC enzymes in the regulation of cell proliferation and differentiation in a wide variety of cell types

(reviewed in (Nishizuka, 1995)). It is clear that PKC enzymes stimulate cell proliferation/differentiation by activating intracellular signalling cascades that regulate transcription factors, leading to increased expression of immediate early response genes (e.g. c-Fos, c-Jun, c-Myc), that control cellular proliferative/differentiation programmes. The most well characterised of these is the mitogenic Raf→Mek→ERK signalling cascade (Fig. 1.5). Much of the evidence for the involvement of PKC in this cascade derives from the use of phorbol esters: acute treatment with phorbol esters leads to a rapid activation of ERK2 (Hoshi *et al.*, 1989; Rossomando *et al.*, 1989) and Raf (Heidecker *et al.*, 1992; Rapp, 1991) in most cell types.

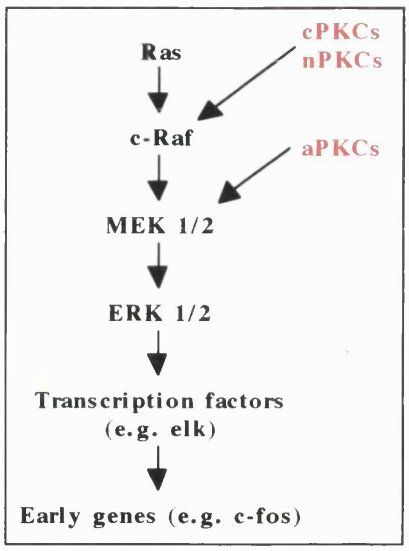


Fig. 1.5. The MAPK cascade. The sequential activation of Raf, MEK and ERK kinases controls gene transcription events that regulate cell proliferentiation & differentiation. The Raf kinase is regulated through interaction with membrane-bound Ras^{GTP} and by phosphorylation events (Marais *et al.*, 1995; Morrison *et al.*, 1993b). Raf subsequently activates MEK, which in turn activates ERK. Active ERK kinases translocate to the nucleus and phosphorylate target molecules such as Elk-1, a protein that forms a ternary complex with serum response factor (SRF), to regulate the serum response element (SRE) found in the promoter/enhancer regions of many growth factor-regulated genes (Treisman, 1994; Treisman, 1996).

By using dominant negative and constitutively activated PKC mutants several groups have now demonstrated that multiple PKC enzymes have the capacity to activate the ERK pathway *in vivo* (Cai *et al.*, 1997; Schonwasser *et al.*, 1998; Soh *et al.*, 1999; Ueda *et al.*, 1996). Interestingly, different PKCs appear to target this pathway at distinct levels, with classical PKCα and novel PKCη using a Raf-dependent pathway to activate MEK and ERK, whilst atypical PKCζ induces MEK and ERK activation in a Raf-independent manner (Schonwasser *et al.*, 1998). PKCs has been shown to phosphorylate c-Raf on several serine sites *in vitro* and *in vivo*, two of which (S259 and S499) have been implicated in the direct activation of Raf by PKC (Carroll and May, 1994; Kolch *et al.*, 1993). However, Schonwasser *et al* have proposed that classical/novel PKCs regulate c-Raf at the level of membrane recruitment and not through phosphorylation events, since constitutively active PKC enzymes can activate c-Raf mutants containing alanine substitutions at S259 or S499 to the same extent as wild-type c-Raf (Schonwasser *et al.*,

1998). Moreover, membrane-targeting of c-Raf apparently bypasses the PKC-dependent step in the regulation of Raf. The mechanism by which atypical PKCζ regulates MEK is unclear at present, with contrasting reports proposing direct (Diaz-Meco *et al.*, 1994) and indirect roles (Schonwasser *et al.*, 1998) for PKCζ in this process. One interesting recent study has suggested that the concerted action of a hierarchical signalling cascade involving PKCλ, PKCε and PKCζ is required to mediate transcriptional activation of the c-Fos promoter in cells expressing oncogenic Ha-Ras (Kampfer *et al.*, 1998).

PKC and the cytoskeleton.

Through pharmacological studies classical/novel PKC enzymes have been implicated in a number of integrin-mediated events including focal adhesion formation, cell spreading, cell migration and cytoskeletal rearrangements (reviewed in (Keenan and Kelleher, 1998; Kolanus and Seed, 1997)). For example, downregulation of classical/novel PKCs by chronic phorbol ester treatment prevents fibronectin-induced stimulation of a Grb2/Ras/Raf/ERK2 cascade, an effect that is reversed by expression of constitutively activated PKCα, δ or ε mutants (Miranti *et al.*, 1999). Recently, Ng *et al* have proposed that PKCα regulates β1 integrin-dependent cell motility through direct association with β1 integrin and control of integrin-trafficking events (Ng *et al.*, 1999b) and a role for cdk6 and classical/novel PKC enzymes in promoting interactions between $\alpha_v\beta_3$ integrin and focal adhesions in the regulation of cell spreading has been demonstrated (Fahraeus and Lane, 1999). However, the precise mechanisms that link PKC enzymes with these integrin-mediated events remain ill-defined.

PKC regulation of PLD.

PKC, in co-operation with Rho-family proteins and Arf-1 (ADP-ribosylation factor-1, a small GTP binding protein), is an important regulator of PLD activity both *in vitro* and *in vivo* (reviewed in (Exton, 1999)). Thus, inhibition of classical/novel PKC enzymes blocks phorbol ester or growth factor-induced activation of PLD in a variety of cell types (Balboa *et al.*, 1994; Gustavsson *et al.*, 1994; Meacci *et al.*, 1999; Yeo and Exton, 1995) and overexpression of $PKC\beta_I$ or PKCα in intact Swiss 3T3 fibroblasts enhances basal and agonist-induced PLD activity (Eldar *et al.*, 1993; Pachter *et al.*, 1992). Furthermore, PDGF-induced PLD activation is markedly reduced in mouse embryo fibroblasts deficient for PLCγ and this is restored by addition of exogenous DAG plus calcium ionophore (Hess *et al.*, 1998). Complexes between PLD1 and PKCα have been detected in phorbol ester-treated

Rat1 fibroblasts and PLD1 is phosphorylated on serine residues under these conditions (Min and Exton, 1998). However, the mechanism by which PLD is activated *in vivo*, and the contribution by PKC enzymes to this process, is poorly understood.

PKC and negative feedback signalling.

In addition to positive signals, PKC is implicated in negative signalling events through the downregulation or desensitisation of cell surface proteins, including receptor tyrosine kinases, G-protein coupled receptors and ion channels (Inglese *et al.*, 1993; Seedorf *et al.*, 1995). PKC also acts as a negative feedback regulator of PLC-mediated $PI(4,5)P_2$ hydrolysis and calcium mobilisation (Levesque *et al.*, 1997; Lopez-Rivas *et al.*, 1987; Mendoza *et al.*, 1986; Ozawa *et al.*, 1993b).

One of the most well characterised of these events is the desensitisation of the EGF-receptor upon exposure to phorbol esters or PDGF. Here, negative regulation of the EGF receptor occurs through specific phosphorylation events, mediated via classical/novel PKC enzymes and MAPK, that decrease the affinity of EGF binding sites and inhibit EGF-induced tyrosine kinase activity of the receptor (Collins *et al.*, 1983; Friedman *et al.*, 1984; Sinnett-Smith and Rozengurt, 1985). Mutational analysis has revealed two residues within the cytoplasmic tail of the EGF-receptor (T654 and T669) that are phosphorylated in response to phorbol esters (Hunter *et al.*, 1984; Morrison *et al.*, 1993a). Phosphorylation at one or other of these sites is necessary but not sufficient however for EGF receptor desensitisation, indicating that other mechanisms are involved.

In vivo functions for PKC.

Despite the recognised importance of PKC in signal transduction, the events occurring downstream of individual PKC enzymes remain poorly defined, probably because most cells express several PKC isoforms and because downstream targets that transduce signals from specific PKC enzymes have not been identified. At present very few studies have attempted to define functional roles of a particular PKC enzyme in an *in vivo* model system.

PKC enzymes have been implicated in cellular proliferation (as discussed above) and Murray *et al* have investigated the role of $PKC\beta_{II}$ in cell proliferation *in vivo* by generating a transgenic mouse that overexpresses $PKC\beta_{II}$ within intestinal epithelial cells (Murray *et al.*, 1999). These mice exhibit hyperproliferation of colon epithelial cells and a high incidence of pre-neoplastic lesions. These responses may be mediated through a

Wnt/adenomatous polyposis coli (APC)/β-catenin signalling pathway, since the $PKC\beta_{II}$-transgenic mice show increased expression of β-catenin and decreased glycogen synthase kinase 3β activity within the colonic epithelial cells. The $PKC\beta_{I/II}$ knockout mouse is characterised by B cell immunodeficiency however, as described below.

In a separate *in vivo* study, mice homozygous for PKCγ deficiency develop normally and are viable but exhibit mild neurological defects (Abeliovich *et al.*, 1993a; Abeliovich *et al.*, 1993b). Normal synaptic transmission is seen unaffected but long-term potentiation (LTP) within the hippocampus is greatly diminished. PKCγ is not required for spatial and contextual learning however, since the mice can learn to carry out hippocampal-dependent tasks. Defects within the peripheral nervous system of $PKC\gamma^{-/-}$ mice have also been noted, where pathogenic pain responses to peripheral nerve injury are reduced (Malmberg *et al.*, 1997). Moreover, phosphorylation of the neuronal PKC substrate RG3 (but not MARCKs or GAP43) is abolished in $PKC\gamma^{-/-}$ mice (Ramakers *et al.*, 1999), which may contribute to the learning defects and impaired hippocampal LTP observed in these mutant mice.

1.3. ANTIGEN RECEPTOR SIGNALLING.

In this thesis several model systems were utilised to study signal transduction pathways regulated by DAG including fibroblasts, lymphocytes and mast cells. As a substantial portion of the work presented here has been performed using lymphocytes and mast cells this model system will be discussed in detail. The salient properties of the other cell model systems will be presented in subsequent sections of this thesis, as appropriate.

1.3.1. Induction of antigen receptor signalling.

The co-ordinated activation of lymphocytes, mast cells and other cells of the immune system ensures successful immune responses are mounted against invading pathogens. Signal transduction pathways triggered by the recognition of foreign antigen by specific receptors on these cells are critical for the secretion of both allergic mediators and regulatory cytokines that serve to amplify the processes of lymphocyte activation and proliferation.

Antigen receptors themselves are multimeric complexes composed of extracellular ligand recognising subunits non-covalently associated with intracellular signal transducing chains (Fig 1.6). Two defining points in the field of antigen receptor signal transduction

came from the discovery of immunoreceptor tyrosine-based activation motifs (ITAMs), characterised by tandem YxxL sequences, within the signalling chains of these receptors (Reth, 1989) and from the observation that antigen receptor engagement induces multiple cytoplasmic protein tyrosine phosphorylation events. The mechanisms underlying antigen receptor signalling have been extensively characterised through both genetic and biochemical approaches (Campbell, 1999; Clements *et al.*, 1999; Kinet, 1999; Kurosaki, 1999; van Leeuwen and Samelson, 1999). An overview of the current knowledge regarding immediate signalling events downstream of antigen receptors is summarised here.

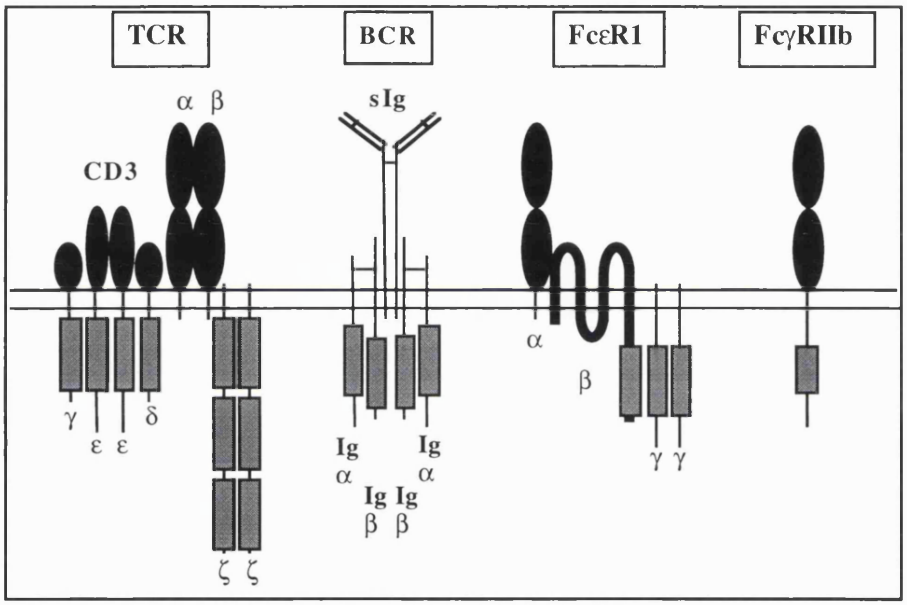


Fig. 1.6. The Antigen Receptor Superfamily.

The physiological ligand for the TCR is foreign peptide bound to major histocompatibility complex (MHC)-encoded molecules presented on the surface of professional antigen presenting cells, such as dendritic cells. Activation of the BCR complex is induced by aggregation of surface immunoglobulin (sIg) through the binding of multivalent foreign antigen. Activation of the antigen receptor present on mast cells, the FcεR1 (the high affinity receptor for IgE) is induced when binding of specific multivalent antigen to receptor-bound IgE results in receptor clustering. The tandem YxxL phosphoacceptor ITAMs are indicated. The homologous region in the FcγRIIb receptor contains a single YxxL motif preceeded by a small hydrophobic residue at the -2 position and confers inhibitory rather than activation signals.

One of the earliest events following antigen receptor engagement is the phosphorylation of tyrosine residues within the signalling chain ITAM motifs of these receptors by activated Src family kinases such as Lyn, Fyn, Blk and/or Lck. Phosphorylation of receptor ITAMs creates specific binding sites for the SH_2 domains of the cytosolic protein tyrosine kinases Syk (B cells and mast cells) and ZAP70 (T cells) which are subsequently tyrosine phosphorylated and activated by the upstream Src

kinases. Recruitment of Syk/ZAP70 to antigen receptors is a key event that triggers a plethora of downstream signalling pathways, including adaptor protein recruitment, second messenger production, activation of GTP-binding proteins and stimulation of other protein kinases, which co-ordinately regulate the complex biological responses required of lymphocytes for successful immune responses.

1.3.2. Signalling pathways initiated by antigen receptors.

Central to antigen receptor signalling is the recruitment of specific adaptor proteins that couple protein tyrosine kinases to downstream signalling cascades involving PI3-Kinase, PLCγ and the small GTPases Ras, Rho and Rac, as summarised in Figure 1.7. In T lymphocytes and mast cells, two major adaptor molecules critical for this function are LAT (a recently identified integral plasma membrane protein, (Zhang *et al.*, 1998a)) and SLP-76 (SH_2-domain containing leukocyte protein of 76 kDa, (Clements *et al.*, 1998; Yablonski *et al.*, 1998)), whilst in B lymphocytes one major adaptor protein, BLNK (B cell linker protein, (Fu *et al.*, 1998; Weinands *et al.*, 1998)) is used to couple to multiple downstream signalling cascades.

PI3-Kinase signalling

Rapid induction of PI3-Kinase activity has been detected in response to engagement of TCR, BCR and FcεR1 antigen receptors (Barker *et al.*, 1995; Gold and Aebersold, 1994; Ward *et al.*, 1996; Yano *et al.*, 1993). Models for the activation of PI3-Kinase by antigen receptors invoke binding of the regulatory subunit of PI3-Kinase to phosphorylated adapters which recruit the catalytic domain to the plasma membrane. One mechanism by which PI3-Kinase appears to be regulated is through binding of the SH_2 domain of the p85α subunit of PI3-Kinase to phosphorylated costimulatory molecules such as CD28 (in T cells, (Ward *et al.*, 1993)) and CD19 (in B cells, (Tuveson *et al.*, 1993)). Alternatively, a proline-rich sequence within p85α may bind directly to the SH_3 domains of Src family kinases (Pleiman *et al.*, 1994).

Genetic evidence for the importance of PI3-Kinase for B lymphocytes has been illustrated by the phenotype of mice lacking the p85α regulatory subunit which show profound defects in B cell function (Fruman *et al.*, 1999; Suzuki *et al.*, 1999). In contrast, p85α-deficient T lymphocytes appear normal, suggesting that an distinct PI3-Kinase isoform is predominant in T cells. Recent studies have defined several targets for PI3-Kinase in antigen receptor-activated cells, including the Tec family tyrosine kinases Btk

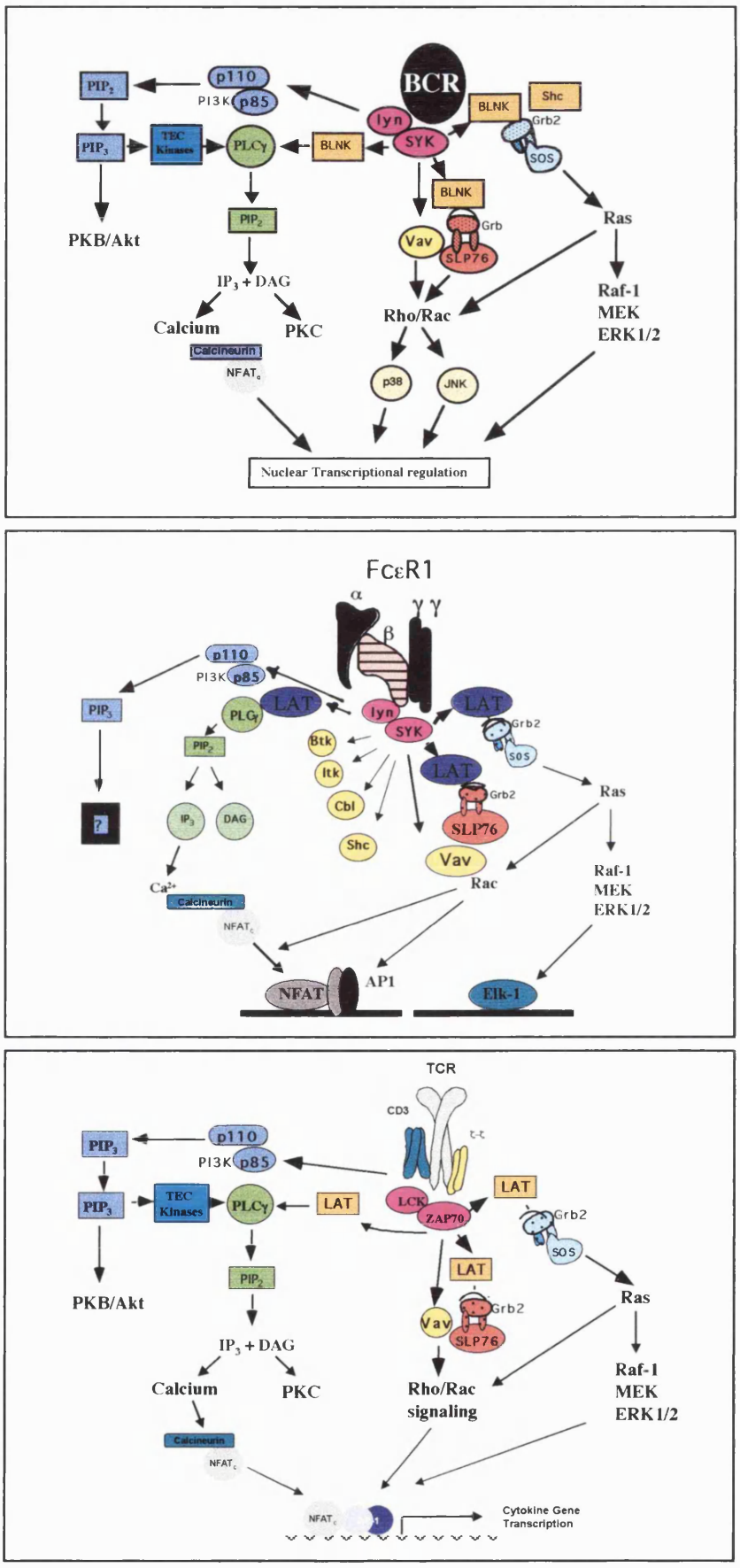


Fig. 1.7. Overview of early antigen receptor signalling.

and Itk (August *et al.*, 1997; Bolland *et al.*, 1998; Li *et al.*, 1997b; Scharenberg and Kinet, 1998b) and a serine/threonine kinase PKB/Akt (Reif *et al.*, 1997). These kinases contain PH domains that bind the 3'phosphoinositide products of PI3-Kinase and recruits these kinases to the plasma membrane for subsequent activation (Astoul *et al.*, 1999; Leevers *et al.*, 1999). Tec kinases play a critical role in the regulation of PLCγ activation, as detailed below. The other PI3-Kinase target, PKB/Akt has several potential functions in antigen receptor signalling: it has been shown to play an important role in the maintenance of cell survival in fibroblasts and epithelial cells, reviewed in (Alessi and Cohen, 1998; Coffer *et al.*, 1998; Hemmings, 1997). PKB/Akt phosphorylates and inactivates glycogen synthase kinase, an enzyme initially identified as a regulator of glycogen metabolism but which also regulates the nuclear export of the nuclear factor of activated T cells (NFAT), a family of transcription factors involved in cytokine gene induction (Beals *et al.*, 1997). In T lymphocytes, PKB/Akt also regulates the activity of E2F transcription factors, which are important components of the mammalian cell cycle machinery (Brennan *et al.*, 1997).

Rho/Rac signalling.

A third class of PH domain-containing proteins regulated by the products of the PI3-Kinase are guanine nucleotide exchange factors (GEFs) for Rho GTPases such as Rac, Rho and CDC42 (Cerione and Zheng, 1996). The best characterised Rho family GEF in lymphocytes is Vav-1. Binding of $PI(3,4,5)P_3$ to the Vav-1 PH domain, co-operates with interactions between the Vav-SH_2 domain and phosphotyrosine motifs in the adaptor protein SLP-76 or the costimulatory receptor CD19 (in T and B cells respectively) to recruit Vav to the plasma membrane (Han *et al.*, 1998; O'Rourke *et al.*, 1998; Tusto *et al.*, 1996). Once at the membrane, Src-family kinases phosphorylate and activate Vav (Crespo *et al.*, 1997; Deckert *et al.*, 1996; Han *et al.*, 1997; Margolis *et al.*, 1992).

Rho-GTPases regulate lymphocyte survival, proliferation and differentiation and also cytoskeletal reorganisations through interactions with multiple effectors that include phosphatidylinositol-4-phosphate-5-kinase (PIP5-Kinase); p21 activated kinase (PAK) and two members of the mitogen-activated protein kinase family, JNK and p38 (reviewed in (Henning and Cantrell, 1998)). For example, Vav-1 deficient T cells exhibit defects in actin polymerisation (Fischer *et al.*, 1998; Holsinger *et al.*, 1998). Additionally, Wiscott-Aldrich immunodeficiency syndrome is characterised by cytoskeletal and cell activation abnormalities in lymphocytes and the defect maps to an effector protein for Cdc42, termed WASP, which has been implicated in the control of actin depolymerisation (Snapper and

Rosen, 1999). Moreover, Cdc42 controls T cell polarisation toward antigen presenting cells (APC), a process that is essential for efficient T cell-APC contact and the directed release of regulatory cytokines (Stowers *et al.*, 1995).

Experiments with active and inhibitory mutants of Rac and Rho have also revealed critical roles for these GTPases in mediating signals from antigen receptors to nuclear transcription events through regulating AP-1 and NFAT transcription factors (Genot *et al.*, 1996; Turner and Cantrell, 1997; Turner *et al.*, 1998)

Ras signalling

The guanine nucleotide binding protein $p21^{Ras}$ rapidly accumulates in its active, GTP-bound form in antigen receptor activated cells and Ras function is essential for cytokine gene induction. The guanine nucleotide binding cycle of Ras is controlled by Sos (the mammalian homologue of the *Drosophila* 'Son of Sevenless' protein) which promotes the formation of active GTP-bound Ras complexes. GTPase activating proteins (GAPs), which stimulate the intrinsic GTPase activity of Ras, subsequently turns off Ras signalling through hydrolysis of bound GTP to GDP. Following antigen receptor engagement, the SH_2 domain of the Grb2 adaptor molecule interacts with tyrosine-phosphorylated residues in the cytoplasmic tail of the LAT/BLNK thereby forming protein complexes that regulate the membrane localisation and catalytic activity of Sos and thus Ras. Activated Ras couples to multiple biochemical effector signalling pathways, including the Raf-1/MEK/ERK1/2 kinase cascade and signalling pathways controlled by the Rac/Rho GTPases, that activate transcription factors involved in cytokine gene induction (DeFranco, 1997; Kurosaki, 1999; van Leeuwen and Samelson, 1999).

PLCγ signalling.

Antigen receptor coupling to PLCγ induces hydrolysis of $PI(4,5)P_2$ and hence controls the production of IP_3 and DAG, as discussed in section 1.1.1. Initial models for TCR regulation of PLCγ were simple and proposed that tyrosine phosphorylation of the enzyme resulted in its activation. Genetic studies have now indicated that antigen receptor activation of PLCγ requires at least three classes of protein tyrosine kinases; Src family kinases, ZAP70/Syk and Tec kinases such as Rlk and Itk in T cells or Btk in B cells and mast cells (Campbell, 1999; Kinet, 1999; Kurosaki, 1999). Current models propose that the adaptor proteins LAT and BLNK are selectively tyrosine phosphorylated by upstream Syk/ZAP70 kinases in response to triggering of antigen receptors and interact with the

SH_2 domain of PLCγ, thereby recruiting it to the plasma membrane for activation by Syk/ZAP70- and Tec-family kinases. At the same time the $PI(3,4,5)P_3$ product of PI3-Kinase binds to the PH domain of Btk and Itk kinases, bringing them to the plasma membrane, where autophosphorylation events and transphosphorylation by Src kinases results in full Tec kinase activity (August *et al.*, 1997; Li *et al.*, 1997b; Scharenberg *et al.*, 1998a). In T lymphocytes one Tec family member, Rlk, lacks a PH domain and is instead recruited to the membrane via palmitoylation of a cysteine-string motif (Debnath *et al.*, 1999). Once localised to the plasma membrane, PLCγ is tyrosine phosphorylated and activated by Tec family kinases and by ZAP70/Syk.

Analysis of a SLP-76 deficient Jurkat T cell line has also demonstrated an unequivocal role for the adaptor protein SLP76 in the regulation of PLCγ, but quite why is not yet established (Yablonski *et al.*, 1998). SLP76 could potentially co-ordinate Tec/PLCγ interactions. Alternatively, SLP76 has been shown to regulate the formation of Vav/Rac complexes in antigen receptor-activated T cells (Bubeck-Wardenburg *et al.*, 1998). Recent evidence indicates that Vav induces Rac-dependent activation of phosphatidylinositol 4-phosphate 5-kinase (PIP5-Kinase) in B lymphocytes (O'Rourke *et al.*, 1998). PIP5-Kinase functions to replenish plasma membrane $PI(4,5)P_2$ pools through phosphorylation of PI4P. If a similar Vav-Rac-PIP5-Kinase pathway occurs in T cells, then defects in $PI(4,5)P_2$ biosynthesis could explain the PLCγ signalling defects observed in SLP76-negative T cells. In support of this model, Vav-deficient T lymphocytes also show reduced calcium mobilisation following TCR-activation (Fischer *et al.*, 1998; Holsinger *et al.*, 1998)

The potency of calcium and PKC signalling pathways for lymphocyte activation is underlined by the ability of pharmacological agents which elevate intracellular calcium levels and activate PKC (calcium ionophores and phorbol esters respectively) to mimic many aspects of antigen receptor triggering, including activation of transcription factors, T and B cell proliferation and differentiation, positive selection of thymocytes, protection from Fas-mediated apoptosis in B cells and secretion of inflammatory mediators in mast cells (Buccione *et al.*, 1994; Crabtree and Clipstone, 1994; DeFranco, 1997; Ozawa *et al.*, 1993a; Rao, 1994; Su *et al.*, 1994; Takahama and Nakauchi, 1996).

Elevation of intracellular calcium levels is critical during the initial phases of antigen receptor activation both for induction of cytokine gene expression in B & T lymphocytes and mast cells and also for controlling cytolytic functions of T cells (Crabtree, 1999; Golstein and Smith, 1977; Rao *et al.*, 1997). Calcium elevation leads to the activation of

both calmodulin-dependent protein kinase II and calmodulin-activated serine/threonine phosphatase calcineurin. One important role for calcium is in the regulation of NFAT (nuclear factor of activated T cells) proteins. NFAT dephosphorylation is mediated by calcineurin, which results in the nuclear migration of NFAT and induction of specific gene transcription events (Choi *et al.*, 1994; Crabtree, 1999; Lee and Oliver, 1995; Millard *et al.*, 1988; Venkataraman *et al.*, 1994).

Receptor-induced calcium elevations often occur as oscillating spikes of varying duration, interval and amplitude (Berridge, 1993). Strikingly, variations in these calcium waves have a dramatic impact upon calcium-regulated signalling pathways in lymphocytes and mast cells. Thus, a large transient increase in intracellular calcium concentration activates JNK and the transcriptional activity of NFκB, whereas nuclear translocation of the NFAT transcription factor requires sustained, low-amplitude calcium signalling (Dolmetsch *et al.*, 1997). Moreover, caged-IP_3 derivatives and calcium clamping techniques have been used to show that the interval length between calcium oscillations also influences transcription factor activation (Dolmetsch *et al.*, 1998; Li *et al.*, 1998).

Classical and novel PKC enzymes have been implicated in a wide range of antigen-receptor mediated responses through the use of pharmacological activators and inhibitors of PKC or from studies using constitutively active/dominant-negative PKC mutants. These responses include regulation of transcription factor activity and cytokine gene induction, in particular NFAT, NFκB and AP-1 (Baier-Bitterlich *et al.*, 1996; Genot *et al.*, 1995; Ghaffari-Tabrizi *et al.*, 1999; Huo and Rothstein, 1995; Razin *et al.*, 1994; Trushin *et al.*, 1999; Werlen *et al.*, 1998; Xie and Rothstein, 1995). PKC enzymes have also been implicated in the regulation of lymphocyte apoptosis/survival (Cuvillier *et al.*, 1996; Morrow *et al.*, 1999; Toth *et al.*, 1999; Yahata *et al.*, 1999); and in the modulation of cell surface molecules, such as CD4, CD3 & CD69 in T cells (Parolini *et al.*, 1999; Pelchen-Matthews *et al.*, 1993; Swat *et al.*, 1993) and the FcεR1γ chain in mast cells (Germano *et al.*, 1994).

FcεR1-induced secretory responses in mast cells appear to be regulated through PKC since phorbol esters (in combination with calcium ionophore) stimulate mast cell degranulation and PKC inhibitors attenuate FcεR1-induced degranulation (Buccione *et al.*, 1994; Chang *et al.*, 1997; Kimata *et al.*, 1999). Moreover, addition of exogenous PKCα or δ restores FcεR1-induced secretory responses in permeablised mast cells (Ozawa *et al.*, 1993a) and overexpression of $PKC\beta_I$ or η (but not α, δ or ε) in RBL 3H3 mast cells significantly enhances degranulation upon FcεR1-crosslinking (Chang *et al.*, 1997). Brown *et al* have demonstrated that a GFP-tagged PLD1 enzyme translocates from

intracellular secretory granules to the plasma membrane of FcεR1-activated mast cells and that inhibition of PLD1 activity prevents FcεR1-mediated secretory responses (Brown *et al.*, 1998). Since PKC enzymes have been implicated in the regulation of PLD activity (section 1.2.9) a PKC-PLD pathway may control antigen receptor-regulated exocytosis in mast cells.

PKC enzymes are intimately involved in T cell activation and proliferation events through regulating the expression of the cytokine interleukin-2 (IL-2) and its receptor (Wilkinson and Nixon, 1998). IL-2 gene induction requires the co-ordinate action of multiple transcription factors, including NFAT, AP-1, NFκB and Oct-1. Expression of constitutively active PKCε and PKCα mutants in T cells stimulates the transcription factors AP-1 and (in synergy with calcium signals) NFAT. Activated PKCε is also able to induce NFκB activity (Genot *et al.*, 1995). However, PKC inhibitors do not block TCR stimulation of NFAT or AP-1, although PKC activity is essential for the induction of NFκB in T lymphocytes, which could explain the role of PKC in IL-2 gene expression (Williams *et al.*, 1995).

One PKC isoform that may be particularly important for TCR signalling cascades is PKCθ. By using constitutively active mutants of PKC enzymes it has been shown that PKCθ (but not PKCα or ε) induces AP-1 transcriptional activity in a murine thymoma cell line (Baier-Bitterlich *et al.*, 1996). Similarly, PKCθ preferentially synergizes with calcineurin to activate JNK and induce the transcription of reporter genes driven by c-*jun* and IL-2 promoters (Ghaffari-Tabrizi *et al.*, 1999; Werlen *et al.*, 1998). Moreover, PKCθ (but not other PKC isoforms) is specifically recruited to the plasma membrane at the contact zone formed between T cells and antigen presenting cells and is activated during immune stimulation (Monks *et al.*, 1998; Monks *et al.*, 1997). TCR activation by crosslinking antibodies does not induce clustering of PKCθ within this zone however, suggesting that the translocation of PKCθ requires costimulatory signals from antigen presenting cells in addition to TCR signals.

The PKC$\beta_{I/II}$ knockout mouse exhibits B cell immunodeficiency, demonstrating a critical role for this classical PKC enzyme in B cell function (Leitges *et al.*, 1996). B cell development is normal in PKC$\beta_{I/II}^{-/-}$ mice but peripheral spleen and peritoneal cavity B cell numbers are reduced due to a severe decrease in the numbers and frequency of a specific B cell subset, the B-1 B ($D23^{-}IgM^{+}$) cells. Peripheral B cells from PKC$\beta_{I/II}^{-/-}$ mice proliferate poorly *in vitro* in response to BCR crosslinking antibodies. In addition, PKC$\beta_{I/II}^{-/-}$ mice are unable to mount humoral responses to T cell-independent antigens *in vivo* and have reduced serum IgM and IgG3 antibody levels. In contrast, the T cell compartment

within $PKC\beta_{I/II}^{-/-}$ mice is normal. Thus, PKCβ is critical for B cell activation and cellular immune responses but is dispensable for T cell functions, indicating that the TCR and the BCR may use distinct PKC enzymes to mediate signalling events that control effector responses. The phenotype of the $PKC\beta_{I/II}^{-/-}$ mice is similar to the B cell defects observed in XID mice (which contain a point mutation within the Btk PH domain, (Kerner *et al.*, 1995; Khan *et al.*, 1995; Rawlings *et al.*, 1993; Thomas *et al.*, 1993) and also in mice lacking the p85α regulatory subunit of PI3-Kinase (Fruman *et al.*, 1999; Suzuki *et al.*, 1999). This is perhaps not suprising given the linear PI3-Kinase→Btk→PLCγ signalling cascade in B cells described above.

Genetic and biochemical evidence has also suggested a role for PKC enzymes in the regulation of mitogen-activated protein kinase cascades in B lymphocytes. For example, although Ras is critical for ERK activation in antigen receptor signalling cascades, stimulation of B cells with phorbol esters can also promote ERK activation and downregulation of phorbol ester-responsive PKC enzymes eliminates BCR-mediated activation of ERK by the BCR (Jiang *et al.*, 1998). Furthermore, BCR-mediated activation of ERK is significantly inhibited in PLCγ-deficient DT40 B cells but is normal when only calcium mobilisation is disrupted in IP_3-receptor-deficient B cells (Hashimoto *et al.*, 1998). Similarly, BCR-induced activation of two other mitogen-activated protein kinase family members, p38 and JNK also requires PLCγ signalling events (Hashimoto *et al.*, 1998), although p38 and JNK are also targets for the small GTPase Rac.

However, as in many other cellular systems, few immediate targets for PKC in lymphocytes and mast cells have been identified and the contribution of individual PKC enzymes to particular antigen receptor signalling pathways remains mostly unknown.

1.4. ADDITIONAL C1 DOMAIN-CONTAINING PROTEINS.

For many years the biological actions of DAG (and phorbol esters) were thought to be mediated solely through classical or novel PKCs. However, the identification of regulatory C1 domains within proteins distinct from the PKC family has increased the complexity of these phorbol ester/DAG-induced responses. In some cases binding to phorbol ester and/or DAG has been demonstrated for these proteins with a concomitant activation of protein function (summarised in Table 1.1).

Protein	N° of copies	Binding	Comments
DAG kinases	2	No	C1 not required for DAG binding or catalysis
chimaerins	1	Yes	Phorbol ester stimulates GAP activity against Rac
Munc-13	1	Yes	Homologue of *Caenorhabditis elegans* Unc-13. C1 regulates neurotransmitter release
Raf	1	No	C1 binds to Ras prenyl group stabilising Ras:Raf interactions
Ksr	1	n.d.	kinase suppressor of Ras
RasGRP	1	Yes	C1 mediates plasma membrane localisation and cell transformation

Table 1.1. Mammalian C1 domain-containing proteins.
Summary of mammalian proteins (distinct from PKC) containing consensus C1 domains identified to date. n.d., not determined. Adapted from (Hurley *et al.*, 1997; Ron and Kazanietz, 1999a).

The Munc-13 proteins are enriched in synaptosomes and contains a C1 domain that bind DAG and phorbol esters with high affinity (Betz *et al.*, 1998). Phorbol ester binding induces translocation of Munc13 to the plasma membrane of presynaptic vesicles where it regulates neurotransmitter release in a manner independent of that induced by PKC (Betz *et al.*, 1998; Duncan *et al.*, 1999). Phorbol ester-induced interactions between Munc13 and Doc2 (a presynaptic protein forming part of the exocytotic machinery) are thought to be involved in this process (Duncan *et al.*, 1999).

In addition a number of regulators of small GTPases contain C1 domains and are regulated by DAG and phorbol esters. For example, the chimaerin proteins contain a C-terminal GTPase-activating domain that regulates Rac, but not Cdc42 or Rho, (Diekmann *et al.*, 1991; Hall *et al.*, 1993; Kozma *et al.*, 1996). The chimaerins also contain a single copy of a functional C1 domain that bind phorbol esters with high affinity (Ahmed *et al.*, 1990; Areces *et al.*, 1994; Caloca *et al.*, 1997) indicating that a link between DAG and Rac signalling events may exist *in vivo*. Indeed, it has been shown that the GAP activity of α1-chimaerin towards Rac is enhanced by phorbol esters, at least *in vitro* (Ahmed *et al.*, 1993).

More recently a search for proteins that enhance Ras signalling led to the identification of a novel member of the Ras GEF family, RasGRP, which is highly expressed within the hematopoietic system and in neuronal tissues (O' Ebinu *et al.*, 1998). RasGRP and a homologous Rap1-specific GEF (CalDAG-GEF1) contain functional C1 domains indicating that these exchange factors may constitute a novel link between DAG and GTPase-regulated signalling pathways *in vivo* (Kawasaki *et al.*, 1998; O' Ebinu *et al.*, 1998; Tognon *et al.*, 1998). Serum-induced DAG or exogenous phorbol ester bind to the

C1 domain of these proteins, inducing their plasma membrane translocation and augmenting their GEF activity towards Ras or Rap1, presumably through regulating contact with these substrates. Indeed, the C1 domain of RasGRP is essential for the transforming activity of this protein and for phorbol ester-induced activation of a Ras/MAPK signalling cascade in RasGRP-expressing Rat2 fibroblasts (Tognon *et al.*, 1998).

Almost invariably, the biological effects of DAG and phorbol esters *in vivo* are attributed to PKC enzymes. However the identification of additional 'non-PKC' DAG/phorbol ester-responsive proteins highlights the need for caution in interpreting these studies.

1.5. PROTEIN KINASE D.

In 1994, the Growth Regulation Laboratory had cloned a novel C1 domain-containing kinase, from a murine Swiss 3T3 cDNA library, that had been named Protein Kinase D (PKD), (Valverde *et al.*, 1994). At the same time the human homologue of PKD was also identified and named Protein Kinase C mu (PKCμ), (Johannes *et al.*, 1994). PKD (916 amino acids) and PKCμ (912 amino acids) have predicted molecular masses of 102 kDa but migrate on SDS-PAGE at ~110 kDa indicating a degree of post-translational modification. PKD/PKCμ is ubiquitously expressed in a wide variety of tissues and cells but particularly high levels are seen in lung, heart, thymus and peripheral blood mononuclear cells, as detected by measurement of mRNA levels or western blot analysis for protein expression (Johannes *et al.*, 1994; Rennecke *et al.*, 1996; Valverde *et al.*, 1994). For simplicity PKD/PKCμ will be referred to as PKD throughout this thesis, unless specifically indicated.

1.5.1. Domain structure of PKD.

PKD is composed of an N-terminal regulatory region and a C-terminal catalytic domain as shown in Figure 1.8. The N-terminus of PKD contains a tandem repeat cysteine-rich zinc finger binding motif, between residues 145-326, that displays homology to the DAG/phorbol ester binding C1 domain of classical and novel PKCs (Johannes *et al.*, 1994; Valverde *et al.*, 1994). Indeed the two C1 domains of PKD (C1A and C1B) show 86% and 81% identity to the described C1 domain consensus sequence, as highlighted in Table 1.2.

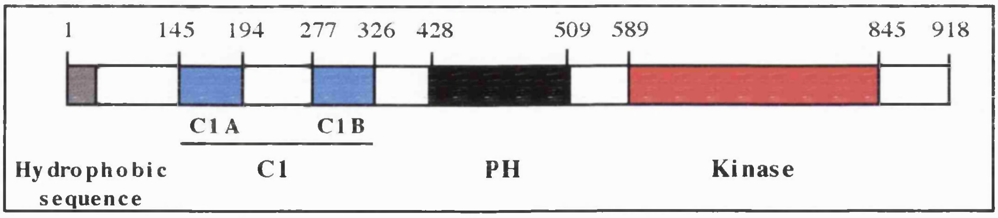


Fig. 1.8. Domain structure of Protein kinase D.
The N-terminal regulatory region of PKD is characterised by a hydrophobic sequence, two DAG/phorbol ester binding C1 domains and a PH domain. The C-terminal region of PKD contains a consensus serine/threonine kinase domain.

C1A	H A L F V H S Y R A P A F C D H C G E ML W G L V R Q G L K C E G C G L N Y H K R C A F KI P N N C
C1B	H T F VI H S Y T R P T V C Q F C K K L L [K] G L F R Q G L Q C K D C R F N C H K R C A P K V P N N C
	* : : * * * : : * : : * * * * : : * : * : *
Consensus	H h h P C C h h h G h b Q G C C h h H b C h C

Table 1.2. Alignment of the PKD C1A and C1B motifs with the C1 domain consensus sequence. Key: uppercase letter are amino-acid residues; h=large hydrophobic; a=aromatic; b=basic; *=identity; :=conserved homology. Consensus sequence taken from (Hurley *et al.*, 1997).The C1A motif is a perfect fit. The C1B motif shows one major violation form the consensus, with a lysine at position 22 (boxed) which could potentially disrupt the structure of the DAG-binding site. However, as discussed in the text PKD binds DAG/phorbol esters with high affinity and in fact the C1B motif acts as the major binding site for PKD.

Consistent with this, a bacterially expressed fusion protein of the isolated PKD C1 domain is able to bind phorbol esters and DAG-analogues *in vitro*, (Valverde *et al.*, 1994). Moreover intact PKD (either purified or transiently expressed in COS-7 cells) also exhibits high affinity phorbol ester binding, K_d=2-10 nM, that is competed by analogues of DAG (Dieterich *et al.*, 1996; Valverde *et al.*, 1994; Van Lint *et al.*, 1995).

As discussed above, the individual cysteine-rich motifs of classical and novels PKCs have non-equivalent roles with respect to DAG/phorbol ester binding. *In vitro* analysis of the binding of [^{3}H]PDBu to a GST fusion protein containing the PKD C1 domain has revealed two distinct classes of binding site of low (K_d=50 nM) and high (K_d=3 nM) affinity respectively, within the PKD C1 domain (Iglesias *et al.*, 1998a), suggesting that the C1A and C1B motifs of PKD are functionally non-equivalent. Indeed, deletion or mutation the PKD C1 domain confirms that the C1B motif is a high affinity binding site which mediates the majority of DAG/phorbol ester binding to PKD both *in vitro*, and *in vivo* (Iglesias *et al.*, 1998a).

In addition to a C1 domain, the N-terminal region of PKD contains a highly hydrophobic amino-acid sequence and, unlike PKCs (including mammalian, *Drosophila* and yeast isoforms), a PH domain. In contrast to the PKC family, PKD does not possess a

typical pseudosubstrate motif upstream of the C1 domain nor a calcium-binding C2 domain (Johannes *et al.*, 1994; Valverde *et al.*, 1994). The extreme N-terminus of PKD contains a hydrophobic sequence that was initially predicted to form a transmembrane domain (Johannes *et al.*, 1994; Valverde *et al.*, 1994). However PKD protein is found associated with both soluble and particulate fractions of cells (Johannes *et al.*, 1995; Nishikawa *et al.*, 1998; Valverde *et al.*, 1994; Van Lint *et al.*, 1995) and deletion of this N-terminal hydrophobic sequence does not alter the subcellular distribution of PKD compared to wild-type PKD, either in resting or in stimulated cells (J. Sinnett-Smith, E. Rozengurt, unpublished observations). Moreover experiments carried out by the Malhotra laboratory have since confirmed that PKD is not a transmembrane protein as PKD can be dissociated from cellular membranes upon urea washing (Jamora *et al.*, 1999).

The catalytic domain of PKD, residues 589-845, contains all 11 distinct subdomains characteristic of protein kinases (Hanks and Hunter, 1995), including an ATP binding motif in subdomain I at residues 596-691 (GSGQFG); a lysine at residue 618 in subdomain II and DFG and APE motifs in subdomains VII and VIII, respectively that are highly conserved in protein kinases. A comparison of the amino-acid sequence of the PKD catalytic domain indicates that PKD is a distinct protein kinase distantly related to calcium-regulated kinases. The rank order of homology within the catalytic domain is: Myosin light chain kinase (*Dictyostelium*, 41% identity)>Ca^{2+}/calmodulin-dependent kinase (CaM kinase) type II> CaM kinase type IV> cAMP-dependent protein kinase>phosphorylase B. In particular the catalytic domain of PKD displays only very low similarity to the highly conserved catalytic subdomains of the PKC family. For example, the motif XXDLKXXN/D in subdomain VI, which is important for guiding substrates into the correct orientation for catalysis in all protein kinases (Hanks and Hunter, 1995) is YRDLKLDN in all PKCs, which differs from that of PKD (HCDLKPEN) in every variable residue. Furthermore, the activation loop of PKD does not contain a consensus PDK1 phosphorylation motif suggesting that unlike other serine kinases, including classical, novel or atypical PKCs, PKB/Akt or $p70^{S6}$kinase, PKD is not regulated by this kinase.

The distant connection between PKD and the PKC family of kinases is further emphasised when the relatedness of the protein sequences of these kinases are compared, as displayed in Figure 1.9. Furthermore, PKD is only very distantly related to the serine kinases PKB/Akt; $p70^{S6K}$ or the archetypal cAMP-dependent protein kinase. Instead PKD is more closely related to calcium-regulated kinases though homology to their catalytic domains.

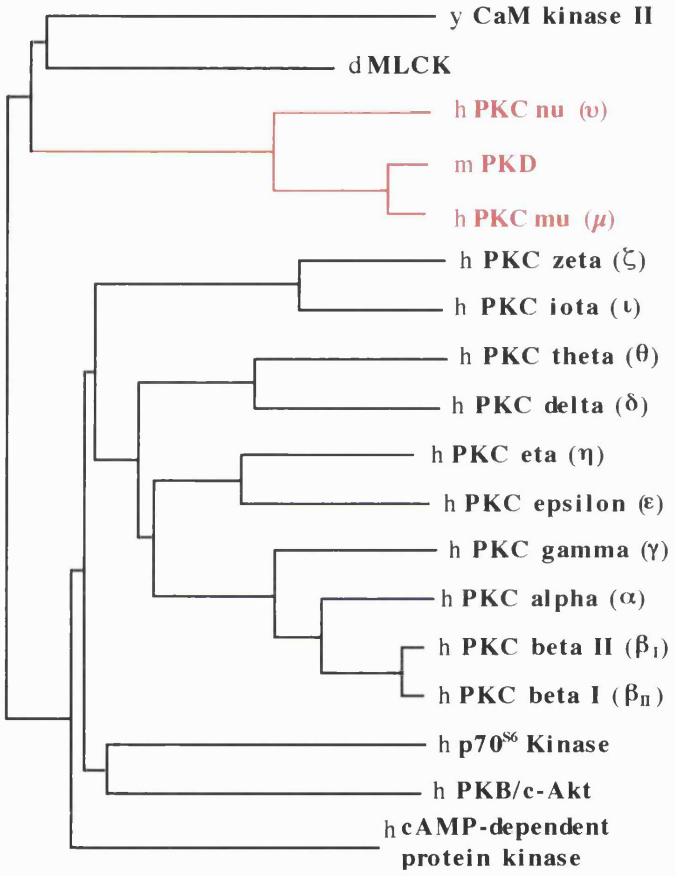


Figure 1.9. Relationship of PKD to other serine/threonine kinases.

PKD (and its human homologue PKCμ) are more closely related to the calcium-regulated kinases CaM Kinase II and myosin light chain kinase (MLCK) than to members of the PKC superfamily or to the serine kinases $p70^{S6}$Kinase, PKB/c-Akt or to the cAMP-dependent protein kinase. The newly described putative serine kinase PKCυ is 70% identical to both murine and human homologues of PKD (extending to 95% in the catalytic domain). A small truncation in the C-terminus of PKCυ, as well as the absence of an N-terminal hydrophobic sequence, distinguishes this kinase from PKD. h=human, m=mouse, y=*S. cerevisiae*, d=*Dictyostelium*. Sequences were aligned using ClustalX software and a distance matrix was prepared from the multiple sequence alignments. The branch lengths are proportional to the estimated divergence along each branch.

Thus PKD cannot be classified as a typical member of the PKC superfamily. However PKCμ, the human homologue of PKD, and a recently identified related kinase

PKCυ (Hayashi *et al.*, 1999), are referred to as PKC enzymes in the literature, presumably because, as discussed above these kinases contain a DAG-binding C1 domain. It would therefore be more correct to refer to PKD, and its related kinases, as a separate protein kinase family.

1.5.2. Regulation of PKD catalytic activity by DAG-analogues and phorbol esters.

The presence of a functional C1 domain within the N-terminal regulatory region of PKD confers enzymatic responsiveness to DAG and phorbol esters, in common with classical and novel PKC enzymes. Thus, DAG-analogues or phorbol esters in combination with phosphatidly-L-serine synergistically stimulate the catalytic activity of purified PKD in *in vitro* peptide phosphorylation assays and in autophosphorylation assays (Johannes *et al.*, 1994; Van Lint *et al.*, 1995). As expected, given the absence of a C2 domain, activation of PKD *in vitro* is independent of calcium.

Although regulated by DAG, the structural differences between the catalytic domains of PKD and classical and novel PKCs highlighted above indicates that these kinases may have distinct functions. This is further confirmed by the finding that activated PKD does not phosphorylate (or only poorly phosphorylates) a number of synthetic peptide substrates utilised by PKC enzymes, including myelin basic protein, histone, MARCKs and a peptide based on the PKCε pseudosubstrate sequence (Dieterich *et al.*, 1996; Valverde *et al.*, 1994; Van Lint *et al.*, 1995). PKD does however phosphorylate Syntide-2 (Dieterich *et al.*, 1996; Valverde *et al.*, 1994; Van Lint *et al.*, 1995), a peptide derived from phosphorylation site-2 of glycogen synthase, which is also a substrate for calmodulin-dependent protein kinases (Lorca and al., 1993; Mochizuki *et al.*, 1993). Analysis of peptides phosphorylated by DAG/phorbol ester-stimulated PKD demonstrated that phosphorylation occurs exclusively on serine residues (Dieterich *et al.*, 1996; Valverde *et al.*, 1994), indicating that PKD is a serine kinase.

1.6. AIMS OF THE THESIS.

This introductory chapter has summarised the regulation of classical and novel PKC enzymes by DAG and tumour promoting phorbol esters and the critical role played by these kinases in mediating cellular responses to these signals. Moreover, the concept that additional cellular proteins are also regulated by DAG and phorbol esters has been introduced. At the start of this thesis, the *in vitro* characterisation of Protein Kinase D as a novel diacylglycerol and phorbol ester-regulated serine kinase had been established. This combined with the distinctive structural features of PKD raised the possibility that some of

the biological actions of DAG *in vivo* could be mediated by Protein Kinase D. The aim of this thesis was to investigate the upstream regulation of Protein Kinase D in intact cells:

(1) To examine the mechanism by which the pharmacological agent bryostatin 1 regulates Protein Kinase D activity.

(2) To probe the molecular basis of Protein Kinase D activation and subcellular localisation under physiological conditions using antigen receptor signalling cascades in lymphocytes and mast cells as a model system.

CHAPTER 2: Materials and Methods

2.1. Reagents.

ECL reagents, ^{125}I-Protein A (15 mCi/ml), [^{3}H]PDBu (0.25 mCi/ml), [^{32}P]orthophosphate (10 mCi/ml) and [γ^{32}P]ATP (370 MBq/ml) were from Amersham International (UK). Protein A-agarose was from Boehringer Mannheim and Protein G sepharose was supplied by the ICRF Research Monoclonal Antibody Service. The PKC-inhibitors GF 109203X and Ro 31-8220 were from LC Laboratories. The MEK-1 inhibitor PD 098059 was from New England Biolabs. LY 294002 was from Zeneca. Cytochalasin D, wortmannin, H89, Keyhole Limpet Hemocyanin, Keyhole Limpet Hemocyanin conjugated to di-nitro phenol (KLH-DNP) and non-radioactive ATP were from Calbiochem-Novabiochem. Phytohaemagglutinin was from Wellcome Diagnostics. Recombinant human IL-2 was from Eurocetus. Ficoll-Hypaque (Lymphoprep) was from Nycomed Pharmacia. Recombinant mouse Immunoglobulin E raised against di-nitro phenol (IgE anti-DNP), rapamycin, microcystin, phosphatidyl-L-serine, PDBu and TPA were from Sigma Aldrich Company (United Kingdom). Purified PKC from rat brain (containing α, β and γ isoforms) was from TCS Biologicals, UK. Fetal calf serum and pre-stained molecular weight markers were from Gibco (UK). Protogel acrylamide solution was from National Diagnostics.

Synthetic oligonucleotides were generated by the ICRF Oligonucleotide Synthesis Unit. L-broth bacterial growth media (0.5% bacto-yeast extract, 1% bacto-tryptone, 1% NaCl, final pH 7.6), Brain Heart Infusion (BHI) media (3.7% BHI powder, final pH 7.6) and PBS [137 mM sodium chloride, 3.3 mM potassium chloride, 1.7 mM potassium dihydrogen orthophosphate (anhydrous), 10 mM disodium hydrogen orthoposphate (anhydrous), final pH 7.4] were from the ICRF Central Cell Services Unit. All other reagents were from standard suppliers or as indicated in the text.

2.2. Antibodies

The following monoclonal antibodies, purified from hybridoma supernatants using Protein A affinity chromatography, were supplied by the ICRF Research Monoclonal Antibody Service: UCHT-1, raised against human CD3ε (Beverley and Callard, 1981); 2C11, raised against murine CD3ε (Leo *et al.*, 1987; Samelson *et al.*, 1987); 9E10, raised against the C-Myc epitope MEQKLISEEDL (Evan *et al.*, 1985) and GFP3E1, raised against

Green Fluorescent Protein (ICRF). A crosslinking monoclonal antibody against CD28, mAb 9.3, generated by Dr. C. June, was obtained from Dr. S. Ward, University of Bath.

The polyclonal antibody (PA 1) raised against the carboxy-terminal 15 residues of murine PKD (EEREMKALSERVSIL) has been described previously (Valverde *et al.*, 1994; Van Lint *et al.*, 1995). A polyclonal antiserum raised against the *cis*-Golgi matrix protein GM130 has been described previously (Nakamura *et al.*, 1995) and was obtained from the Cell Biology Laboratory, ICRF.

Commercial antibodies were as follows: PKD (sc-935), PKCα (sc-208), PKCδ (sc-213), PKCε (sc-214) and PKCζ (sc-216), were from Santa Cruz Biotechnology Inc; phospho-p44/p42 erk1/2 was from Promega, UK; pan-p42 erk2 was from Transduction Labs, UK; phospho-S473 PKB and pan PKB were from New England Biolabs, UK; rabbit anti-mouse F(ab)'2 and Intact IgG were from Zymed.

2.3. Coupling of 9E10 to Protein G sepharose beads.

Protein G sepharose beads (2 ml of a 50:50 slurry, washed x3 in PBS/0.05% azide) were incubated overnight with 2 mg of 9E10 monoclonal antibody at RT. The next day the beads were washed sequentially in PBS/0.05% azide (x3); 100 mM boric acid pH 8.5 (x1) and 200 mM triethanolamine pH 8.4 (x1). The beads were then incubated with 30 ml of freshly prepared 20 mM dimethylpimeliate/HCl (dissolved in 200 mM triethanolamine pH 8.4), for 45 min at RT. The beads were finally incubated for 1 h in 20 mM ethanolamine pH 8 at RT before they were washed x3 in PBS/0.05% azide, diluted 1:5 with non-coupled, washed, Protein G beads and stored as a 50:50 slurry in PBS/0.05% azide at 4°C. The efficiency of the coupling procedure was assessed by SDS-PAGE and commassie blue staining analysis of samples taken from various stages of the protocol. Aliquots (30µl, ~1.5µg of antibody) were used to immunoprecipitate Myc-tagged proteins from whole cell lysates.

2.4. cDNA expression constructs.

All constructs were purified using Quaigen maxiprep kits or by CsCl density gradient centrifugation before use in transfection experiments. Constitutively active PKCη and PKCε mutants (containing pseudosubstrate motif deletions) were generously provided by Peter Parker, Protein Phosphorylation Laboratory, ICRF. A constitutively active PI3-Kinase mutant in which the 110 kDa catalytic subunit of PI3-Kinase is localised to the plasma membrane of cells by fusion to the extracellular and transmembrane regions of

CD2 has been previously described (Reif *et al.*, 1996). The NAGT1-GFP cDNA construct, containing the N-terminal and transmembrane regions of N-acetylglucosaminyltransferase fused to GFP has been described previously (Shima *et al.*, 1997) and was generously provided by David Shima, Cell Biology Laboratory, ICRF.

The following plasmids encoding different PKD constructs were used in this thesis, all derived from a pcDNA3 mammalian expression vector containing wild-type murine PKD cDNA (bases -125 to 3179):

Construct	Description	Reference
PKD	Wild-type	(Van Lint *et al.*, 1995)
PKD K618M	Kinase dead. ATP binding site mutation	(Zugaza *et al.*, 1996)
PKD ΔC1	C1 domain deletion. Constitutively active	(Iglesias *et al.*, 1998a)
PKD P287G	Point mutation within the C1B motif.	(Iglesias *et al.*, 1998a)
PKD ΔPH	Deletion of the entire PH domain. Constitutively active	(Iglesias & Rozengurt, 1998c)
PKD S744/S748E	Activation loop mutant. Constitutively active	(Iglesias *et al.*, 1998b)
PKD D733A	Kinase deficient mutant.	(Iglesias *et al.*, 1998b)

2.5. Maxiprep purification of plasmid DNA.

Plasmid DNA was prepared by a CsCl purification technique. A 400 ml culture of *E. coli* was grown overnight in BHI-medium, containing 50 μg/ml ampicillin. Bacteria were pelleted by centrifugation (3350g , 4°C, 20 min) and resuspended in 40 ml Solution I (50 mM glucose, 25 mM Tris pH 7.4, 10 mM EDTA). Bacteria were lysed by the addition of 80 ml Solution II (1% SDS, 0.2 M NaOH) and denatured proteins, chromosomal DNA and cellular debris were precipitated by adding 40 ml Solution III (5 M KAc pH 5). Precipitates were removed by centrifugation (3350g , 20 min, 4°C) and plasmid DNA in the supernatant was precipitated with 150 ml isopropanol. After centrifugation (3350g , 20 min, 4°C) the DNA pellet was resuspended in 5 ml dH_2O and contaminating RNA was precipitated on ice by adding 2.5 ml of ammonium acetate (7.5 M stock solution). Samples were centrifuged, as above, and plasmid DNA in the supernatant was precipitated with ethanol at -20°C for >1 h. After centrifugation (3350g, 20 min, 4°C) plasmid DNA pellets were resuspended in 8.5 ml dH_2O and ethidium bromide (0.5 ml of 10 mg/ml solution). 8.8g of CsCl was dissolved in this solution and was divided equally between two heat-sealable ultracentrifugation tubes before centrifugation in a vertical rotor (Beckman Vti 65.2) at 385, 000 rpm for 4 h at 20°C. The plasmid DNA was removed using a syringe and the ethidium bromide extracted with water-saturated *n*-butanol. Plasmid DNA was precipitated with 2-

3 volumes of ethanol at -20°C for 1 h, centrifuged (~ 3350g, 20 min, 4°C), resuspended in 0.5 ml dH_2O and the concentration measured by optical density (at 260/280 nm).

2.6. Cell culture.

All cell types were maintained by regular passaging and culturing in a humidified atmosphere containing 5-10% CO_2 at 37°C. Foetal calf serum was routinely heat-inactivated by incubation at 55°C for 1 h. RPMI-1640 and DMEM cell culture media (supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin) were produced by the ICRF Central Cell Services.

Human peripheral blood-derived T lymphoblasts were prepared as follows: T lymphocytes were isolated from peripheral human blood by discontinuous Ficoll-Hypaque gradient sedimentation. 25 ml of blood was overlaid onto 15 ml of Lymphoprep and centrifuged at 800g for 30 min in a swing-out rotor. The leukocyte intermediate layer was isolated and washed x3 in RPMI-1640 medium before culturing in RPMI-1640 medium containing 10% fetal bovine serum, 2 µg/ml phytohaemagglutinin and 2 ng/ml IL-2 for 3-4 days. The resulting T lymphoblasts were subsequently maintained in RPMI-1640 medium containing 10% fetal bovine serum and 2 ng/ml IL-2, splitting 1:2 every 48 h. T lymphoblasts were quiesced by washing three times in RPMI-1640 and culturing in RPMI-1640, supplemented with 10% fetal calf serum, without IL-2 for 48 h before experiments. The human Jurkat T leukaemia cell line JH6.2 (Gillis and Watson, 1981), was maintained in RPMI-1640 medium containing 10% fetal calf serum.

The BALB/c mouse B lymphoma A20 cell line (Kim *et* al, 1979) was maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 50 µM 2β-mercaptoethanol. The rat basophilic leukaemia cell line, RBL 2H3, was obtained from Dr J. Rivera (National Institute of Allergic, Musculoskeletal and Skin Diseases, NIH, Bethesda, MD, USA). RBL 2H3 cells were maintained as monolayer cultures in DMEM medium supplemented with 10% fetal calf serum. Cells were passaged or prepared for experiments by detaching from culture flask substratum using cell scrapers. Simian SV40-transformed COS-7 kidney cells (Gluzman, 1981) were maintained in DMEM containing 10% foetal calf serum. For transfection purposes COS-7 cells were plated (24 h before transfection) in 90 mm dishes, at $6x10^5$ cells/dish in DMEM containing 10% foetal calf serum. Stock cultures of murine Swiss 3T3 fibroblasts (Todaro and Green, 1963) were maintained in DMEM medium supplemented with 10% foetal calf serum. For experimental purposes, cells were plated in 90 mm dishes at $6x10^5$ cells/dish in DMEM containing 10%

foetal bovine serum and used after 6-8 days, when the cells were confluent and quiescent. Secondary cultures of mouse embryonic fibroblasts (ICRF Cell Services Laboratory) were seeded in 90 mm dishes at $6x10^5$ cells/dish in DMEM containing 10% foetal bovine serum. They were switched down to 0.5% foetal bovine serum after 3-4 days and were used after 24 h when the cells were confluent and quiescent.

For long term storage, exponentially growing cells were washed and resuspended in 90% fetal calf serum/10% dimethylsulphoxide at ~$1x10^7$ cells/ml. Cells were then slowly frozen to -70°C over several days before long term storage in liquid nitrogen. Cells were recovered by rapid thawing to 37°C and washed with several changes of pre-warmed medium before culturing.

2.7. Transient transfection of cells.

Transient transfection of cells was carried out using a Beckman Gene-Pulser electroporation apparatus. JH6.2, A20 or RBL 2H3 cells ($1.5 x10^7$/0.5 ml) were pulsed in 0.4 cm cuvettes with 10-20 μg of cDNA plasmid (final concentration) at 310v and 960 μF, before diluting with 5 ml of complete medium. Cells were allowed to recover overnight before stimulation as indicated. For transient transfection of COS-7 cells, 4 x 10^6/0.8 ml cells were electroporated with 5μg of cDNA at 450v and 250 μF, with the cells incubated on ice for 5 min before and following electroporation. COS-7 cells were plated on three 6 cm dishes in complete medium and used for stimulation after 48 h.

Alternatively, in some experiments exponentially growing COS-7 cells, 40-60% confluent, were transfected with cDNA constructs using Lipofectin (Life Technologies), according to the manufacturer's instructions.

2.8. Cell stimulation, lysis and acetone precipitation of total cell proteins.

T and B cells were washed twice before they were resuspended at the required density in RPMI-1640 medium. Antigen receptors were triggered using F(ab)'2 or Intact fragments of anti-mouse IgG (in the case of the BCR) or the crosslinking CD3ε antibody, UCHT-1 (in the case of the TCR) at the indicated concentrations and times.

RBL 2H3 cells were detached from tissue culture flasks and stimulated at the required cell density in suspension (unless other wise indicated). Cells were primed for 1 h with 1 μg/ml IgE anti-DNP in DMEM/10% foetal calf serum before antigenic crosslinking of bound monomeric IgE was performed using 500 ng/ml of KLH-DNP conjugate, as described in (Turner and Cantrell, 1997).

Adherent fibroblasts were rinsed twice with DMEM before stimulation with various agonists, as indicated, for different times.

Following stimulation, all cell types were lysed for 10-20 min at 4°C in a buffer containing 50 mM Tris/HCl, pH 7.4; 2 mM EGTA; 2 mM EDTA; 1 mM dithiothreitol; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 1 mM 4-(2'-aminoethyl)-benzenesulfonyl fluoride hydrochloride and 1% Triton X-100. Lysates were then clarified by centrifugation at 20,800g for 10 min at 4°C. Total cellular proteins were precipitated from the supernatant with ice-cold acetone (in a ratio of 0.5 ml lysate to 0.7 ml acetone) at -20°C for 1 h. Precipitated proteins were pelleted in a microfuge and air-dried before they were resuspended in 2x SDS-PAGE reducing sample buffer (1 M Tris-HCl, pH 6.8, 0.1 mM Na_3VO_4, 6% SDS, 0.5 M EDTA, 4% 2-β mercaptoethanol, 10% glycerol, 0.01% bromophenol blue).

2.9. Cell fractionation Assays

COS-7 cells were washed twice in ice-cold PBS and resuspended in isotonic buffer (25 mM Tris pH 7.4, 250 mM sucrose, 2.5 mM MgAc, 10 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM 4-(2'-aminoethyl)-benzenesulfonyl fluoride hydrochloride). Cells were lysed by disruption using a 1 ml Dounce homogenisor and nuclei/unbroken cells were pelleted by centrifugation at 800g x 1 min. Cytosolic (soluble) and membrane (pellet) fractions were prepared from the resulting supernatant by ultracentrifugation at 100, 000 g for 20 min at 4°C. Equal protein samples from each fraction were extracted for 10 min in 5x SDS-PAGE sample buffer and analysed by SDS-PAGE and western blotting.

For fractionation of A20 B lymphoma cells, the cells were washed twice in ice-cold PBS and resuspended in 1 ml hypotonic buffer (10 mM Tris pH 7.4, 2 mM EGTA, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM 4-(2'-aminoethyl)-benzenesulfonyl fluoride hydrochloride). The cells were subjected to two rapid freeze-thaw cycles (on dry ice). The cell suspension was then homogenised on ice using a Dounce Homogenisor (40 strokes) and intact cells, nuclei and other debris were pelleted by centrifugation at 500 g for 5 min. Soluble and particulate fractions were generated by ultracentrifugation at 100, 000 g for 30 min at 4°C. Proteins in the soluble fraction were precipitated with ice-cold acetone whilst proteins in the pellet were harvested straight into 5x SDS-PAGE reducing sample buffer. Samples were then analysed by SDS-PAGE and western blotting.

2.10. *In vitro* kinase assays

Endogenous PKD was immunoprecipitated from lysates at 4°C for 2 h with the PA-1 antiserum (1:100 dilution of serum) directed against the C-terminal 904-918 residues of PKD, previously described (Valverde *et al.*, 1994; Van Lint *et al.*, 1995), and recovered with Protein-A agarose beads.* Myc-tagged PKD proteins were immunoprecipitated using a 9E10 monoclonal antibody covalently coupled to Protein-G sepharose. Immunocomplexes were washed twice in lysis buffer (described above) and once in kinase buffer (30 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$). PKD autophosphorylation was determined by incubating immunocomplexes with 20 µl of kinase buffer containing 100 µM [γ-^{32}P]-ATP, final concentration at 30°C for 10 min. Reactions were terminated by the addition of 2x SDS-PAGE sample buffer and the samples were analysed by SDS-PAGE and autoradiography.

Exogenous substrate phosphorylation by PKD was measured by the incorporation of [γ-^{32}P]-ATP into synthetic peptides. *In vitro* kinase assays were carried out as described above, but with the addition of substrate peptides (2.5 mg/ml final concentration) to the reaction mix. Reactions were terminated by the addition 100 µl of 75 mM orthophosphoric acid and the supernatant was spotted onto Whatman P-81 phosphocellulose paper. The papers were washed thoroughly in 75 mM orthophosphoric acid, dried and radioactivity incorporated into the peptides was determined by Cerenkov counting. PKD substrate peptides used in this thesis were synthesised by the ICRF Peptide Synthesis Unit: Syntide-2 (PLARTLSVAGLPGKK) a peptide based on phosphorylation site II of glycogen synthase and a peptide based on the C-terminal 15 residues of murine PKD (EEREMKALSERVSIL). K_m values of PKD for peptides were determined by plotting reciprocal values of phosphate incorporation into increasing concentrations of synthetic peptide against reciprocal values of the peptide concentrations (Lineweaver-Burke plot). The intercept with the x-axis of this double reciprocal plot was used to calculate the K_m of PKD for the peptide.

2.11. *In vitro* activation of PKD.

PKD was eluted from (washed) immunocomplexes by competition with 2x volume of kinase buffer, pH 8 containing 0.75 mg/ml (final concentration) of immunising peptide at 4°C for 30 min. The eluted PKD was then mixed with micelles containing different lipids (as indicated in Figure legends) and *in vitro* kinase assays carried out, as described above. Lipid micelles were freshly prepared by drying lipids (dissolved in ethanol) under nitrogen

*The C-terminal epitope of PKD recognised by this antibody is modified by an autophosphorylation event upon cellular stimulation (as discussed in Chapter 4), reducing the affinity of this antibody for PKD in western blotting experiments. However, the amount of PA-1 antibody used for immunoprecipitation studies is sufficient to recover 90-95% of PKD from cell lysates (data not shown).

gas at RT, resuspending in kinase buffer and sonicating on a medium setting for 3 x 30 sec on ice.

2.12. ^{32}Pi Labelling

Swiss 3T3 fibroblasts were washed twice in phosphate-free DMEM and incubated at 37°C with this medium containing 200 μCi/ml of carrier-free orthophosphate (Pi) for 18h. Cells were then stimulated with different agonists as indicated in Figure legends and lysed before PKD was immunoprecipitated using the PA-1 antiserum and analysed by SDS-PAGE and autoradiography.

2.13. SDS-PAGE and Western Blotting.

Proteins were resolved under reducing conditions by 8% SDS-PAGE using Protogel 30% acrylamide, 0.8% bis-acrylamide stock solutions. Stacking gels were 5% acrylamide. The running buffer used contained 25 mM Tris pH 8.3, 190 mM glycine and 3.5 mM SDS. Resolved proteins were transferred to polyvinylidene difluoride membranes (Millipore) by electroblotting in 10 mM CAPS pH 11 buffer (or alternatively in a buffer containing 200 mM glycine, 25 mM Tris, 0.01% SDS and 20% methanol) at 4°C, 75v for 4h. Membranes were blocked using 5% non-fat milk (w/v) in PBS for either 1 h at RT or overnight at 4°C. Western blot analysis of proteins was performed with specific antisera, as indicated below, diluted in PBS/0.5% non-fat milk/0.05% azide. Secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit or donkey anti-mouse IgG, diluted 1:5000 in PBS/0.05% Tween-20. Washes between antibody incubations were generally 3 x 5 min of approximately 100 ml PBS/0.05% Tween-20. Immunoreactive bands were visualised by ECL. In some experiments the secondary layer was ^{125}I-coupled Protein-A and immunoreactive bands were detected by autoradiography. Where indicated, membranes were stripped of antibodies by incubating for 30 min at 55°C (with agitation) in a buffer containing 100 mM 2β-mercaptoethanol, 2% SDS, 62.5 mM Tris pH 6.7. The membrane was washed x3 in PBS/0.05% Tween-20 before blocking and western blotting was repeated.

Western blotting primary antibody conditions:

PKD (sc-935)	200 ng/ml	(2 h, RT)
PKD (PA-1)	1:1000 dilution of serum	(2 h, RT)
pS916 PKD	1:1000 dilution of serum	(ON, 4°C)
GFP	1μg/ml	(ON, 4°C)
9E10	1μg/ml	(2 h, RT)

PKC (α, δ, ε, ξ)	200 ng/ml	(ON, 4°C)
pS473 PKB/Akt	1:1000 of supplied stock	(ON, 4°C)
pan PKB/Akt	1:1000 of supplied stock	(2 h, RT)
pT202/Y204 erk 1/2	1:5000 of supplied stock	(ON, 4°C)
pan erk 2	1:5000 of supplied stock	(2 h, RT)

2.14. Generation of anti-phosphopeptide antisera.

A phosphopeptide corresponding to the C-terminal 15 amino-acids (residues 904-918) of murine PKD (EEREMKALSERVS^{916}IL) was synthesised with serine 916 as a phosphorylated residue. The peptide was then coupled to keyhole limpet hemocyanin using a glutaraldehyde coupling method, to cross-link NH_2 groups (Collawn and Paterson, 1997). Briefly, 4 mg of phosphopeptide was dissolved with 4 mg of Keyhole Limpet Hemocyanin (KLH) in 0.1 M $NaHCO_3$, in a final volume of 2 ml. 0.05% ultra pure glutaraldehyde was added, the pH adjusted to 8 with NaOH and then mixed overnight in the dark, at RT. The following day glycine ethyl ester (pH 8) was added to a final concentration of 0.1 M and incubated at RT for 30 min to block residual non-coupled glutaraldehyde. Coupled phosphopeptide-KLH proteins were then precipitated with 5 volumes of cold acetone at -70°C for 30 min and pelleted by centrifugation at 10, 000 x g for 5 min. The pellet was then dried and resuspended in sterile PBS at a concentration of 1 mg/ml. The KLH-coupled phosphopeptides were used to generate rabbit antisera using standard immunisation techniques which was carried out by the ICRF Biological Resources Unit. Rabbit antisera were screened for antigen reactivity by ELISA and Western blot analysis.

2.15. ELISA assays

Microtiter plates were coated with 2.5 pmol of various peptides overnight at 4°C in a final volume of 50 µl in 0.1M sodium bicarbonate, pH 9.6. The plates were then washed (x3) in PBS and subsequently blocked with 100 µl PBS containing 0.1% gelatin for 1 h at RT and then washed (x1) in PBS. The plates were incubated for 1 h at RT with serial (2-fold) dilutions of specific antisera, followed by x4 washes in PBS containing 0.05% Tween-20. This was followed by a 1 h incubation with donkey anti-rabbit-horse-radish peroxidase (1:5000 dilution). Plates were again washed (x4) in PBS/0.05% Tween-20 and then incubated with ABTS substrate (10mg ABTS [2,2'-azino-bis(3-ethylbenthiazoline-6-sulphonic acid)]; 0.64 g Citric acid; 25 µl of 30% H_2O_2, 33 ml dH_2O, pH 4.3). Immunoabsorbance was detected by reading the OD_{405} after a 30 min development period.

2.16. DNA Subcloning.

Plasmid DNA (1 μg) was digested in a final volume of 10 μl (in the appropriate buffer) at 37°C for 2 h, using 1 unit of enzyme (final concentration). DNA fragments were separated on agarose gels using TAE buffer (0.04 M Tris-acetate, 2 mM EDTA, pH 8). For subcloning purposes, desired fragments were isolated using a Qiagen Gel Purification Kit (according to the manufacturer's instructions). DNA fragments were ligated into the desired plasmid using T4 DNA ligase (Gibco). Typically, a 3 molar excess of DNA fragment was used relative to the plasmid, using ≤100 ng of DNA in total, in a final volume of 10 μl. Ligation reactions were performed at RT for 2 h.

Ligation reactions were then used to transform DH5α *E. coli*. Circularised plasmid DNA was mixed with 100 μl competent *E. coli* cells and incubated on ice for 30 min. The cells were then heat-shocked at 45°C for 45 sec and returned to ice for 5 min. 200 μl L-Broth medium was added and the cells were left to recover at 37°C for 30 min before plating onto LB-agar plates, containing 50 μg/ml ampicillin, and incubating at 37°C overnight.

Single colonies obtained from transformation reactions were grown overnight in 5 ml L-Broth (containing 50 μg/ml ampicillin). Plasmid DNA was isolated from 2 ml aliquots using QIAprep spin miniprep kits (Qiagen), according to the manufacturer's instructions. Colonies containing the desired plasmid cDNA were identified by restriction enzyme digestion.

2.17. Preparation of heat-shock competent DH5α *E. coli*.

A single DH5α colony was picked from a minimal plate and grown overnight in 25 ml L-broth. The culture was subsequently diluted 1:200 in fresh L-broth and grown to logarithmic phase (OD_{550}=~0.4). The cells were chilled on ice, collected by centrifugation at 4,000 rpm for 10 min at 4°C and resuspended in 160 ml of ice-cold transformation buffer I (30 mM KAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol, final pH 5.8). After a 30 min incubation on ice the cells were pelleted by centrifugation at 4,000 rpm for 10 min at 4°C and resuspended in 30 ml of transformation buffer II (10 mM PIPES, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol, final pH 6.5) for 30 min on ice. Aliquots of 400μl were snap frozen on cardice and stored at -70°C.

2.18. DNA mutagenesis

An N-terminally Myc-tagged wild-type PKD construct was generated by ligating a double stranded oligonucleotide encoding the c-Myc-epitope (MEQKLISEEDL) in frame to

the N-terminus of wild-type PKD within a pcDNA3 mammalian expression construct. Briefly, 10 μg of sense and antisense oligonucleotides encoding the Myc-epitope (bold) linked to the N-terminal 15 residues of murine PKD and containing 5'-*ClaI* and 3'-*SacII* restriction enzyme overhangs (underlined) were annealed in TE buffer (0.04 M Tris, 2 mM EDTA, pH 8) containing 75 mM NaCl, at 95°C for 10 min, before being slowly cooled slowly to RT overnight. The resulting double-stranded oligonucleotide was ligated between the *ClaI/SacII* sites of wild-type pcDNA3-PKD. This N-terminally Myc-tagged PKD construct was then used for experimental purposes and for carboxy-terminal mutational analysis.

Myc-PKD sense:

^{5}CGATACC**ATGGAACAAAAACTCATCTCAGAAGAGGATCT**GAGCGTCCCTCCGCT GCTGCGACCGCCTAGCCCGCTCCTGCCCGCCGC$^{3'}$.

Myc-PKD antisense:

^{5}GGCGGGCAGGAGCGGGCTAGGCGGTCGCAGCAGCGGAGGGACGCT**CAGATCC TCTTCTGAGATGAGTTTTTGTTC**CATGGTAT$^{3'}$.

A Myc-PKD mutant lacking the C-terminal 23 residues (PKD ΔCT) was generated by introducing a double-stranded oligonucleotide encoding a stop codon into the *Nhe1* site of wild-type pcDNA3-Myc PKD. Briefly, an inverse palindromic double stranded oligonucleotide containing STOP codons in both directions and with overhanging *Nhe1* restriction sites (underlined) was ligated into the *Nhe1* site of wild-type pcDNA3 Myc-PKD:

ΔC-terminal oligonucleotide:

GCTAGCTGAAGATCTTCAGCTAGC

Single point mutations at the S916 residue of PKD were generated using a PCR-based technique. Briefly, a PCR fragment containing mutant nucleotides (in bold) encoding a serine to alanine or a serine to glutamic acid substitution at the 916 site (PKD S916A and PKD S916E, respectively) was obtained using the oligonucleotides shown below. PCR reactions were performed in a total volume of 100 μl, containing 100 ng of template cDNA (pcDNA3-Myc-PKD wild-type); 2 μl of dNTP mix (dATP, dCTP, dGTP and dTTP, each 10 mM); 10 μl of 10x native *pfu* reaction buffer (Stratagene); 10 μl of sense and antisense oligonucleotide primers (each 10 μM stock concentration) and 5 units of native *Pfu* DNA polymerase (Stratagene). Mineral oil was overlaid to prevent evaporation. The PCR conditions used consisted of an initial 95°C melting step, followed by 25 cycles of melting, annealing and extension [95°C (1 min), 55°C (2 min) and 72°C (1 min)]. A final 10 min chase at 72°C was included. The resulting PCR fragments were purified using a Qiagen PCR Purification Kit (according to the manufacturer's instructions) and were digested with

Nhe1 and *Xba1* restriction enzymes (underlined) and used to replace the original pcDNA3-Myc PKD *Nhe1/Xba1* fragment.

S916A sense:

$^{5'}$AGTGCTAGCCACAGCGACAGTCCTGAGGCTGAAGAGAGAGAGATGAAAGCCCT CAGTGAGCGTGTC**GCC**ATCCTCTGA$^{3'}$.

S916A antisense:

$^{3'}$CCCTCTAGAACTAGTCCGCGGGGATCC$^{5'}$.

S916E sense:

$^{5'}$AGTGCTAGCCACAGCGACAGTCCTGAGGCTGAAGAGAGAGAGATGAAAGCCCT CAGTGAGCGTGTC**GAA**ATCCTCTGA$^{3'}$.

S916E antisense:

$^{3'}$CCCTCTAGAACTAGTCCGCGGGGATCC$^{5'}$.

Chimeric fusion proteins between Green Fluorescent Protein (GFP) and different PKD mutants were constructed by subcloning PKD constructs, in frame, into the *EcoR1* restriction enzyme site of the pEFplink2-GFP_{C3} mammalian expression vector provided by S. Cleverley (Lymphocyte Activation Laboratory, ICRF).

All cDNA constructs generated were checked by restriction enzyme analysis and DNA sequencing.

2.19. Automated DNA sequencing.

DNA sequencing was carried out using the ABI prism dye terminator sequencing kit (Perkin Elmer). cDNA regions of interest were amplified by PCR using an oligonucleotide primer and fluorescently labelled terminator bases to generate single-stranded, labelled cDNA fragments that are then analysed by gel electrophoresis and computer processing. Briefly, the PCR reaction mix consisted of 300-500 ng of cDNA template; 3.2 pmole of primer; 8 µl of Terminator Ready Reaction Mix (Perkin Elmer) in a final volume of 20 µl. PCR reaction cycles (25) were (a) a 30 sec denaturing step (96°C); (b) a 15 sec annealing step (at the required annealing temperature, 48-60°C) and (c) a 4 min extension step (60°C). Amplified cDNA was precipitated with 0.3M NaAc and 2.5 volumes of absolute ethanol at 4°C for 10 min and recovered by centrifugation at 14 000 rpm for 30 min at 4°C. DNA pellets were rinsed with 70% ethanol and air dried. Samples were analysed by the staff of the ICRF Equipment Park, using a ABI Prism 310 Genetic Analyser.

2.20. Cell imaging and Immunofluorescence.

Cells transiently transfected with GFP constructs were grown on sterile glass coverslips overnight, stimulated as appropriate and then fixed in 4% paraformaldehyde for 20 min at RT. Nuclear staining of nucleic-acids using propidium iodide, was carried out by permeabilization of cells in 0.1% Triton X-100 followed by sequential incubation with 500 μg/ml RNase A (37°C, 10 min) and 0.1 μg/ml propidium iodide (RT, 10 min). Coverslips were washed in PBS after each stage before mounting in 15 μl Gelvatol (Monsanto Chemcicals) onto glass slides and allowed to set for at least 4 h. Staining of lipid rafts was carried out by incubating fixed cells with 5 μg/ml Cholera Toxin B subunit/Biotin (Sigma) for 20 min at RT, followed by incubation with 5 μg/ml Streptavidin-Alexa^{568} conjugate (Molecular Probes) for 20 min at RT. The cells were washed in PBS after each stage and mounted in Gelvatol for subsequent confocal imaging.

Immunofluorescence staining for intracellular proteins was carried out by fixing coverslips in ice-cold methanol (-20°C) for 8 min before permeabilization with PBS/0.1% Triton X-100 at RT for 4 min. Aldehyde groups were quenched by incubating with PBS/0.1% sodium borate for 10 min at RT followed by blocking with PBS/0.2% gelatin (10 min, RT). Primary and secondary antibodies (diluted in PBS/0.2% gelatin) were applied, as indicated in Figure legends, with a 30 min incubation in PBS/0.2% gelatin at 4°C in between. Coverslips were washed x3 in PBS between stages. Coverslips were mounted onto glass slides using 15 μl Gelvatol. For live cell imaging, transfected cells were plated onto 35 mm sterile glass dishes overnight (MatTek Corp, USA). The cells were incubated in phenol-red free DMEM medium and placed inside a prewarmed (37°C) chamber under the confocal microscope. Images were then taken at various time points before and during stimulation with different agonists.

All confocal images were obtained using a Zeiss Axiovert-100M inverted confocal microscope. GFP fluorescence was excited with an argon laser, emitting at 488 nm and images were acquired using a 63xNA1.4 oil immersion objective and Zeiss LSM 510 software. For dual fluorescence analysis, images were corrected for contribution of propidium iodide or Alexa^{568} to the GFP signal.

2.21. [^{3}H]Phorbol 12,13-dibutyrate (PDBu) binding assays.

[^{3}H]PDBu binding to transfected COS-7 cells was performed by incubating cells in DMEM containing 1mg/ml bovine serum albumin and 20 nM [^{3}H]PDBu at 37°C for 30 min. The cells were then rapidly washed with ice-cold PBS containing 1mg/ml bovine serum

albumin and lysed with NaOH-SDS. Bound radioactivity was determined by liquid scintillation counting. Non-specific binding was determined in the presence of 10 μM unlabeled PDBu.

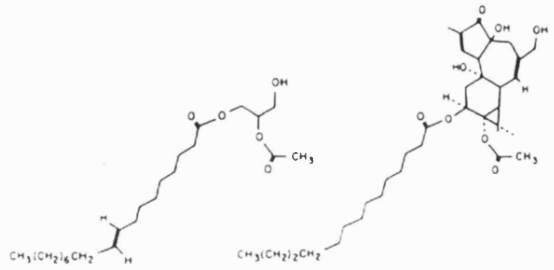
CHAPTER 3 : Activation of PKD by bryostatin 1

3.1 INTRODUCTION

In vivo, classical and novel PKCs are activated by membrane localised lipid co-factors such as DAG and phosphatidyl-L-serine. However, a number of pharmacological agents can also bind to and activate these PKC isoforms, the most well characterised of which are the tumour promoting phorbol esters. Phorbol esters have been successfully used to characterise functions for classical and novel PKCs *in vivo*, including roles in the mitogenesis and proliferation of cells, differentiation and apoptosis (see Chapter 1). Atypical PKC enzymes are not regulated by DAG or phorbol esters however, but are activated by other lipid second messengers, including ceramide (Muller *et al.*, 1995) and the PI3-Kinase lipid products PI-3,4-P_2 and PI-3,4,5-P_3 (Nakanishi *et al.*, 1993; Palmer *et al.*, 1995b; Singh *et al.*, 1993; Toker *et al.*, 1994). PI-3,4-P_2 and PI-3,4,5-P_3 are also able activate novel PKC enzymes, *in vitro*. PKD, through the presence of a regulatory cysteine-rich C1 domain, is activated by phorbol esters and DAG analogues in the presence of phosphatidyl-L-serine *in vitro* but not by phosphoinositides or ceramide (Dieterich *et al.*, 1996; Valverde *et al.*, 1994; Van Lint *et al.*, 1995).

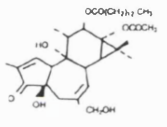
3.1.1. Bryostatins: Non-classical activators of PKC enzymes.

Pettit and co-worker's first identified bryostatin 1 as a potent anti-neoplastic activity present in the marine bryozoan *Bugula nertitina* in the late 1960's (Pettit *et al.*, 1970). Subsequently, bryostatin 1 and several other related compounds were purified for analysis (Blumberg and Pettit, 1992; Pettit *et al.*, 1982). The bryostatins possess potent anti-neoplastic activity in both *in vitro* and *in vivo* model systems (Hornung *et al.*, 1992; Jones *et al.*, 1990; Pettit *et al.*, 1982; Schuchter *et al.*, 1991). In addition, the bryostatins also display several immunopotentiating properties, including the induction of cytokine secretion and stimulating the growth of multipotential progenitor cells (Leonard *et al.*, 1988; May *et al.*, 1987; Sredni *et al.*, 1990; Trenn *et al.*, 1988). In this regard, bryostatin 1 has entered clinical trials as a potential therapeutic agent, particularly for melanoma (Philip *et al.*, 1993; Prendiville *et al.*, 1993) although significant side effects may limit the use of bryostatins as therapeutic agents. Interestingly modified bryostatins, which have decreased affinity for PKC, but which retain their growth inhibitory properties are less toxic in animal models (Szallasi *et al.*, 1996a).

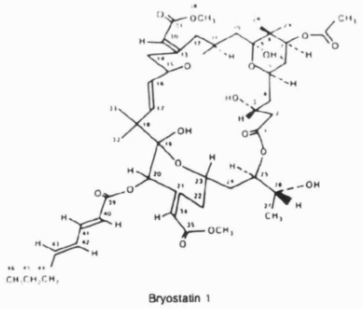


1-oleoyl-2-acetylglycerol
(OAG)

12-0-tetradecanoylphorbol 13-acetate
(TPA)



Phorbol 12, 13-dibutyrate
(PDBu)



Bryostatin 1

Fig. 3.1. Structures of phorbol esters, bryostatin 1 and DAG-analogues.
X-ray crystallography studies have shown the importance of the C20 hydroxy group of the phorbol esters for binding to PKC (Zhang, 1995). In addition, computer modelling suggests that the C19 and C20 hydroxy groups of phorbol esters are important for PKC binding (Wender, 1986; Nakamura, 1989). These groups correspond to the C19 and C26 hydroxy groups of the bryostatins. Changes to these groups dramatically reduces the potency of the bryostatins for PKC binding/activation (Wender, 1988; Lewin, 1991; Rando, 1992; Kazanietz, 1994; Szallasi, 1996). Structures taken from (Kraft, 1986; Ramsdell, 1986).

The bryostatins are macrocyclic lactones whose overall structure is distinct from that of the phorbol esters. Nevertheless, the 3-dimensional arrangement of key functional groups within the bryostatins display similarity to that of phorbol esters (Fig. 3.1). The bryostatins can therefore compete with phorbol esters for binding to PKC and can directly activate PKC *in vitro* (Berkow and Kraft, 1985; Kraft *et al.*, 1986; Ramsdell *et al.*, 1986; Smith *et al.*, 1985). The bryostatins (as well as the phorbol esters) bind to and activate classical and novel PKCs to similar extents *in vitro*, with no distinction between different isoforms (Kazanietz *et al.*, 1994a; Lewin *et al.*, 1992).

Given that both bryostatins and phorbol esters bind to the same cellular target *in vivo* (that is PKC) it is not surprising that many biological effects of these two classes of compounds are similar. These include the induction of mitogenesis in Swiss 3T3 fibroblasts (Smith *et al.*, 1985) and the activation of polymorphonuclear leukocytes (Berkow and Kraft, 1985). However, biological responses induced by the bryostatin 1 will often differ dramatically from those induced by phorbol esters, as summarised in Table 3.1. For example, in many systems bryostatin 1 can only induce a subset of phorbol ester responses and will antagonise those phorbol ester effects that it does not induce. Thus, bryostatin 1 is inactive as a tumour promoter and is able to block phorbol ester-induced tumour promotion *in vitro* and *in vivo* (Hennings *et al.*, 1987; Lu *et al.*, 1997a). Bryostatin 1 can also antagonise the effects of phorbol esters on the differentiation of HL60 promyelocytic leukemia cells (Kraft *et al.*, 1986); the inhibition of chemically induced differentiation of Friend erythroleukemia cells (Dell'Aquila *et al.*, 1987) and the proliferation of human T lymphocytes (Isakov *et al.*, 1993). In addition, many of the biological actions of bryostatin 1 show a characteristic biphasic dose-response relationship that is not observed with phorbol esters (Table 3.1).

The molecular mechanism(s) underlying the differential effects of bryostatin 1 and the phorbol esters remain poorly understood. However, bryostatin 1 binds to PKCs with greater affinity than phorbol esters (Lewin *et al.*, 1992) and differences between the activation/downregulation of PKC enzymes induced by bryostatin as compared to phorbol esters have been reported. For example, bryostatin 1 is more effective than phorbol esters at inducing the downregulation of certain PKC isoforms in T lymphocytes and in breast cancer cell lines (Isakov *et al.*, 1993; Kennedy *et al.*, 1992). In addition, bryostatin 1 displays a unique pattern of PKCδ downregulation in a number of cell types (Szallasi *et al.*, 1996a; Szallasi *et al.*, 1994; Szallazi *et al.*, 1994). However, it is unclear whether all the unusual biological effects mediated by bryostatin but not phorbol esters can be attributed

Response	Assay	References
Simple agonist effects	PKC activity	A
	Proliferation	B
	Neutrophil activation	C
Antagonism of phorbol ester effects - negligible agonist effects	Differentiation (+ and -)	D
	Proliferation (+ and -)	E
	Inhibition of cell-cell communication	F
	Tumour promotion	G
Biphasic bryostatin dose-response	PKCδ downregulation	H
	c-Jun upregulation	H
	Differentiation	I
	Proliferation (+ and -)	J
	Cytokine secretion	K
Effects unique to Bryostatin	Rapid PKCα downregulation	L
	Lamin B phosphorylation	M

Table 3.1 - Heterogeneity of bryostatin versus phorbol ester effects_

[A] (Berkow and Kraft, 1985; Kraft *et al.*, 1986; Smith *et al.*, 1985); [B] (Gschwendt *et al.*, 1988; Kraft *et al.*, 1988; Smith *et al.*, 1985); [C] (Berkow and Kraft, 1985); [D] (Dell'Aquila *et al.*, 1987; Gschwendt *et al.*, 1988; Hocevar *et al.*, 1992; Kraft *et al.*, 1986; Szallazi *et al.*, 1994); [E] (Isakov *et al.*, 1993; Kennedy *et al.*, 1992; Levine *et al.*, 1991; Mackanos *et al.*, 1991); [F] (Pasti *et al.*, 1988); [G] (Hennings *et al.*, 1987; Lu *et al.*, 1997a); [H] (Szallasi *et al.*, 1996a; Szallasi *et al.*, 1994); [I] (Stone *et al.*, 1988); [J] (Dale and Gescher, 1989; Jones *et al.*, 1990); [K] (Sredni *et al.*, 1990); [L] (Jalave *et al.*, 1993); [M] (Fields *et al.*, 1988).

to a differential regulation of PKC enzymes. Blumberg and co-workers have reported that a bryostatin 1 derivative, which is 60-fold less potent at inducing PKC activation/downregulation than native bryostatin 1, is able to inhibit cell growth to the same extent as wild-type bryostatin 1 (Szallasi *et al.*, 1996a). This suggests that at least some of the biological effects of bryostatins may be independent of PKC. The divergent biological effects elicited by phorbol esters and bryostatin 1 in certain systems prompted an investigation into whether bryostatin 1 can regulate PKD.

3.1.2. Aims.

(1) Determine whether bryostatin 1 can regulate the activity of PKD.

(2) Compare the regulation of PKD by PDBu and bryostatin 1.

(3) Determine the mechanism underlying PKD activation by bryostatin 1 *in vivo.*

3.2. RESULTS.

To enable an investigation into the regulation of PKD by bryostatins, Dr. George R. Pettit (Cancer Research Institute, Arizona State University) generously supplied bryostatin1.

3.2.1. Bryostatin 1 induces PKD activity *in vitro* and *in vivo*.

In initial experiments to determine whether bryostatin 1 was able to activate PKD *in vitro,* COS-7 fibroblasts were transiently transfected with a cDNA construct containing wild-type PKD. 72 h post-transfection the cells were lysed and PKD was immunoprecipitated with the PA-1 antiserum (directed against the C-terminal 15 residues of PKD). PKD was then eluted from immunocomplexes by competition with excess immunising peptide and incubated with different activating co-factors in the presence of [γ-^{32}P]ATP. PKD activity (as measured by autophosphorylation) was then analysed by SDS-PAGE and autoradiography. As shown in Figure 3.2a, bryostatin 1 was able to directly activate PKD *in vitro* in a phospholipid-dependent manner. The degree of PKD activation induced by bryostatin 1 was comparable to that induced by the phorbol ester PDBu.

To determine whether bryostatin 1 could also regulate PKD activity *in vivo*, COS-7 cells were transiently transfected with a wild-type PKD expression construct and subsequently treated with either PDBu or bryostatin 1 before lysis and PKD immunoprecipitation. The resulting immunocomplexes were incubated with [γ-^{32}P]ATP and then analysed by SDS-PAGE and autoradiography. Stimulation of intact cells with bryostatin 1 or with PDBu was found to increase the catalytic activity of PKD, as measured by autophosphorylation, which was maintained during cell lysis and immunoprecipitation and which could be measured by *in vitro* kinase assays in the absence of any further lipid co-factors (Fig. 3.2b). These results indicate that bryostatin 1 regulates the catalytic activity of PKD, *in vitro* and *in vivo*, in a manner comparable to that induced by phorbol esters.

3.2.2. The bryostatin 1-inducible kinase activity in PA-1 immunoprecipitates is PKD.

Control experiments were subsequently performed to confirm that the ~110 kDa kinase activity observed in PA-1 immunoprecipitates prepared from bryostatin 1-stimulated cells was indeed PKD, and not a non-specifically associating kinase. COS-7 cells were

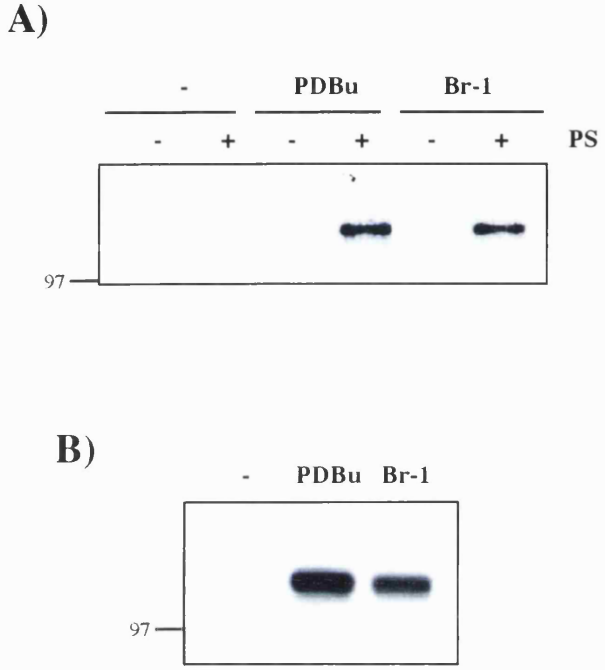


Fig. 3.2. Bryostatin 1 induces PKD catalytic activity *in vitro* and *in vivo*.
(a) COS-7 cells were transiently transfected with pcDNA3-PKD, as detailed in Chapter 2. After 72 h, viable cells were lysed and PKD immunoprecipitated using the PA-1 antiserum. PKD was then eluted from the immunocomplexes and incubated with [γ-^{32}P]-ATP in the presence of 50 nM bryostatin 1 (Br-1); 200 nM PDBu and 125 μg/ml phosphatidyl-L-serine (PS) as indicated for 10 min at 30°C. PKD autophosphorylation was analyzed by SDS-PAGE and autoradiography. Results shown are representative of three independent experiments.
(b) COS 7 cells were transiently transfected with pcDNA3-PKD and after 72 h treated with either 50 nM bryostatin 1 for 30 min or 200 nM PDBu for 10 min and lysed. PKD was immunoprecipitated with the PA-1 antiserum and PKD activity was measured by *in vitro* kinase assays, SDS-PAGE and autoradiography (IVK). Results show PKD autophosphorylation and are representative of three independent experiments.

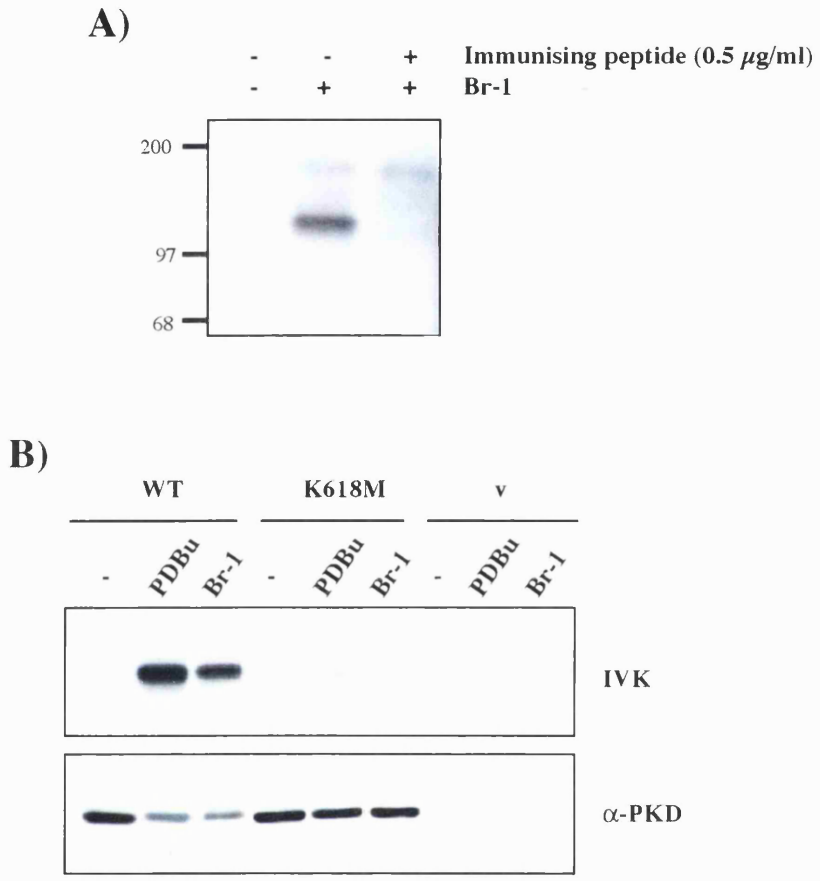


Fig. 3.3. The bryostatin 1-inducible kinase activity in PA-1 immunoprecipitates is PKD.
(a) COS-7 cells (transiently transfected with pcDNA3-PKD) were left unstimulated (-) or were incubated with 50 nM bryostatin 1 for 30 min (+) and lysed. PKD was immunoprecipitated with the PA-1 antiserum, either in the presence (+) or absence (-) of excess immunizing peptide, as indicated. PKD autophosphorylation was measured by *in vitro* kinase assays, SDS-PAGE and autoradiography. Similar results were seen in two independent experiments.
(b) COS-7 cells were transiently transfected with different expression vectors: pcDNA3 (v), pcDNA3-PKD wild-type (WT) or pcDNA3-PKD K618M (K618M). After 72 h, cultures were treated with 50 nM bryostatin 1 for 30 min or 200 nM PDBu for 10 min, lysed and PKD immunoprecipitated with the PA-1 antiserum. Upper panel, PKD activity (as measured by autophosphorylation) was determined in *in vitro* kinase assays followed by SDS-PAGE and autoradiography (IVK). Lower panel, PKD expression levels were determined by SDS-PAGE and western blotting of whole cell lysates using a specific PKD antibody (sc-935). Results shown are representative of three independent experiments.

transiently transfected with a wild-type PKD construct and after 72 h stimulated with bryostatin 1. The cells were subsequently lysed, PKD immunoprecipitated and *in vitro* kinase assays performed. As shown in Figure 3.3a, the kinase activity induced by bryostatin 1 was completely abrogated by the addition of the immunising peptide to cell lysates during PKD immunoprecipitation.

In addition, COS-7 cells were transfected with either wild type (WT) or kinase-defective (K618M) PKD expression constructs. The transfected COS-7 cells were stimulated with either bryostatin 1 or PDBu and subsequently lysed before PKD was immunoprecipitated using the PA-1 antiserum. *In vitro* kinase assays were performed and PKD autophosphorylation was analysed by SDS-PAGE and autoradiography. In parallel, lysates from these cells were analysed by western blotting with a specific PKD antibody. Bryostatin 1 treatment, like PDBu, resulted in the activation of wild-type PKD (Fig. 3.3b). However, no inducible kinase activity was observed in COS-7 cells transfected with the kinase-deficient PKD mutant (K618M), despite similar expression levels of these two PKD proteins (Fig. 3.3b). In addition, no kinase activity was detected in COS 7 cells (which express extremely low levels of endogenous PKD protein) transfected with the control vector pcDNA3 (Fig. 3.3b). These results verified that the bryostatin 1-induced kinase activity measured in PKD immunoprecipitates was indeed due to the activation of PKD.

3.2.3. Kinetics of PKD activation by bryostatin 1.

To investigate the regulation of endogenous PKD by bryostatin 1 a model fibroblast cell line, Swiss 3T3, was used. This cell line has been characterised extensively and serum depravation induces cell senescence whilst addition of serum or other pure mitogenic factors causes the cells to re-enter the cell cycle thus promoting DNA synthesis and cell proliferation (Rozengurt, 1980). As a consequence Swiss 3T3 fibroblasts have provided a useful model system with which to study the regulation of different intracellular signalling pathways.

To investigate the kinetics of bryostatin 1-induced PKD activation, quiescent cultures of Swiss 3T3 fibroblasts were treated with either PDBu or bryostatin 1 for various times (1-30 min), before the cells were lysed, PKD immunoprecipitated and PKD activity measured in *in vitro* kinase assays. PDBu treatment led to a rapid increase in PKD activity which was detectable within 1 min and was maximal within 10 minutes of treatment (in agreement with previous observations (Zugaza *et al.*, 1996)). In contrast,

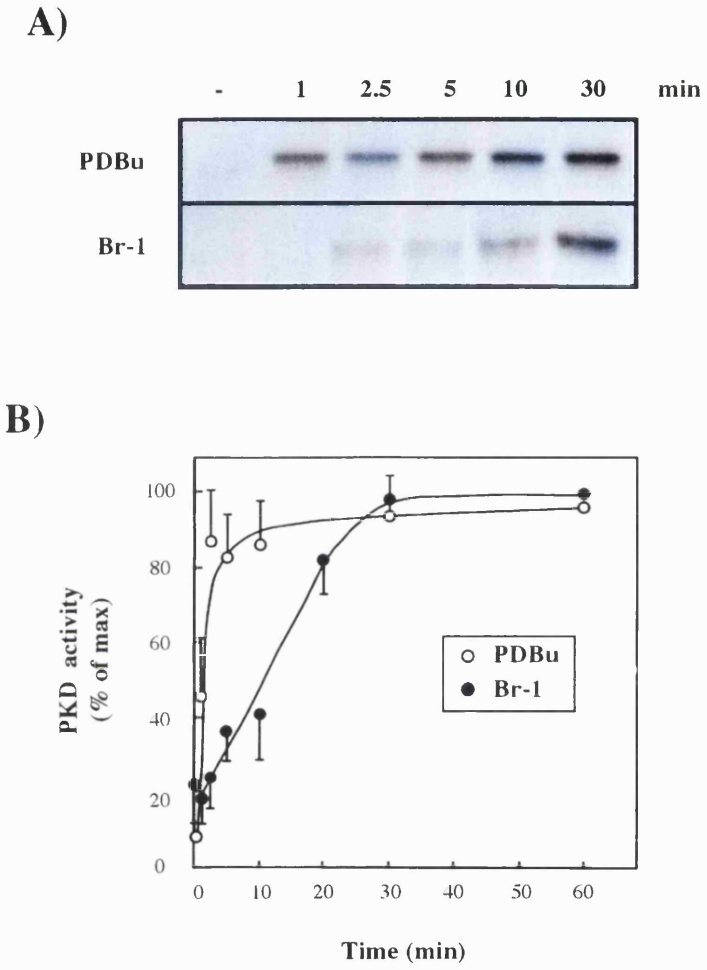


Fig. 3.4. Early kinetics of PKD activation induced by bryostatin 1 and PDBu.
Confluent and quiescent cultures of Swiss 3T3 fibroblasts were incubated in the presence of either 10 nM bryostatin 1 or 200 nM PDBu for various times as indicated before lysis. PKD was immunoprecipitated with the PA-1 antiserum and subjected to *in vitro* kinase assays, SDS-PAGE and autoradiography. Upper panel, representative autoradiograms showing PKD autophosphorylation. Lower panel, PKD autophosphorylation induced by PDBu and bryostatin 1 was quantified by scanning densitometry and the results are shown expressed as a percentage of the maximum response induced by either bryostatin 1 or PDBu, as appropriate. Data are the mean ± s.e. of three independent experiments.

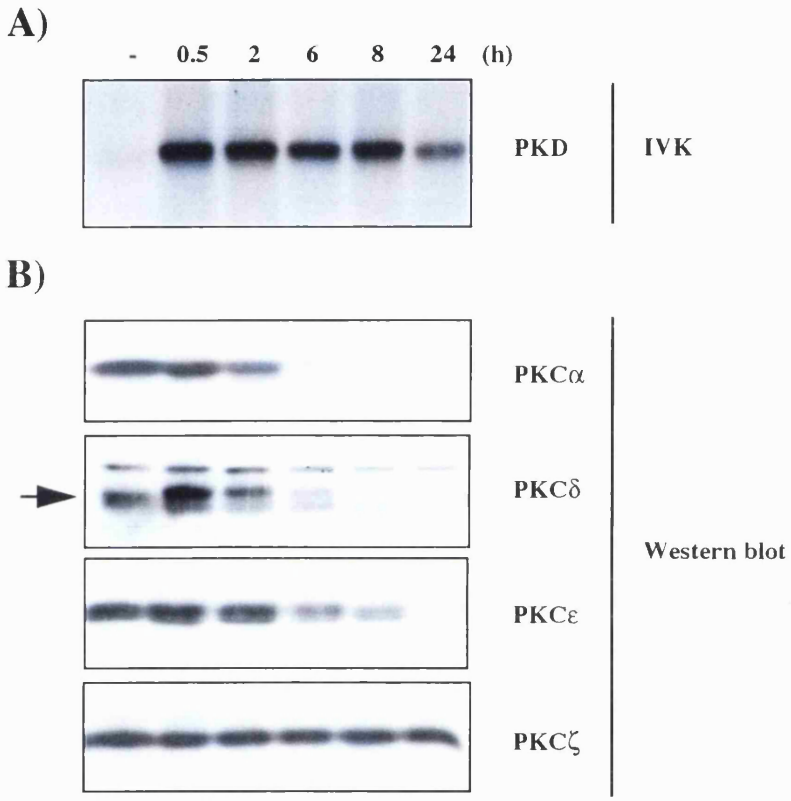


Fig. 3.5. Prolonged bryostatin 1 treatment does not downregulate PKD.
(a) Confluent and quiescent cultures of Swiss 3T3 fibroblasts were incubated with 10 nM bryostatin 1 for various times (0-24 h) and subsequently lysed before PKD was immunoprecipitated using the PA-1 antibody. PKD activity (autophosphorylation) was measured by *in vitro* kinase assay, SDS-PAGE and autoradiography.
(b) Parallel cultures of Swiss 3T3 fibroblasts, treated as in (a) were lysed directly into 2x SDS-PAGE sample buffer and analysed by SDS-PAGE and western blotting with different isoform-specific PKC antibodies, as indicated. The arrow indicates PKCδ.
Similar results were seen in 2 separate experiments.

PKD activity induced by bryostatin 1 was delayed, with a maximal response only occurring ~30 minutes after stimulation (Fig. 3.4).

Downregulation of classical and novel PKC isoforms following prolonged stimulation *in vivo* with phorbol esters or other PKC-specific activators such as bryostatin 1, has long been established (Rodriguez-Pena and Rozengurt, 1984). This process may serve to terminate the biological actions of PKCs and occurs as the net result of increased proteolysis with no change in the rate of synthesis (Young *et al.*, 1987). Suprisingly, long-term exposure of Swiss 3T3 fibroblasts to bryostatin 1 did not result in the downregulation of PKD activity. Instead a high level of PKD activity was maintained for at least 8 h following stimulation of fibroblasts with bryostatin 1 and significant PKD activity was still detectable after 24h of continuous stimulation (Fig. 3.5a). This contrasted with the downregulation of bryostatin 1-sensitive PKC enzymes present in these cells (PKC alpha, delta and epsilon), which occurred 2-8 h following exposure to bryostatin 1 (Fig. 3.5b). The only PKC isoform not downregulated was the atypical PKCξ isoform, which is not activated by DAG, phorbol esters or bryostatin 1 (Toker, 1998). Similar results demonstrating the lack of PKD downregulation following prolonged stimulation of HeLa cells with phorbol esters has also been described (Johannes *et al.*, 1995). These data indicate that PKD is resistant to the mechanisms(s) controlling PKC downregulation.

3.2.4. Bryostatin 1 induces a biphasic activation of PKD.

The dose-response curve for bryostatin 1-mediated activation of PKD was also investigated by treating quiescent cultures of Swiss 3T3 cells with increasing concentrations (0.3-100 nM) of bryostatin 1 before PKD immunoprecipitation and *in vitro* kinase assays. Stimulation of intact cells with bryostatin 1 was found to induce a striking dose-dependent increase in PKD activity (Fig.3.6a), with half-maximal and maximal stimulation achieved at ~1 nM and 5-10 nM, respectively. Maximal PKD activation induced by bryostatin 1 was comparable to that induced by the phorbol ester PDBu (Fig. 3.6a). Strikingly, higher concentrations of bryostatin 1 (>10 nM) activated PKD to a lesser extent. The biphasic pattern of PKD activity was not due to the induction of PKD degradation by high concentrations of bryostatin 1 since western blot analysis demonstrated equivalent PKD protein levels at all bryostatin 1 concentrations used (Fig. 3.6a).

Bryostatin 1-mediated activation of PKD was also demonstrated when PKD activity was measured by the phosphorylation of an exogenous substrate peptide, Syntide-2, a previously described *in vitro* substrate for PKD (Valverde *et al.*, 1994; Van Lint *et al.*,

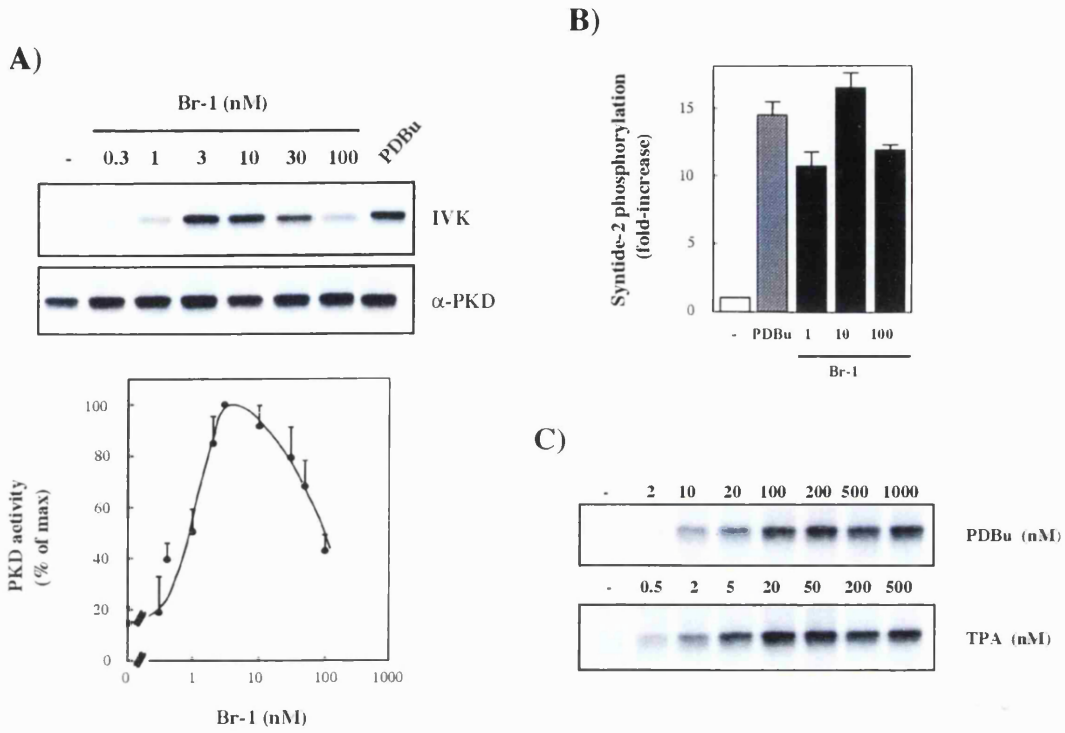


Fig. 3.6. Bryostatin 1, unlike phorbol esters, induces biphasic PKD activation in Swiss 3T3 fibroblasts.
(a) Confluent and quiescent cultures of Swiss 3T3 fibroblasts were incubated with increasing concentrations of bryostatin 1 (0-100 nM) for 30 min or with 200 nM PDBu for 10 min and lysed. PKD was immunoprecipitated from the lysates using the PA-1 antiserum and subjected to *in vitro* kinase assays, SDS-PAGE and autoradiography (IVK). Alternatively, lysates were incubated at 37°C for 30 min (to active endogenous protein phosphatases) before PKD was immunoprecipitated and analysed by SDS-PAGE and western blotting using a specific PKD antibody (sc-935). One representative experiment in shown (upper panel) and the mean ± s.e. increase in PKD autophosphorylation from 3 independent experiments (as quantified by scanning densitometry) are shown (lower panel), expressed as a percentage of the maximum response induced by bryostatin 1.
(b) Confluent and quiescent cultures of Swiss 3T3 fibroblasts were incubated in the presence of either increasing concentrations of bryostatin 1 for 30 min (solid bars), 200 nM PDBu for 10 min (hatched bar) or were left unstimulated (open bar). The cells were lysed and PKD immunoprecipitated with the PA-1 antiserum. PKD activity was measured in a syntide-2 phosphorylation assay as described in Chapter 2. Results shown are expressed as the fold increase in syntide-2 phosphorylation over that induced by PKD isolated from resting cells and are the mean ± s.e. of two independent experiments, each performed in duplicate.
(c) Confluent and quiescent cultures of Swiss 3T3 fibroblasts were stimulated with increasing concentrations of either PDBu or TPA for 10 min, lysed and PKD immunoprecipitated with the PA-1 antibody. PKD activity (as assessed by autophosphorylation) was analysed by *in vitro* kinase assays, SDS-PAGE and autoradiography. Results shown are representative of two independent experiments.

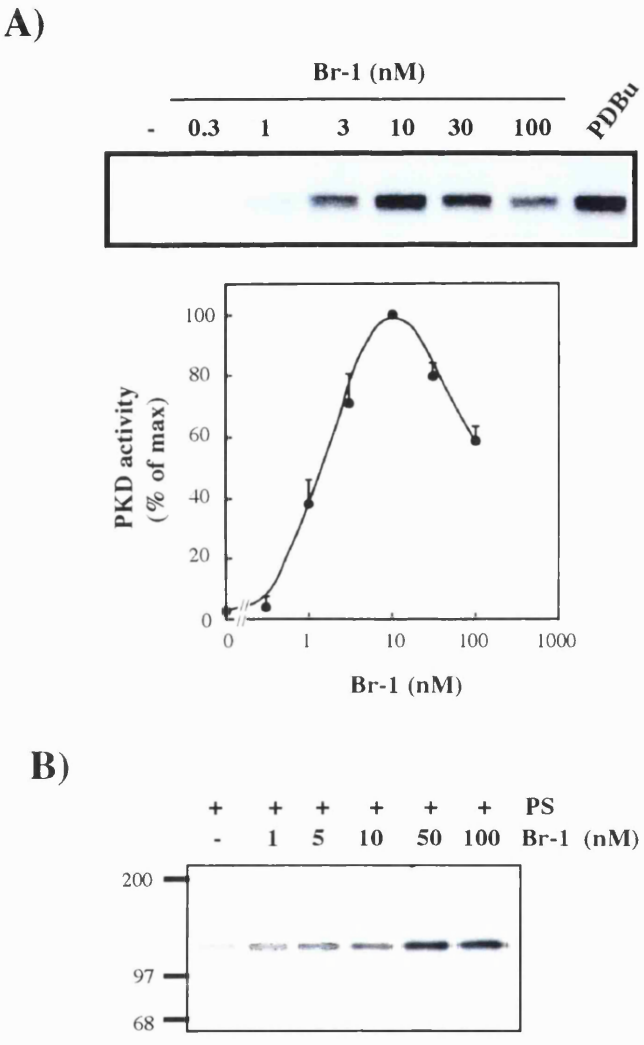


Fig. 3.7. Bryostatin 1 induces biphasic PKD activation in mouse embryo fibroblasts but not during direct *in vitro* stimulation.

(a) Confluent and quiescent cultures of MEFs were treated with increasing concentrations of bryostatin 1 (0-100 nM) for 30 min or with 200 nM PDBu for 10 min as indicated, lysed and PKD immunoprecipitated with PA-1 antiserum. PKD activity, as shown by autophosphorylation, was determined by *in vitro* kinase assays followed by SDS-PAGE, autoradiography and scanning densitometry. Results shown are the mean ± s.e. of three independent experiments (lower panel). A representative autoradiogram is shown (upper panel).

(b) COS-7 cells were transiently transfected with pcDNA3-PKD and after 72 h viable cells were lysed and PKD immunoprecipitated using the PA-1 antiserum. PKD was then eluted from immunocomplexes and activated *in vitro* by incubation with [γ-^{32}P]-ATP plus increasing concentrations of bryostatin 1 (0-100 nM) in the presence of 125 μg/ml phosphatidyl-L-serine (PS), as indicated for 10 min at 30°C, followed by SDS-PAGE and autoradiography. Results shown are representative of two independent experiments.

1995). As shown in Figure 3.6b, a biphasic pattern of syntide-2 phosphorylation was detected in PKD immunoprecipitates (prepared from cells stimulated with increasing concentrations of bryostatin 1), comparable to the biphasic pattern of PKD autophosphorylation shown in Fig. 3.6a.

In contrast, phorbol esters did not induce a biphasic activation of PKD (Fig. 3.6c). The maximal effects of either PDBu or TPA on PKD activity (achieved at 100 nM and 20 nM, respectively) were not effected by increasing the concentrations of these agents by 10- and 25-fold respectively. Thus, bryostatin 1, unlike phorbol esters, induces a biphasic activation of PKD in intact Swiss 3T3 cells.

To confirm that the biphasic pattern of PKD activity induced by bryostatin 1 was not restricted to the Swiss 3T3 fibroblast cell line, experiments were performed using murine embryonic fibroblasts (MEF). As shown in Fig. 3.7a, a dose-dependent biphasic activation of PKD induced by bryostatin 1 was observed in quiescent cultures of secondary MEF, with a maximum response occurring at ~10 nM. Thus, the biphasic activation of PKD by bryostatin 1 was not restricted to immortalised cell lines. Strikingly however the dose-response curve for the direct activation of PKD by bryostatin 1 and phosphatidyl-L-serine *in vitro* was not biphasic (Fig. 3.7b), indicating that the biphasic regulation of PKD by bryostatin 1 is not due to a direct effect on PKD.

3.2.5. Bryostatin 1 antagonises the activation of PKD induced by phorbol esters.

The biphasic bryostatin 1 dose-response curve of PKD activation suggested that bryostatin 1 could potentially, at high concentrations, antagonise the activation of PKD mediated by phorbol esters. To examine this possibility, confluent and quiescent cultures of Swiss 3T3 cells were treated with either PDBu alone or with PDBu in combination with increasing concentrations of bryostatin 1. PKD activation induced by bryostatin 1 alone followed the same biphasic pattern observed previously and PDBu-induced PKD activity was not effected by the simultaneous addition of either 1 or 10 nM bryostatin 1 (Fig. 3.8). However, PKD activation induced by PDBu in the presence of a high concentration (100 nM) of bryostatin 1 was reduced to levels seen with 100 nM bryostatin 1 alone (Fig. 3.8). A similar antagonism of PKD catalytic activity was observed when Swiss 3T3 fibroblasts were pre-treated with 100 nM bryostatin 1 (but not with 1 or 10 nM bryostatin 1) for 30 min before stimulation with 200 nM PDBu (data not shown). These results demonstrate that high concentrations of bryostatin 1 can antagonise phorbol ester-induced activation of PKD in intact cells.

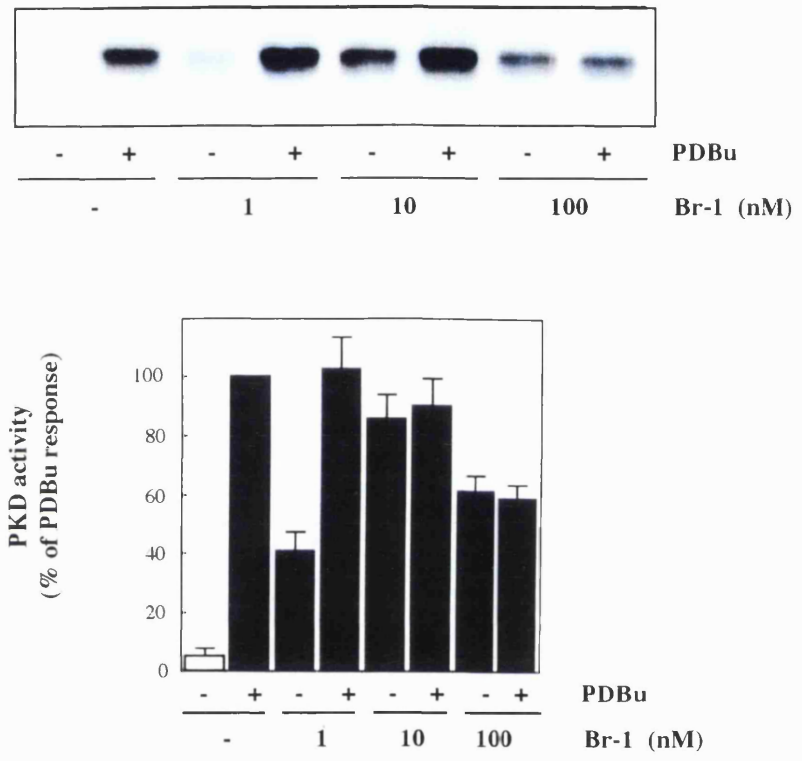


Fig. 3.8. Effect of bryostatin 1 on phorbol ester-induced PKD activation.
Confluent and quiescent cultures of Swiss 3T3 fibroblasts were left unstimulated (-) or were treated with various concentrations of bryostatin 1 (1-100 nM) for 30 min in the presence (+) or absence (-) of 200 nM PDBu as indicated. The cells were lysed and PKD immunoprecipitated with the PA-1 antiserum. Samples were then analysed by *in vitro* kinase assays, SDS-PAGE and autoradiography. A representative autoradiogram is shown (upper panel) along with the mean ± s.e. of PKD activity (as assessed by scanning densitometry) from three independent experiments, expressed as the % of PKD activity induced by PDBu treatment alone (lower panel).

3.2.6. Regulation of bryostatin 1-induced PKD activation through phosphorylation.

As indicated above, PKD activity induced by phorbol esters or bryostatin 1 was maintained during cell lysis and immunoprecipitation and which could be measured by *in vitro* kinase assays in the absence of additional lipid cofactors. This contrasts with the regulation of classical or novel PKC isoforms, which require the presence of lipid cofactors for catalytic activity. The sustained nature of PKD activity implies that PKD is covalently modified during its activation *in vivo* which locks the enzyme in an active conformation. In agreement with this hypothesis active PKD (following stimulation with either bryostatin 1 or PDBu) was consistently observed to migrate more slowly upon SDS-PAGE and western blot analysis than non-stimulated PKD (Fig. 3.9a).

A common strategy used to control the activity of many protein kinases is through the phosphorylation of specific serine, threonine or tyrosine residues. Importantly treatment of PKD, immunoprecipitated from bryostatin 1-stimulated cells, with alkaline phosphatase prevented the reduced electrophoretic mobility of active PKD in SDS-PAGE indicating that PKD is modified by phosphorylation upon activation *in vivo* (Fig. 3.9a). To examine further the role of phosphorylation in the regulation of PKD activity, Swiss 3T3 cells were metabolically labelled with ^{32}Pi and subsequently stimulated with either bryostatin 1 or PDBu. As shown in Figure 3.9b (left), bryostatin 1 induced an 3-4 fold increase in the incorporation of ^{32}Pi into PKD over that seen for control non-stimulated cells, which was comparable to that induced by PDBu treatment. In addition, PKD isolated from ^{32}Pi-labelled Swiss 3T3 cells treated with a high concentration (100 nM) of bryostatin 1 displayed ~50% lower ^{32}Pi incorporation than PKD isolated from cells stimulated with 10 nM bryostatin 1 (Fig. 3.9b, right). These results indicate that PKD is indeed phosphorylated upon activation by bryostatin 1 or phorbol esters, and that the pattern of PKD phosphorylation *in vivo* mimics the biphasic pattern of bryostatin 1-induced PKD activity measured by *in vitro* kinase assays.

To determine the significance of these phosphorylation event(s) the effect of protein phosphatases on bryostatin 1-induced PKD activity was investigated. Lysates prepared from bryostatin 1-stimulated Swiss 3T3 fibroblasts were incubated at 37°C for 30 minutes prior to PKD immunoprecipitation and *in vitro* kinase assays. As can be seen in Figure 3.9c, incubation of cell lysates at 37°C led to the activation of endogenous protein phosphatases resulting in a marked decrease in PKD activity. However, addition of microcystin (a potent inhibitor of PP1 and PP2A phosphatases) under the same conditions

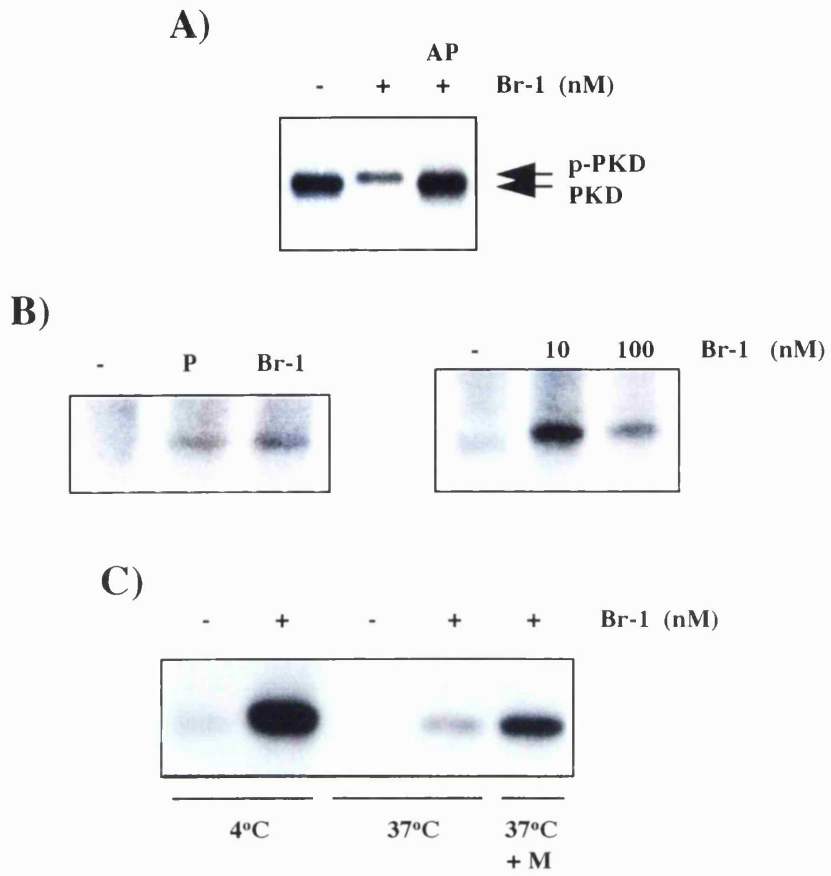


Fig. 3.9. Bryostatin 1 induces PKD phosphorylation: correlation with PKD activity.
(a) Quiescent Swiss 3T3 fibroblasts were left unstimulated (-) or were treated with 10 nM bryostatin 1 for 30 min (+) before PKD was immunoprecpitated using the PA-antiserum. Samples were analysed by SDS-PAGE and western blotting with a specific C-terminal PKD antibody (sc-935). In certain cases the PKD immunoprecipitates were treated with 10 U/ml alkaline phosphatase (AP) for 30 min at 37°C before western blot analysis.
(b) Confluent and quiescent Swiss 3T3 fibroblasts were incubated for 18 h with carrier-free ^{32}Pi in phosphate-free medium and were then left unstimulated (-) or were treated with either 10 nM bryostatin 1 (Br-1) for 30 min or 200 nM PDBu (P) for 10 min (left). Alternatively, the cells were left unstimulated (-) or were treated with either 10 or 100 nM bryostatin 1 for 30 min (right). In both cases the cells were lysed, PKD immunoprecipitated with PA-1 antiserum and *in vivo* PKD phosphorylation was analysed by SDS-PAGE and autoradiography. Results shown are representative of at least two independent experiments.
(c) Confluent and quiescent Swiss 3T3 cells were incubated with (+) or without (-) 10 nM bryostatin 1 for 30 min and lysed. Lysates were then incubated with either 1 μM microcystin (+M) or an equivalent volume of solvent for 30 min at 37°C. Alternatively lysates were kept at 4°C. PKD was immunoprecipitated with the PA-1 antibody and PKD activity (autophosphorylation) was analysed by *in vitro* kinase assays, SDS-PAGE and autoradiography. Results shown are representative of three individual experiments.

preserved substantial PKD activity (Fig. 3.9c). Together, these data indicate that phosphorylation event(s) are important for regulating the activity of PKD in response to bryostatin 1.

3.2.7. Bryostatin 1 activates PKD through a PKC-dependent signailing pathway *in vivo*.

Potential signalling pathways regulated by bryostatin 1 that could mediate the phosphorylation and activation of PKD *in vivo* were subsequently investigated. Inhibition of a variety of intracellular signalling kinases, including $p70^{S6K}$ (rapamycin); PI3-kinase (wortmannin); Protein Kinase A (H-89) and $p42/p44^{MAPK}$ (using the MEK-1 inhibitor PD 098059) did not effect the activation of PKD by bryostatin 1 (Fig. 3.10a). Similarly, disruption of the actin cytoskeleton and inhibition of $p125^{FAK}$ tyrosine phosphorylation by cytochalasin D had no effect on the subsequent activation of PKD induced by bryostatin 1 (Fig. 3.10a). However, pre-treatment of Swiss 3T3 cells with GF 109203X (also known as bisindolylmaleimide I) or Ro 31-8220, potent inhibitors of classic and novel isoforms of PKC (Toullec, 1991; Yeo and Exton, 1995) did prevent the subsequent activation of PKD by bryostatin 1 (Fig. 3.10b).

It was therefore possible that bryostatin 1-sensitive classical and novel PKC isoforms could be involved in the regulation of PKD activation *in vivo*. To investigate this further Swiss 3T3 fibroblasts were pre-treated with increasing concentrations of GF 1 or Ro 31-8220 before stimulation of the intact cells with bryostatin 1. Alternatively these inhibitors were added directly to PKD during *in vitro* kinase assays. As shown in Figure 3.11a, pre-treatment of intact cells with GF 1 or Ro 31-8220 led to a marked dose-dependent reduction in the subsequent activation of PKD elicited by either bryostatin 1 or by PDBu. In striking contrast, neither GF I nor Ro 31-8220 inhibited the activity of PKD (induced by stimulating intact cells with bryostatin 1 or PDBu) when they were added directly to *in vitro* kinase assays, at concentrations identical to those used for pretreating cells (Fig. 3.11a).

To verify that these PKC inhibitors were not directly affecting the activity of PKD, COS-7 cells were transfected with a wild-type PKD expression construct, lysed and PKD immunoprecipitated with the PA-1 antiserum. PKD was then eluted from the immunocomplexes by competition with the immunising peptide and incubated with [γ-^{32}P]ATP in the presence or absence of phosphatidyl-L-serine and bryostatin 1 as indicated. As demonstrated in Figure 3.11b, addition of GF 1 to this reaction mixture did

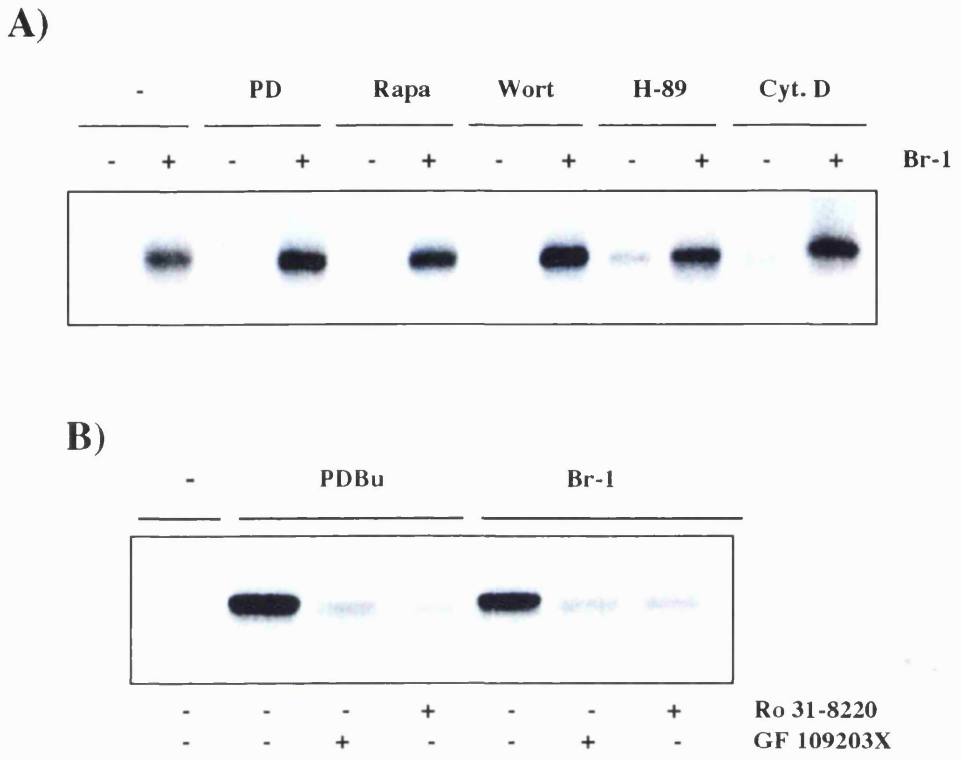


Fig. 3.10. Bryostatin 1-induced activation of PKD is blocked by inhibitors of classical/novel PKCs.

(a) Confluent and quiescent cultures of Swiss 3T3 fibroblasts were pretreated with either 10 μM PD 098059 (PD); 20 nM rapamycin (Rapa); 50 nM wortmannin (Wort); 60 μM H-89; 2.5 μM cytochalasin D (Cyt. D) or an equivalent volume of solvent (-) for 1 h, as indicated. The cells were then incubated for a further 30 min in the absence (-) or presence (+) of 10 nM bryostatin 1. The cells were then lysed, immunoprecipitated with PA-1 antiserum and PKD activity (autophosphorylation) was measured by *in vitro* kinase assays, SDS-PAGE and autoradiography. Results shown are representative of two independent experiments.

(b) Confluent and quiescent cultures of Swiss 3T3 fibroblasts were pretreated with either 3.5 μM GF 109203X or 2.5 μM Ro 31-8220 or an equivalent amount of solvent (-) for 1 h, as indicated. Subsequently the cells were left unstimulated (-) or were treated with 10 nM bryostatin 1 for 30 min (+) and PKD activity measured by *in vitro* kinase assays, as described in (a). Results are representative of four independent experiments.

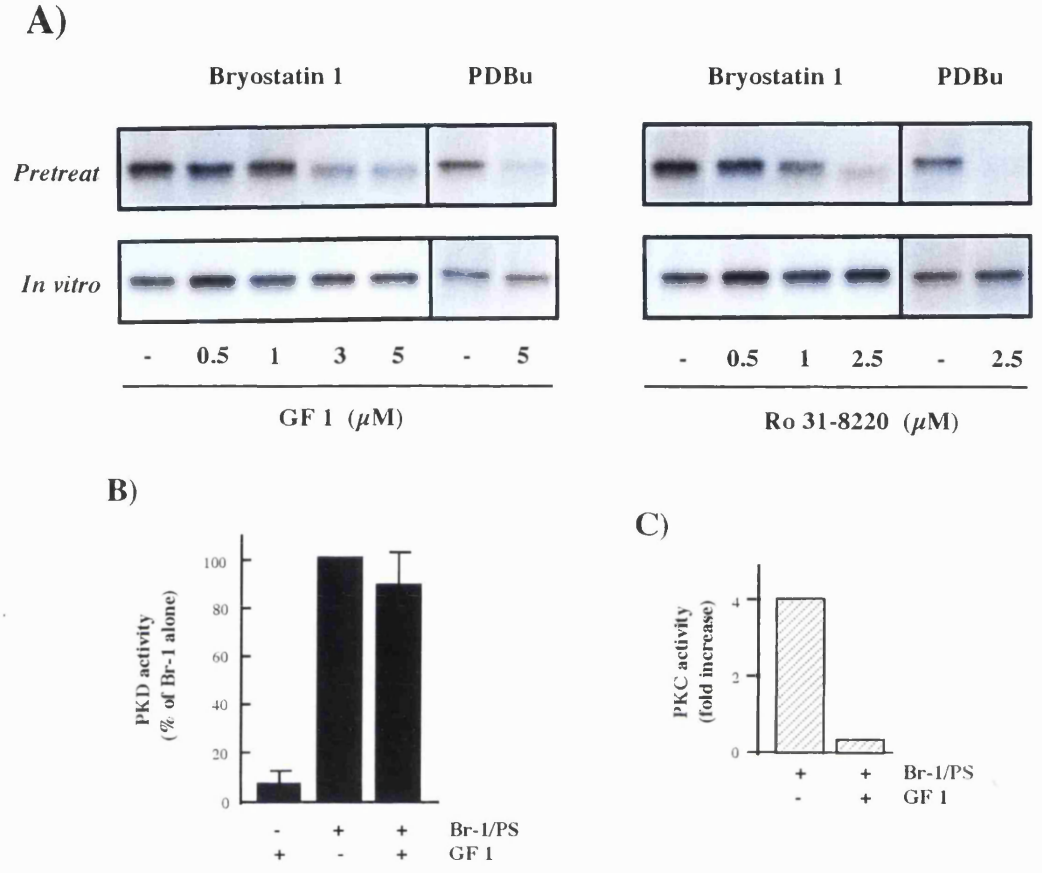


Fig. 3.11. Bryostatin 1 induces PKD activity through a PKC-dependent pathway in intact cells.

(a) *Upper panels*: Confluent and quiescent cultures of Swiss 3T3 fibroblasts were pretreated with increasing concentrations of GF 109203X; Ro 31-8220 or solvent (-) as indicated for 1 h. Cells were subsequently stimulated with either 10 nM bryostatin 1 for 30 min or 200 nM PDBu for 10 min, lysed and PKD immunoprecipitated with the PA-1 antiserum. PKD activity (autophosphorylation) was determined by *in vitro* kinase assays, SDS-PAGE and autoradiography. *Lower panels*: Parallel cultures of Swiss 3T3 fibroblasts were treated with either 10 nM bryostatin 1 for 30 min or 200 nM PDBu for 10 min, lysed and PKD immunoprecipitated using the PA-1 antiserum. PKD activity was determined by *in vitro* kinase assays carried out in the absence (-) or presence of either GF 109203X, Ro 31-8220 or solvent (-) added directly to the phosphorylation mixture at concentrations identical to those used to pretreat intact cells. Samples were then analysed by SDS-PAGE and autoradiography. Results shown are representative of three independent experiments.

(b) COS 7 cells were transiently transfected with a pcDNA3-PKD expression construct and after 72 h lysed. PKD was immunoprecipitated with the PA-1 antibody and subsequently eluted from the immunocomplexes. *In vitro* kinase assays was performed by incubating eluted PKD with [γ-^{32}P]-ATP in the presence of 50 nM bryostatin 1 (Br-1); 125 μg/ml phosphatidyl-L-serine (PS) and 3.5 μM GF 109203X (GF1) as indicated at 30°C for 10 min, followed by SDS-PAGE, autoradiography and scanning densitometry. Results are expressed as a percentage of the maximum PKD activity induced by bryostatin 1/PS and are the mean ± s.e. of three independent experiments.

(c) 5 ng purified PKC (containing a mixture of α, β and γ isoforms) was activated *in vitro* with 50 nM bryostatin 1 (Br-1) and 125 μg/ml phosphatidyl-L-serine (PS) (in PKD kinase buffer containing 1 mM $CaCl_2$) for 10 min at 30°C in the absence (-) or presence (+) of 3.5 μM GF 109203X (GF1). PKC activity was measured in a syntide-2 phosphorylation assay. The data (mean of duplicate points, range <5%) is shown expressed as the fold increase in syntide-2 phosphorylation over that observed with control non-activated samples. Similar results were observed in a second independent experiment.

not prevent direct *in vitro* activation of PKD by bryostatin 1 and phosphatidyl-L-serine. In contrast, activation of purified PKCs by bryostatin 1 and phosphatidyl-L-serine was strikingly inhibited by the inclusion of GF 1 during *in vitro* kinase assays (Fig. 3.11c). Taken together these results indicate that bryostatin 1-mediated PKD activation occurs through a PKC-dependent pathway *in vivo.*

3.3. DISCUSSION.

Bryostatin 1 binds to and activates classical and novel PKC isoforms, although its biological actions frequently differ from those of other PKC activators such as phorbol esters (Blumberg and Pettit, 1992). The results presented in this chapter demonstrate that bryostatin 1, in common with phorbol esters, stimulates PKD catalytic activity in Swiss 3T3 fibroblasts, in secondary mouse embryo fibroblasts and in COS-7 cells transfected with a PKD expression vector. *In vitro*, PKD is directly activated by bryostatin 1 in combination with phosphatidyl-L-serine. Strikingly however, the activation of PKD, following treatment of intact cells with bryostatin 1 or PDBu, is controlled by specific phosphorylation event(s) that are regulated through a PKC-dependent signalling cascade *in vivo*.

3.3.1. Bryostatin 1 induces a biphasic activation of PKD *in vivo.*

Importantly, specific aspects of the regulation of PKD by bryostatin 1 were different from those induced by phorbol esters. Thus, the activation of PKD induced by bryostatin 1 was significantly delayed compared to that induced by the phorbol ester PDBu. Moreover, high concentrations of bryostatin 1 (e.g. 100 nM) were able to antagonise PKD activity induced by PDBu. Moreover, the activation of PKD mediated by bryostatin 1 followed a striking biphasic dose-response pattern which reflected the biphasic pattern of PKD phosphorylation induced by bryostatin 1.

As addressed in section 3.1.1., many of the biological responses induced by bryostatin 1 are characterised by a biphasic dose-response relationship (with a typical maximal effect occurring at ~10 nM) and suppression of phorbol ester-induced effects at higher concentrations. These include stimulation of growth of JB6 cells (Kraft *et al.*, 1988); sensitisation of human cervical carcinoma cells to cis-diamminedichloroplatinum (II) (Basu and Lazo, 1992); induction of cytokine secretion in mononuclear cells (Sredni *et al.*, 1990) and the induction of c-Jun protein in NIH 3T3 cells (Szallasi *et al.*, 1994).

The mechanism(s) underlying the biphasic biological effects induced by bryostatin 1 remain poorly understood at present. However, given that PKD activity is regulated through a PKC-dependent signalling pathway *in vivo*, control of these responses must lie upstream of PKD, potentially at the level of bryostatin 1 sensitive PKC enzymes. In support of this, the dose-response curve for the direct *in vitro* activation of PKD by bryostatin 1 was not biphasic. Interestingly, Blumberg and co-workers have described the biphasic down-regulation of PKCδ in response to chronic bryostatin 1 treatment, where high concentrations of bryostatin 1 specifically prevent phorbol ester-induced PKCδ

downregulation (Szallasi *et al.*, 1994). They subsequently went on to demonstrate that the catalytic domain of PKCδ was sufficient to confer this protective effect (Lorenzo *et al.*, 1997), indicating that this domain contains an isotype specific determinant involved in regulating this unique effect of bryostatin 1.

Many of the mechanisms underlying the unusual biological actions of the bryostatins remain unclear. However the biphasic, PKC-dependent activation of PKD by bryostatin 1 raises the intriguing possibility that at least some of the biphasic biological effects induced by bryostatin 1 could be mediated by active PKD.

3.3.2. Bryostatin 1-induced PKD activity is regulated by phosphorylation event(s) *in vivo*.

Stimulation of Swiss 3T3 fibroblasts with bryostatin 1 induces a persistent activation of PKD that is detectable by *in vitro* kinase assays (either autophosphorylation and syntide-2 phosphorylation assays) in the absence of any further activating agents. As PKD activity, induced by bryostatin 1 stimulation of intact cells, is maintained during cell lysis and immunoprecipitation it is likely that PKD is covalently modified upon activation *in vivo* and is locked in a catalytically active form, as demonstrated by the retarded mobility of activated PKD on SDS-PAGE analysis. The results presented in this chapter show that stimulation of intact Swiss 3T3 cells with bryostatin 1 markedly enhances the incorporation of ^{32}Pi into PKD in metabolically labelled cells. Furthermore, it was observed that treatment of bryostatin 1-stimulated Swiss 3T3 cell lysates with a protein phosphatase inhibitor (microcystin) partially prevented the inactivation of PKD by endogenous protein phosphatases. These results suggest a link between PKD phosphorylation and activity and are in agreement with several other studies which have also implicated a role for phosphorylation event(s) in the regulation of PKD activity in intact cells (Rennecke *et al.*, 1996; Van Lint *et al.*, 1998; Zugaza *et al.*, 1996).

3.3.3. PKC-dependent activation of PKD by bryostatin 1 *in vivo*.

Two distinct mechanisms could have accounted for PKD phosphorylation after bryostatin 1 treatment: one possibility was that an autophosphorylation event was responsible. Alternatively, trans-phosphorylation of PKD by an upstream bryostatin 1-sensitive kinase could regulate the phosphorylation of, and thus the activity of, PKD *in vivo*. Using different inhibitors that discriminate between different intracellular signalling cascades it was shown that GF I and Ro 31-8220 (inhibitors of classical and novel PKCs)

were able to specifically block bryostatin 1- or PDBu-induced PKD activation. Importantly, neither GF I09203X nor Ro 31-8220 inhibited PKD activity when added directly to *in vitro* kinase assays, demonstrating that these PKC inhibitors have no direct inhibitory effect on PKD. These results indicate that persistent PKD activation, induced by bryostatin 1 treatment of intact cells, is mediated through a PKC-dependent pathway.

Since the data described in this chapter has been generated the role of PKC enzymes as upstream regulators of PKD has been the subject of intensive investigation. Thus, both pharmacological (phorbol esters; bryostatin 1) and physiological stimuli which increase intracellular DAG levels (for example, neuropeptide agonists; endothelin; PDGF; angiotensin II) activate PKD via a PKC-dependent pathway in a variety of cell types, as demonstrated by the use of PKC-specific inhibitors (Paolucci and Rozengurt, 1999; Van Lint *et al.*, 1998; Zugaza *et al.*, 1996; Zugaza *et al.*, 1997). It should be noted that the specificity of GF I09203X and Ro 31-8220 as specific inhibitors of PKC enzymes has been questioned recently. For example GF I09203X and Ro 31-8220 will also inhibit $p70^{S6}$ Kinase and Rsk-2 (Alessi, 1997c) as well as CDC-2 kinase, Src and the cAMP-dependent protein kinase (Goekjian and Jirousek, 1999), at similar concentrations to those which inhibit classical and novel PKCs. Moreover, Ro 31-8220 (but not GF 109203X) has been reported to stimulate the stress-activated protein kinase JNK1 and induce the expression of c-Jun independently of PKC (Beltman *et al.*, 1996). However distinct inhibitors of these other kinases do not block the activation of PKD by various agonists *in vivo* (Chapter 3 and (Zugaza *et al.*, 1996; Zugaza *et al.*, 1997)) indicating that classical/novel PKC enzymes function as upstream kinases for PKD.

Indeed several elegant genetic studies have now confirmed the regulation of PKD via a PLC-DAG-PKC signalling cascade *in vivo.* Van Lint and co-workers demonstrated that PLCγ is both necessary and sufficient for the activation of PKD by using various effector mutants of the PDGF receptor stably expressed in A341 fibroblasts (Van Lint *et al.*, 1998). A PDGF receptor mutant that lacks a PLCγ docking site (but with intact PI3-Kinase, SHP-2 and Ras-GAP binding sites) is unable to activate PKD and a PDGF receptor mutant containing only the PLCγ docking site activates PKD as efficiently as the wild-type receptor. Moreover, PKD is not activated following BCR ligation in PLCγ-deficient B cells (Sidorenko *et al.*, 1996). In addition, co-expression of constitutively activated mutants of classical (β_I) or novel (ε, η), but not atypical (ξ), PKCs with wild-type PKD in COS-7 cells increases the basal level of PKD activity to maximal levels in the absence of extracellular

stimuli (Zugaza *et al.*, 1996). Thus classical/novel PKC enzymes are necessary and sufficient to regulate PKD activity.

The activity of multiple serine/threonine kinases is regulated by specific phosphorylation events within the catalytic domain of these kinases (Hanks and Hunter, 1995). Recently, two critical activating phosphorylation sites within the activation loop of PKD have recently been identified at residues serine 744 and serine 748 (Iglesias *et al.*, 1998b). Mutation of S744 and S748 to neutral non-phosphorylatable alanine residues produces a kinase-dead PKD molecule, whilst a double glutamic acid mutation at these residues (to mimic phosphorylated residues) results in a constitutively activated kinase (Iglesias *et al.*, 1998b). Moreover, data obtained using a kinase-defective PKD mutant indicates that these two activation loop sites are trans-phosphorylated by an upstream kinase *in vivo* (Iglesias *et al.*, 1998b), a process that is blocked upon inhibition of classical and novel PKC enzymes (Waldron *et al.*, 1999a). Thus, phosphorylation of the PKD activation loop S744 and S748 residues, via a PKC-dependent mechanism, is both necessary and sufficient to activate PKD (Iglesias *et al.*, 1998b; Waldron *et al.*, 1999a).

Although two phosphorylation sites within the activation loop of PKD (at S744 and S748) have been identified and characterised it is clear that a number of other residues within PKD are also subject to phosphorylation *in vivo.* The data presented in Chapter 4 identifies an autophosphorylation site located at the carboxy-terminus of PKD and describes the generation of a phospho-specific antibody against this site which provides a valuable tool with which to study the regulation of PKD activity *in vivo.*

CHAPTER 4 : Characterisation of S916 as a PKD autophosphorylation site

4.1. INTRODUCTION

The activity and/or stability of many intracellular serine/threonine kinases is frequently regulated by protein phosphorylation events. These phosphorylation events can either play a direct role in the activation process, as occurs for PKB/Akt (Alessi and Cohen, 1998; Downward, 1998) or can function indirectly to release an autoinhibitory domain, for example $p70^{S6}$ kinase (Pullen and Thomas, 1997). In contrast, phosphorylation events for the PKC enzymes are priming steps that ensure maximal catalytic competency upon subsequent ligand-induced activation (see section 1.2.5). The catalytic activity of PKD is also regulated by two critical phosphorylation sites located within the activation loop of PKD, at residues S744 and S748 (Iglesias *et al.*, 1998b; Waldron *et al.*, 1999a), as discussed in section 3.3.3. These sites are not autophosphorylation sites but are rather transphosphorylated by a PKC-dependent signalling cascade (Iglesias *et al.*, 1998b; Waldron *et al.*, 1999a).

Phosphoamino-acid analysis indicates that active PKD (stimulated *in vitro* or *in vivo*) is subject to multiple serine phosphorylation events (Johannes *et al.*, 1995; Johannes *et al.*, 1994; Sidorenko *et al.*, 1996). Two-dimensional tryptic phosphopeptide mapping of ^{32}Pi-labelled, activated PKD has demonstrated that there are at least 8 other major, as yet unknown, phosphorylation sites within PKD besides the activation loop sites S744 and S748 (Iglesias *et al.*, 1998b). These include sites that are basally phosphorylated and sites that are inducibly phosphorylated, either through transphosphorylation (by a distinct upstream kinase) or autophosphorylation (*in cis* or *in trans*) mechanisms.

Nishikawa and co-workers have described the optimal peptide substrate sequences for a number of different PKC isoforms, including that of PKD (Nishikawa *et al.*, 1997). Using an oriented peptide library they determined that PKD preferentially phosphorylates peptides which contain a leucine residue five positions (-5) upstream from the phosphorylated serine; an arganine residue at position -3 and hydrophobic residues at positions -1 and +1 to +4, respectively (Table 4.1). Peptides containing this optimal PKD substrate phosphorylation sequence are not efficiently phosphorylated by other members of the PKC superfamily (Nishikawa *et al.*, 1997).

	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6
Optimal PKD sequence[A]		L	V	R	Q	M	**S**	V	A	F	F	F	
GS peptide[B]		L	S	R	T	L	**S**	V	A	A	L	L	
Syntide-2[C]	P	L	A	R	T	L	**S**	V	A	G	L	P	G
ε-peptide[D]	P	R	K	R	Q	G	**S**	V	R	R	R	V	

TABLE 4.1. The consensus PKD autophosphorylation motif is found within a number of PKD substrate peptides. [A]The optimal PKD substrate sequence described by (Nishikawa *et al.*, 1997). [B]GS and [C]Syntide-2 peptides are based on phosphorylation site II of Glycogen Synthase. [D]ε-pseudosubstrate peptide. The predicted optimal substrate sequence for PKD contains a leucine residue at position -5 upstream of the phosphorylated serine; an arganine residue at position -3 and hydrophobic residues at positions -1 and +1 to +4. The sequences of the GS and Syntide-2 peptides match these criteria and are known to be good *in vitro* substrates for PKD (Dieterich *et al.*, 1996; Valverde *et al.*, 1994; Van Lint *et al.*, 1995). Poor *in vitro* substrates for PKD, e.g. the ε-pseudosubstrate peptide (Valverde *et al.*, 1994), lack a critical leucine residue at position -5. Moreover, the PKC substrate MARCKS is only poorly phosphorylated by PKD (Dieterich *et al.*, 1996) and none of the phosphorylation sites in this protein contain a leucine residue at position -5.

Preliminary observations from the Rozengurt laboratory and from (Rennecke *et al.*, 1996) have indicated that the carboxy-terminal region of PKD is covalently modified in active PKD. Phosphorylation events are known to occur within the carboxy-terminal region of many serine/threonine kinases, including PKC enzymes, PKB/Akt, p70S6 Kinase. The experiments described in this chapter were designed to investigate a potential role for phosphorylation event(s) within the carboxy-terminus of PKD.

4.1.1. Aims.

1) Identify potential *in vivo* phosphorylation site(s) in the carboxyl-tail of PKD.
2) To identify mechanisms regulating these phosphorylation event(s).
3) To establish a system to assay PKD phosphorylation/activity *in vivo*.

4.2. RESULTS.

4.2.1. Post-translational modification of the C-terminal region of PKD upon activation.

Previous studies have demonstrated the reduced immunoreactivity of C-terminal PKD antibodies for activated versus quiescent PKD protein in western blotting experiments (Rennecke *et al.*, 1996) and the Rozengurt laboratory (unpublished data). This observation is illustrated in Figure 4.1 where cell lysates, prepared from murine Swiss 3T3 fibroblasts treated with increasing concentrations of PDBu, were analysed by western blotting using the C-terminal PA-1 PKD antibody. The data shows that as PKD was activated by increasing concentrations of PDBu, the immunoreactivity of the PA-1 antibody for PKD decreased (Fig. 4.1b). Similar results were observed when whole cell extracts prepared from PDBu-treated B cells were analysed by western blotting using a commercial antibody (sc-935) directed against the C-terminus of PKD (Fig. 4.1c).

The differential recognition of quiescent versus activated PKD by the C-terminal antibodies was not due to PKD degradation since increasing levels of PKD catalytic activity (as measured by PKD autophosphorylation) were observed under these conditions (Fig. 4.1b/c). Furthermore, a monoclonal antibody directed against the N-terminus of PKD is able to recognise PKD isolated from resting and from phorbol ester-treated cells equally well (Rennecke *et al.*, 1996). Experiments where activated PKD was dephosphorylated by incubation with protein phosphatases have indicated that phosphorylation event(s) are responsible for mediating the impaired recognition of active PKD by C-terminal antibodies (see Figure 3.9a and (Rennecke *et al.*, 1996)). Thus, the C-terminal region of PKD undergoes a conformational change upon activation as the result of a phosphorylation event(s), which masks the epitope recognised by C-terminal PKD antibodies.

Given that active PKD is phosphorylated on multiple serine residues *in vivo* a plausible explanation for the above observation was that specific residue(s) within the C-terminus of PKD are phosphorylated during the activation process. A single serine residue present at the extreme carboxy-terminus of PKD, within the epitope recognised by C-terminal antibodies, is conserved between the murine and human homologues of PKD, as indicated in Table 4.2.

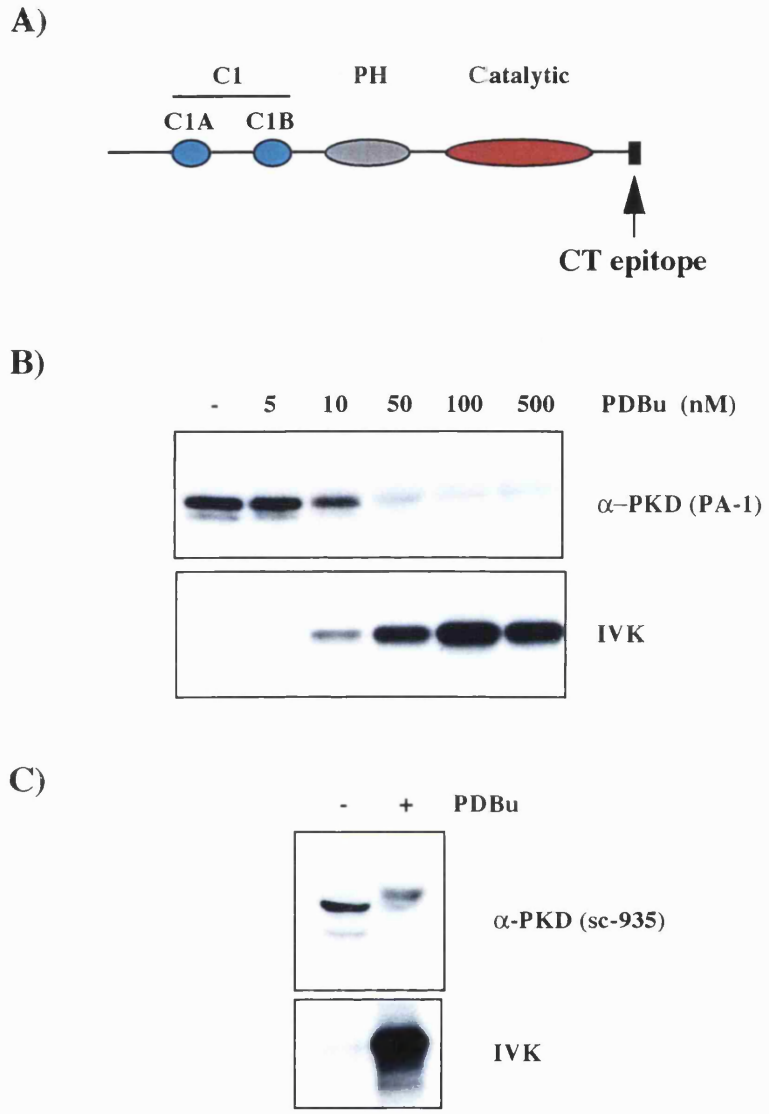


Fig. 4.1. Reduced recognition of active PKD by C-terminal antibodies.
(A) Schematic representation of the epitope of interest (residues 904-918) in murine PKD recognised by C-terminal PKD antibodies. (CT = C terminal).
(B) Confluent and quiescent Swiss 3T3 fibroblasts were left unstimulated (-) or were treated with increasing concentrations of PDBu (5-500 nM) for 10 min before the cells were lysed. Either total cell lysates were subjected to SDS-PAGE and western blot analysis with the PA-1 antibody (upper panel) or PKD was immunoprecipitated with the PA-1 antibody and analysed by *in vitro* kinase assays, SDS-PAGE and autoradiography. PKD activity was measured by autophosphorylation (lower panel).
(C) Quiescent A20 B lymphocytes were left unstimulated (-) or treated with 200 nM PDBu for 10 min (+) before the cells were lysed and subjected to SDS-PAGE and western blot analysis with a commercial C-terminal PKD antibody (sc-935), (upper panel). Alternatively, PKD was immunoprecipitated from the lysates (using the PA-1 antibody) and PKD activity (as shown by autophosphorylation) was measured by *in vitro* kinase assays (lower panel).

PKD (murine)	E	E	R	E	M	K	A	L	S	E	R	V	**S**	I	L^{918}
PKCμ (human)	E	E	T	E	M	K	A	L	G	E	R	V	**S**	I	L^{912}

TABLE 4.2. Alignment of the C-terminal regions of murine and human PKD, recognised by the PA-1 antiserum. The position of a conserved serine residue at position 916 (m) or 910 (h) is highlighted.

Moreover, the amino-acid sequence surrounding this residue, at serine 916, shows striking homology to the described optimal substrate sequence for PKD, with S916 surrounded by a leucine residue at position -5, a basic arganine residue at position -2 and also hydrophobic residues at positions -1 and +1/2. This raised the possibility that serine 916 may be an *in vivo* autophosphorylation site for PKD.

4.2.2. PKD phosphorylates a synthetic peptide from the C-terminus of PKD *in vitro*.

To test the hypothesis that serine 916 may be an autophosphorylation site for PKD, experiments were carried out to determine whether activated PKD could phosphorylate a synthetic peptide corresponding to the C-terminal region of PKD surrounding the S916 site (residues 904-918) *in vitro*. As shown in Figure 4.2a, activated PKD, immunoprecipitated from phorbol ester-treated cells, was able to efficiently phosphorylate both this C-terminal PKD peptide and the Syntide-2 peptide (a known *in vitro* substrate for PKD) in *in vitro* kinase assays. Linear kinetics of C-terminal peptide phosphorylation by activated PKD were observed during *in vitro* kinase assays (Fig. 4.2b) and phosphorylation of the C-terminal peptide by active PKD was dependent on the peptide substrate concentration, exhibiting saturation at ~1 mM (Fig. 4.2c). In contrast, non-active PKD immunoprecipitated from resting cells could not phosphorylate the C-terminal PKD peptide or the Syntide-2 peptide (Fig. 4.2a). These results indicate that S916 could potentially be a PKD autophosphorylation site.

A number of different experimental approaches could have been used to determine whether the serine 916 was phosphorylated *in vivo*. Mass spectrometry; tryptic phosphopeptide mapping and site directed mutagenesis are all widely used techniques to identify *in vivo* phosphorylation sites within proteins. More recently, the generation of phosphorylation state-specific antibodies as a method to identify and characterise putative phosphorylation sites has been described for a variety of proteins (Czernik *et al.*, 1991), including serine/threonine kinases such as $p70^{S6K}$ (Weng *et al.*, 1998); PKB/Akt; MAP

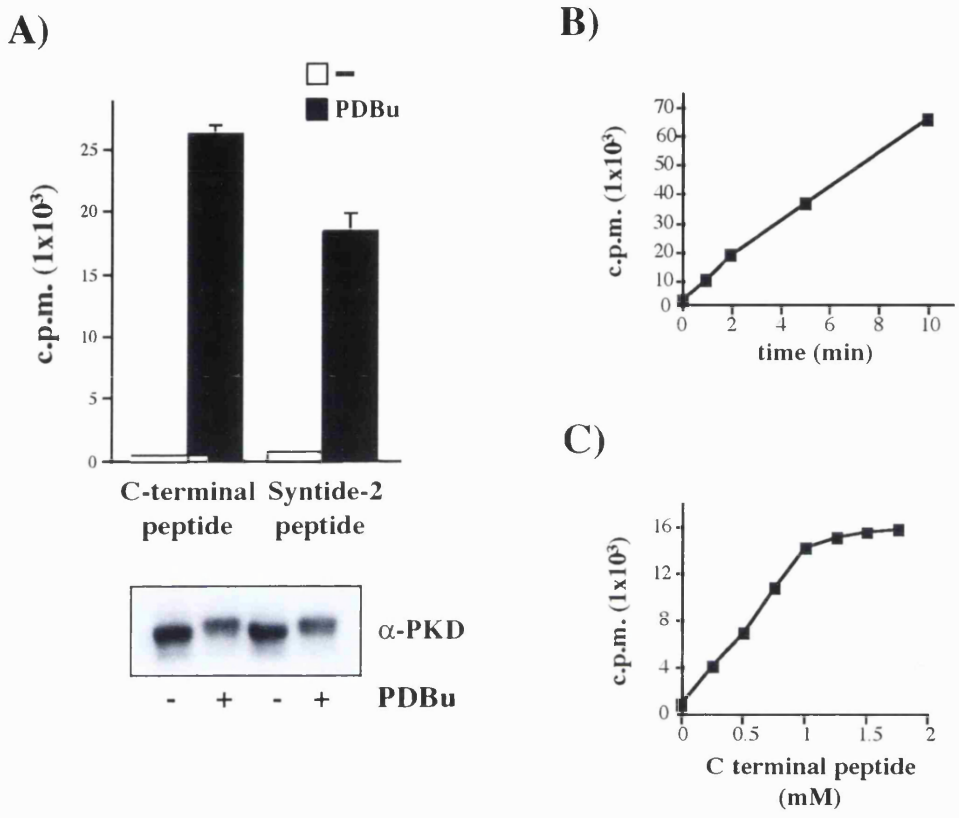


Fig. 4.2. Active PKD phosphorylates a synthetic peptide of the C-terminus of PKD *in vitro*.
COS-7 cells were transiently transfected with an N-terminally Myc-tagged wild-type PKD construct, the generation of which is described in Chapter 2.
(A) Cells were left unstimulated (white bars) or treated with 50 ng/ml PDBu for 10 min (black bars). PKD was immunoprecipitated from whole cell lysates using a 9E10 mAb (which recognises the Myc epitope) and phosphorylation of the Syntide-2 peptide or of a peptide corresponding to the C-terminal 904-918 residues of murine PKD was assayed in *in vitro* kinase assays. Peptides were used at 2.5 mg/ml, final concentration. Results represent the mean ± s.e. c.p.m. incorporated into the peptides from two independent experiments, each performed in triplicate. Western blot analysis of cell lysates (sc-935) shows equivalent PKD expression levels.
(B) ^{32}P-γATP incorporation into 1 mM of the C-terminal peptide, by PDBu-activated PKD, was linear over the 10 minute assay period. Similar results were observed in 2 independent experiments.
(C) ^{32}P-γATP incorporation into different amounts of the C-terminal peptide (0-1.5 mM), after assaying for 10 minutes, by active PKD isolated from phorbol ester-treated cells was saturable at ~1 mM in 4 individual experiments.

Kinases and members of the PKC family (Le Good *et al.*, 1998; Ng *et al.*, 1999a). An antibody that would specifically recognise PKD molecules phosphorylated on S916 would allow the question of whether the S916 residue of PKD is phosphorylated *in vivo* to be addressed. Importantly, generation of an antibody specifically reactive against phosphorylated, active PKD would also provide an invaluable tool for studying the *in vivo* regulation of PKD activity. This would reduce the requirement for experiments involving radioactivity, such as immunocomplex kinase assays (which are an *in vitro* measure of PKD activity) or *in vivo* labelling techniques. For these reasons an attempt was made to generate an antiserum that would selectively react with PKD molecules phosphorylated on serine 916. This approach, in combination with site-directed mutagenesis allowed an investigation into potential phosphorylation events(s) at the carboxy-terminal serine 916 residue of PKD.

4.2.3. Generation of a phospho-antibody directed against the S916 residue of PKD.

A phosphopeptide corresponding to the C-terminal 15 residues of murine PKD (^{904}EEREMKALSERVS^{916}IL^{918}) was synthesised by the ICRF Peptide Synthesis Department, where the residue corresponding to serine 916 was phosphorylated (Fig. 4.3a). This S916 phosphopeptide was then coupled to Keyhole Limpet Hemocyanin and used to immunise rabbits, according to standard immunisation techniques (Collawn and Paterson, 1997).

The immunoreactivity of the resulting antiserum for different murine PKD C-terminal peptides was then examined by ELISA assays. As demonstrated in Figure 4.3b, the antiserum raised against the S916-phosphopeptide displayed a strong immunoreactivity with the S916-phosphopeptide as compared to the non-phosphorylated peptide. Importantly, the pS916 antiserum showed very low crossreactivity with a C-terminal peptide that was phosphorylated at the S912 position (Fig. 4.3b). These data indicated that an antiserum had been generated that could specifically recognise a PKD C-terminal peptide phosphorylated at the serine 916 position *in vitro*.

4.2.4. The pS916 antiserum specifically recognises PDBu-activated PKD ectopically expressed in COS-7 fibroblasts.

Subsequently, experiments were carried out to determine whether the pS916 antiserum could recognise intact PKD isolated from quiescent or PDBu-stimulated cells in

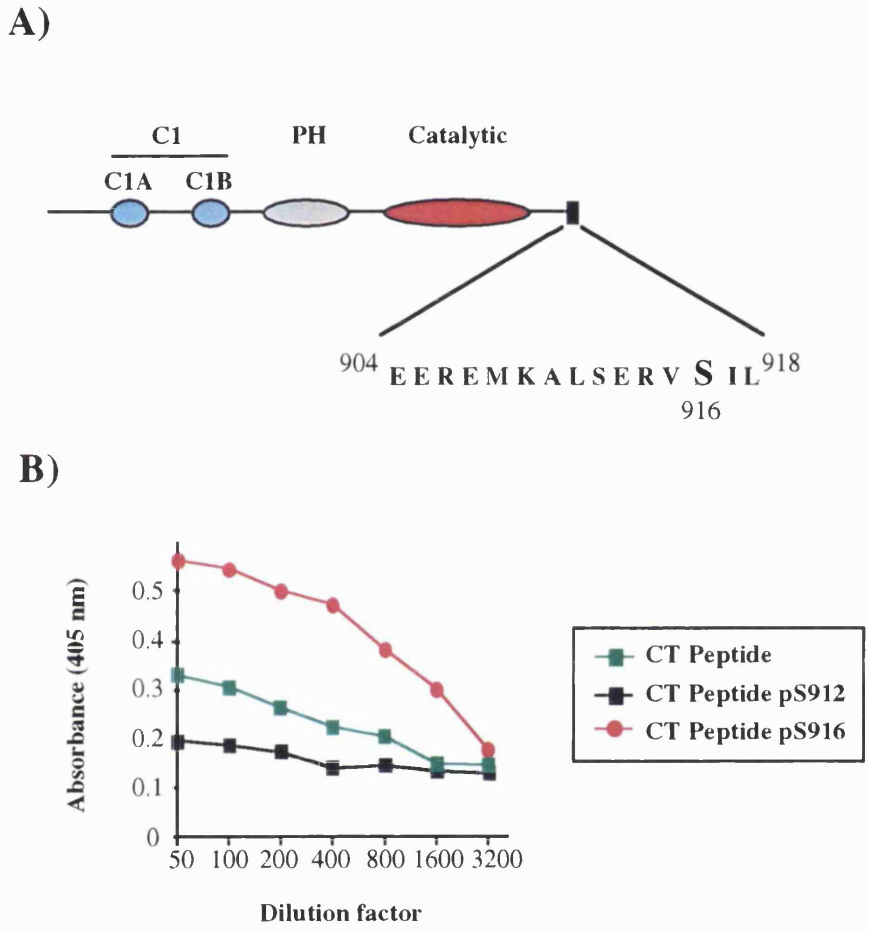


Fig. 4.3. Generation of a phospho-specific antibody against the C-terminal S916 residue of PKD.

(A) Schematic representation of murine PKD showing the C-terminal amino-acid sequence of interest. The S916 residue is indicated.

(B) A synthetic phosphopeptide based on this C-terminal sequence, with serine 916 as the phosphorylated residue, was used to generate a site-specific phospho-PKD antibody. ELISA immunoreactivity of the pS916 antiserum with different C-terminal PKD peptides (either non-phosphorylated; S912 phosphorylated or S916 phosphorylated) is shown. Results are representative of two independent experiments. (CT = C-terminal peptide).

western blotting experiments. COS-7 cells (which express very low levels of endogenous PKD) were transiently transfected with a cDNA construct encoding wild-type PKD with an N-terminal Myc-tag, constructed as described in section 2.18. The cells were subsequently left unstimulated or were treated with PDBu prior to lysis. The data shown in Figure 4.4 indicates that the pS916 antiserum strongly recognised a single protein band which migrated at the expected size for Myc-tagged PKD (~115kDa) both in whole cell lysates and in anti-Myc immunoprecipitates prepared from PDBu-treated COS-7 cells. In contrast, the pS916 antiserum only very weakly recognised PKD isolated from unstimulated COS-7 cells (Fig. 4.4). The ability of the pS916 antibody to recognise ectopically expressed, active PKD was consistent with the hypothesis that the S916 residue of PKD is indeed phosphorylated *in vivo* upon activation of PKD.

4.2.5. The S916 antibody recognises an *in vivo* autophosphorylation site for PKD.

Having established that the pS916 antiserum recognises active PKD phosphorylated *in vivo* in response to PDBu-treatment, the mechanism controlling this phosphorylation event was addressed. Two alternative mechanisms were possible: phosphorylation of PKD on the site recognised by the pS916 antisera could occur as the result of transphosphorylation by an upstream kinase (as occurs for the activation loop sites S744 and S748). Alternatively this site could be phosphorylated by an autophosphorylation mechanism following stimulation of PKD catalytic activity. To differentiate between these two potential mechanisms the ability of the pS916 antibody to recognise different PKD mutants that display different activity states was assessed.

A catalytically inactive PKD mutant (D733A) has been described previously, in which the functionally critical aspartic acid at position 733 within the DFG motif of the PKD kinase domain has been mutated to alanine (Iglesias *et al.*, 1998b). The data presented in Figure 4.5, demonstrates that this kinase-dead PKD mutant, isolated from either quiescent or PDBu-stimulated cells was not reactive with the pS916 antibody. Similarly, a catalytically inactive PKD mutant in which the ATP binding site of PKD is mutated (K618M) (Zugaza *et al.*, 1996) was not recognised by the pS916 antiserum either in non-stimulated or in PDBu-treated cells (data not shown). In contrast, active wild-type PKD stimulated by PDBu showed strong immunoreactivity with the pS916 antiserum (Fig. 4.5).

In addition the reactivity of the pS916 antiserum for different constitutively active mutants of PKD was examined: namely a deletion mutant lacking the entire PH domain

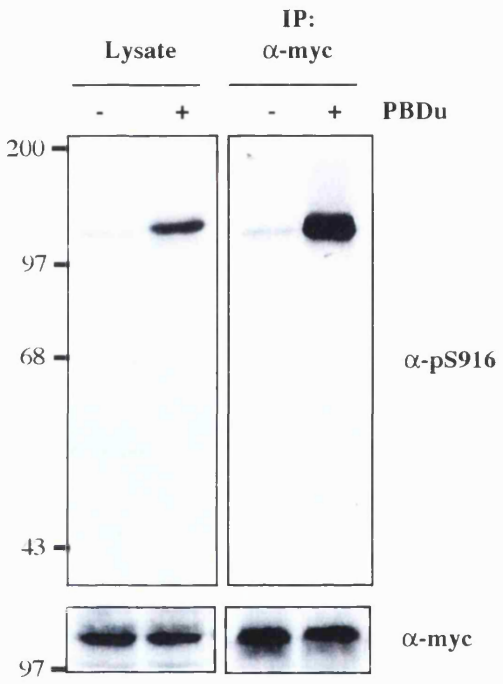


Fig. 4.4. The pS916 antibody recognises phorbol ester-activated PKD transiently expressed in COS-7 cells.

COS-7 fibroblasts were transiently transfected with wild-type Myc-PKD and used for experimental purposes 48 h later. Cells were left unstimulated (-) or were treated with 50 ng/ml PDBu for 10 min (+) before lysis. Subsequently, either total cellular proteins were acetone precipitated from the lysates (left), or PKD was immunoprecipitated using a 9E10 mAb (right). Samples were analysed by SDS-PAGE and western blotting with the pS916 antibody (upper panel). Reprobing with a 9E10 monoclonal antibody confirmed equal loading of protein samples (lower panel). Similar results were obtained in two independent experiments.

Molecular weight markers (kDa) are indicated on the left.

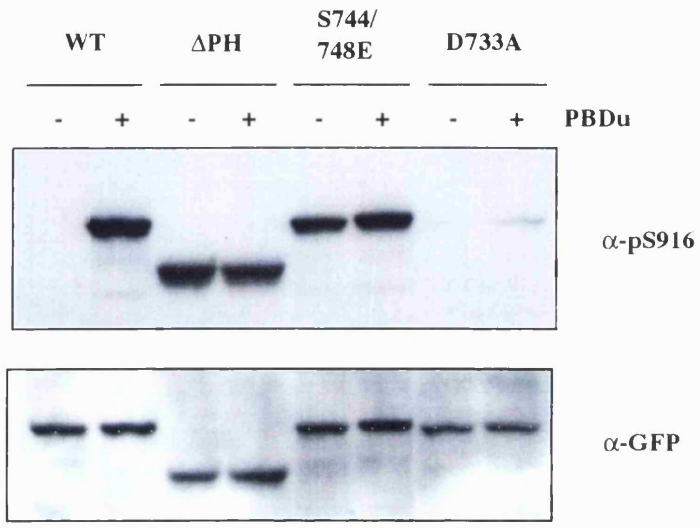


Fig. 4.5. S916 is an *in vivo* PKD autophosphorylation site.
COS-7 cells were transiently transfected with either wild-type PKD (WT) or different PKD mutants, either lacking the PH domain (ΔPH), or containing single or double amino-acid substitutions within the activation loop (D733A and S744/748E, respectively). All the PKD proteins were fused to a N-terminal GFP tag, as described in Chapter 2. After 48 h, cells were left unstimulated (-) or were treated with 50 ng/ml PDBu for 10 min (+). Whole cell lysates were prepared and analysed by SDS-PAGE and western blotting. Upper panel, western blot analysis with the pS916 antibody. Lower panel, reprobe with a GFP mAb (ICRF). Similar results were obtained in two independent experiments.

(ΔPH) and a mutant containing acidic substitutions at residues S744 and S748 (S744/748E), critical activating phosphorylation sites within the activation loop of PKD. The constitutive activity of these mutants has been previously described (Iglesias and Rozengurt, 1998c; Iglesias *et al.*, 1998b). The results presented in Fig. 4.5, show that in contrast to wild-type PKD, which has negligible reactivity with the pS916 antibody in non-stimulated cells, these two constitutively active PKD mutants exhibited a high basal level of immunoreactivity with the pS916 antiserum that was not further increased by phorbol ester treatment. An activated PKD deletion mutant lacking the entire DAG/phorbol ester binding C1 domain (Iglesias and Rozengurt, 1999) was also maximally phosphorylated on the site recognised by the pS916 antiserum in resting cells (data not shown). Collectively, these data demonstrate that the pS916 antibody recognises an *in vivo* autophosphorylation site for PKD.

4.2.6. The pS916 antiserum specifically recognises endogenous, active PKD in B cells.

The data presented above demonstrates that the pS916 antiserum selectively recognises a PKD autophosphorylation site that is regulated in response to phorbol ester treatment in a transient transfection system. It was therefore important to determine whether endogenous PKD present in cells was also inducibly recognised by the pS916 antiserum after phorbol ester treatment or in response to physiological agonists.

The pattern of PKD expression is ubiquitous but is highest in tissues of hematopoetic origin, including the thymus and peripheral blood lymphocytes (Johannes *et al.*, 1994; Rennecke *et al.*, 1996; Sidorenko *et al.*, 1996). It has also been demonstrated that the catalytic activity PKD is regulated by antigen receptors in both T and B lymphocytes (Sidorenko *et al.*, 1996). Therefore lymphocyte model systems were chosen to study the regulation this autophosphorylation event *in vivo.* In initial experiments, cells from a murine A20 B lymphocyte cell line were left unstimulated or were treated with 50 ng/ml PDBu for 10 minutes before whole cell lysates were prepared and western blot analysis carried out, firstly with the pS916 antiserum and subsequently with a pan C-terminal PKD antibody. As shown in Figure 4.6a, the pS916 antiserum showed no immunoreactivity with protein extracts prepared from unstimulated B cells but specifically recognised a single protein band migrating at ~110-120 kDa in extracts prepared from PDBu-treated B cells. Reprobing with a pan C-terminal PKD antibody demonstrated that the pS916-immunoreactive protein pocessed an identical electrophoretic mobility in SDS-PAGE

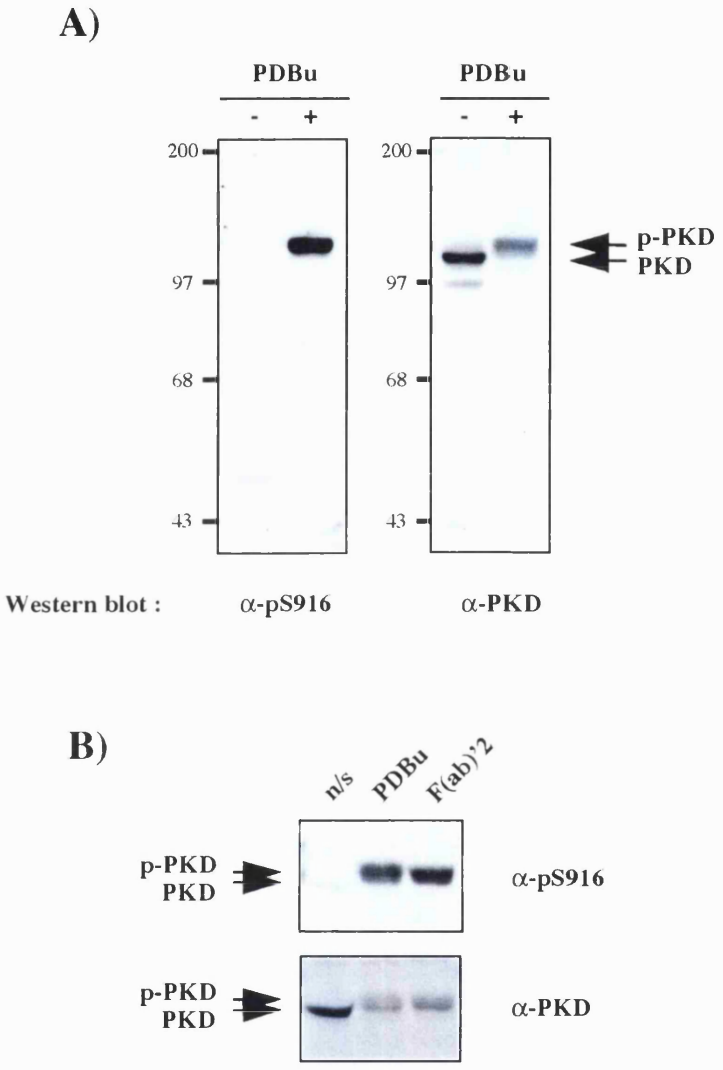


Fig. 4.6. The pS916 antibody recognises endogenous active PKD present in phorbol ester- and antigen receptor-stimulated B lymphocytes.

(A) A20 B lymphocytes were left untreated (-) or were treated with 50 ng/ml PDBu for 10 min (+) before lysis and acetone precipitation of total cellular proteins. Proteins were separated by SDS-PAGE and western blotted with the pS916 antibody (left panel) and subsequently reprobed with a pan C-terminal PKD antibody (sc-935), (right panel). Similar results were obtained in three independent experiments.

(B) A20 B lymphocytes were left untreated (n/s) or were treated with either 50 ng/ml PDBu for 10 min or 10 μg/ml F(ab)'2 anti-mouse IgG for 2 min before lysis and acetone precipitation of total cellular proteins. Proteins were separated by SDS-PAGE and western blotted with the pS916 antibody (upper panel) and subsequently reprobed with a pan C-terminal PKD antibody (sc-935), (lower panel). Similar results were obtained in four independent experiments.

as phorbol ester-activated PKD, which itself migrates more slowly than non-activated PKD (Fig. 4.6a).

Since the pS916 antiserum could selectively recognise endogenous active PKD isolated from pharmacologically-stimulated B cells, the immunoreactivity of the pS916 antibody for PKD isolated from antigen receptor-activated lymphocytes was investigated. The B cell antigen receptor (BCR) complex activates PKD (Sidorenko *et al.*, 1996) and so pS916 immunoreactivity under these conditions was examined. As indicated in Figure 4.6b, the pS916 antiserum strongly reacted with PKD isolated from B cells in which the BCR complex had been stimulated with F(ab)'2 fragments of anti-mouse IgG, but not with PKD isolated from non-stimulated cells. These results confirmed that the pS916 antiserum could selectively and potently react with the endogenous active PKD present in both phorbol ester- and antigen receptor-stimulated B cells. Importantly, the pS916 antiserum did not cross-react with other endogenous cellular proteins, including members of the PKC-superfamily (which are ~80 kDa in size).

4.2.7. Specificity of the pS916 antibody for PKD phosphorylated on S916.

Whilst the data presented in Figures 4.4-4.6 revealed that the pS916 antibody was selectively reactive with phosphorylated active PKD (either endogenous or ectopically expressed), it did not prove that this antibody was recognising PKD molecules that were phosphorylated on S916. Active PKD is phosphorylated on multiple serine residues *in vivo* and so the possibility existed that the pS916 antibody was also reactive to other phosphorylated serine residues. To exclude this possibility the specificity of the pS916 antiserum for the carboxy-terminal serine 916 residue of PKD was investigated in detail.

As shown in Figure 4.7, the reactivity of the pS916 antiserum for activated PKD was completely blocked by competition with the pS916 immunising phosphopeptide in western blot analyses. In contrast a phosphopeptide (containing a phosphorylated S744 residue) from the activation loop of PKD did not block the interaction of the pS916 antibody with activated PKD (Fig. 4.7). A low level of weak reactivity of the pS916 antiserum for PKD isolated from quiescent cells was variably observed in western blotting experiments (see for example Figs. 4.4 and 4.7). The ELISA data presented previously in Figure 4.3 indicated that the pS916 antiserum could also weakly crossreact with a non-phosphorylated C-terminal PKD peptide. In agreement with these results, the non-phosphorylated C-terminal PKD peptide was found to weakly compete the binding of the pS916 antiserum to active PKD in western blot analyses (Fig. 4.7). Taken together, these

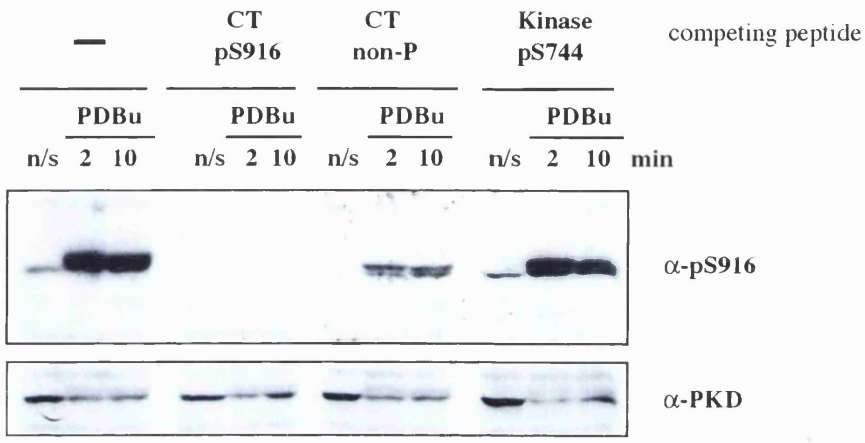


Fig. 4.7. Specificity of the pS916 antibody - I

Lysates from unstimulated (n/s) A20 B lymphocytes or from A20 B lymphocytes treated with 50 ng/ml PDBu for 2 or 10 min were analysed by SDS-PAGE and western blotting with either the pS916 antibody alone or with the pS916 antibody plus 5 μg/ml (final concentration) of different competing peptides, as indicated (upper panel). Reprobing with a pan C-terminal PKD antibody (sc-935) confirmed equivalent loading of protein samples (lower panel). Data are representative of two independent experiments.

(CT = C-terminal; Kinase = kinase domain).

data would suggest that the pS916 antiserum specifically recognises the phosphorylated carboxy-terminal region of active PKD, but that the pS916 antiserum can weakly cross-react with non-active PKD.

To further confirm the specificity of the pS916 antibody for the carboxy-terminal S916 site its reactivity against two PKD mutants was assessed: one containing a deletion of the C-terminal 23 residues (896-918) of PKD (ΔCT), thus lacking the serine 916 residue, and one containing a single amino-acid substitution where the serine at position 916 was replaced by a neutral non-phosphorylatable alanine residue (S916A). The generation of these constructs, each with an N-terminal Myc-tag, is detailed in section 2.18 and they are shown schematically in Figure 4.8a. A20 B lymphoma cells, transiently expressing either Myc-PKD wild type; Myc-PKD ΔCT or Myc-PKD S916A, were left unstimulated or were treated with PDBu or with F(ab)'2 to activate the BCR complex. Whole cell lysates were subsequently prepared and analysed by SDS-PAGE and western blotting. The Myc-tagged PKD proteins migrated more slowly than endogenous PKD upon SDS-PAGE allowing discrimination between the endogenous PKD proteins and the ectopically expressed Myc tagged-PKD proteins. As shown in Figure 4.8b, the pS916 antibody did not react with the endogenous or the Myc-tagged PKD proteins isolated from non-stimulated cells but as expected the pS916 antiserum did react with the endogenous PKD present in BCR- or PDBu-stimulated B cells (Fig. 4.8b). The pS916 antibody also reacted strongly with Myc-tagged wild type PKD isolated from stimulated B cells, but was unable to recognise either the ΔCT or S916A Myc-PKD proteins isolated from F(ab)'2- or PDBu-treated B cells (Fig. 4.8b). Equivalent expression levels of all three Myc-PKD proteins was confirmed by western blot analysis with a monoclonal 9E10 antibody, reactive with the Myc epitope tag (Fig. 4.8b). The selective loss of reactivity of the pS916 antibody for the C-terminal PKD mutants confirmed that the pS916 antibody was indeed specifically recognising phosphorylated S916 residues within the C-terminus of PKD in antigen receptor- and phorbol ester-activated B cells. Moreover, mutation of the S916 site to an alanine residue prevented the reduced recognition of active PKD (compared to non-activated PKD) by C-terminal PKD antibodies (Fig. 4.8c). In contrast, the loss of this C-terminal S916 phosphorylation site did not effect the retarded mobility shift of activated PKD upon gel electrophoresis (Fig. 4.8b) indicating that multiple additional PKD trans- and auto-phosphorylation sites exist that remain to be identified.

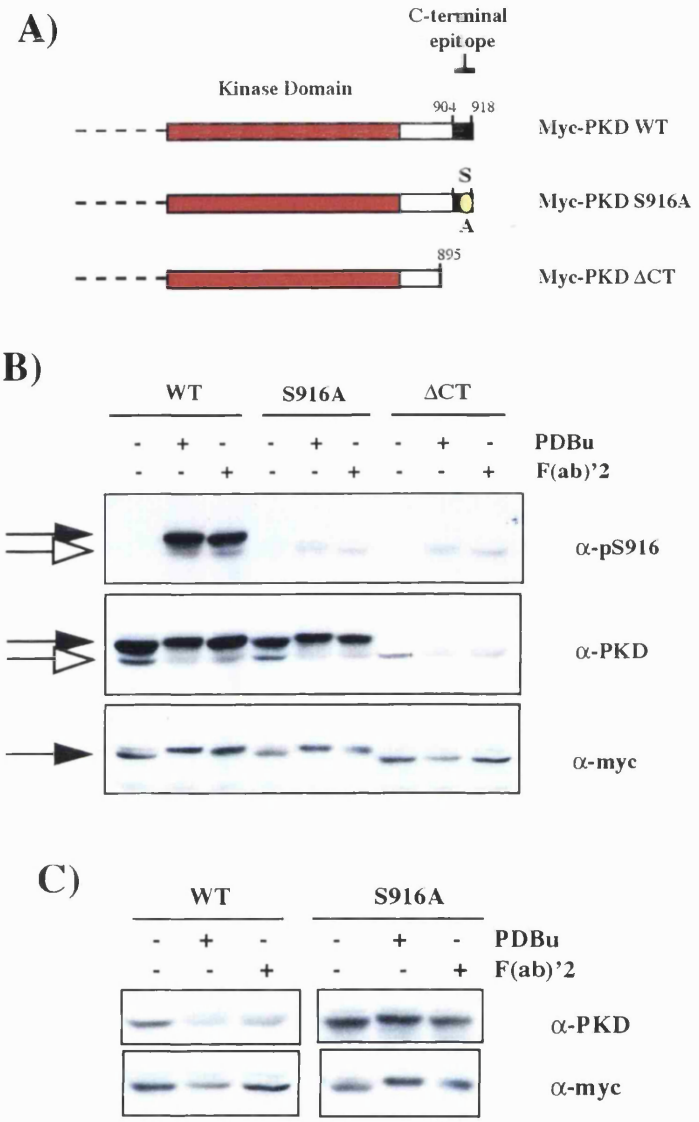


Fig. 4.8 Specificity of the pS916 antibody - II

(A) Schematic representation of Myc-PKD C-terminal PKD mutants. An N-terminally Myc-tagged PKD construct was generated as described in Chapter 2. Myc-PKD ΔCT (lacking the extreme C-terminal 23 residues of PKD) and Myc-PKD S916A (where the C-terminal serine 916 residue was replaced by a non-phosphorylatable alanine) were generated as described in Chapter 2.

(B) A20 B lymphocytes were transiently transfected with either wild-type Myc-PKD (WT), Myc-PKD ΔCT or Myc-PKD S916A. Cells were left unstimulated (-) or were treated with either 50 ng/ml PDBu for 10 min or with 10 μg/ml F(ab)'2 anti-mouse IgG for 2 min (+) as indicated. Western blot analysis of whole cell lysates was performed with the pS916 antibody; secondly with a pan C-terminal PKD antibody (sc-935) and finally with a 9E10 mAb directed against the Myc epitope. Similar results were obtained in three individual experiments. Black arrows indicate Myc-tagged PKD proteins. White arrows indicate endogenous PKD.

(C) A20 B lymphocytes were transiently transfected with either wild-type Myc-PKD (WT) or Myc-PKD S916A. Cells were left unstimulated (-) or were treated with either 50 ng/ml PDBu for 10 min or with 10 μg/ml F(ab)'2 anti-mouse IgG for 2 min (+) as indicated. Western blot analysis of whole cell lysates was performed with a C-terminal PKD antibody (sc-935) and with the 9E10 mAb. Similar results were obtained in three other experiments.

4.2.8. Analysis of the PKD S916 autophosphorylation site.

Having identified the C-terminal serine 916 residue of PKD as an *in vivo* autophosphorylation site that is specifically recognised by a pS916 antiserum, the function of this phosphorylation event was addressed, using the different Myc-tagged C-terminal PKD mutants. As illustrated in Figure 4.9, both the ΔCT and S916A mutants exhibited a low basal catalytic activity in resting B lymphocytes that was markedly enhanced after stimulation with PDBu or following antigen receptor ligation, comparable to that of wild-type PKD. These results were observed whether PKD activity was measured either by autophosphorylation (Fig. 4.9a) or by exogenous substrate peptide phosphorylation (Fig. 4.9b). Moreover, analysis of the kinetic properties of both wild-type and S916A PKD demonstrated that mutation of serine 916 does not significantly alter the catalytic activity of PKD. Thus, the apparent K_m values for a peptide containing the C-terminal 15 residues of PKD were 2.54±0.6 mM and 1.44±0.47 mM (n=4) for wild-type PKD and S916A PKD, respectively (Fig. 4.10).

In addition, phosphorylation of the S916 residue in PKD was not sufficient to induce PKD activity *in vivo*. Replacement of the serine residue at position 916 with a glutamic acid residue (the negative charge mimics a phosphorylated residue) did not effect the low basal catalytic activity of PKD, nor did it enhance PDBu-induced activation of PKD above that observed for wild-type PKD (Fig. 4.11a). From these data it can be concluded that S916 phosphorylation is neither sufficient nor required for the induction of PKD catalytic activity *in vivo*.

Finally, to determine whether S916 phosphorylation regulates the stability and/or duration of PKD activity *in vivo*, A20 B lymphoma cells were transiently transfected with either wild-type or S916A Myc-PKD constructs and the kinetics of PKD activity following BCR-ligation was investigated. As shown in Figure 4.11b, both wild-type and S916A PKD were rapidly activated after BCR-ligation to the same extent, a response that was maintained for over 30 min for both kinases. Thus S916 phosphorylation does not appear to control the stability and/or duration of PKD activity, at least over the time period measured in these experiments.

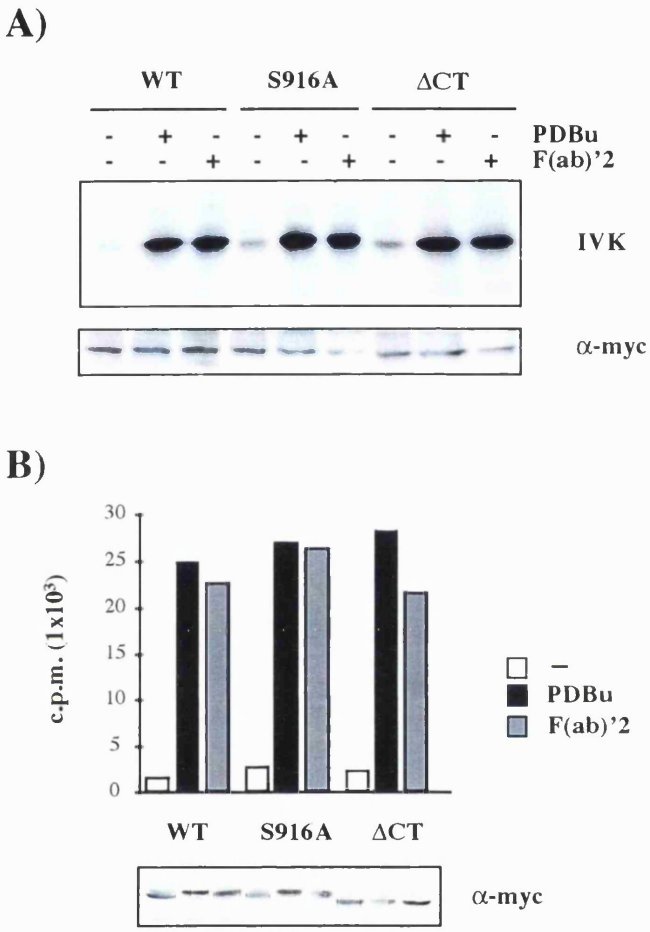


Fig. 4.9. S916 phosphorylation is not required for PKD catalytic activity.
A20 B lymphocytes were transiently transfected with either wild-type Myc-PKD (WT), Myc-PKD S916A or Myc-PKD ΔCT. Cells were left unstimulated (-) or were treated with either 50 ng/ml PDBu for 10 min or with 10 μg/ml F(ab)'2 anti-mouse IgG for 2 min (+) to activate the BCR complex, as indicated. Myc-tagged-PKD proteins were immunoprecipitated from whole cell lysates using 9E10-coupled Protein G-sepharose beads and PKD activity was measured by *in vitro* kinase assays, using either PKD autophosphorylation (**A**) or exogenous substrate phosphoryation (**B**) as a readout for activity. In panel B), the data are the mean c.p.m. incorporated into 2.5 mg/ml of a C-terminal PKD peptide from duplicate points (range <10%). In parallel, lysates were subjected to SDS-PAGE and western blot analysis with the 9E10 mAb to confirm equal protein expression levels. Results are representative of 4 and 2 independent experiments, respectively.

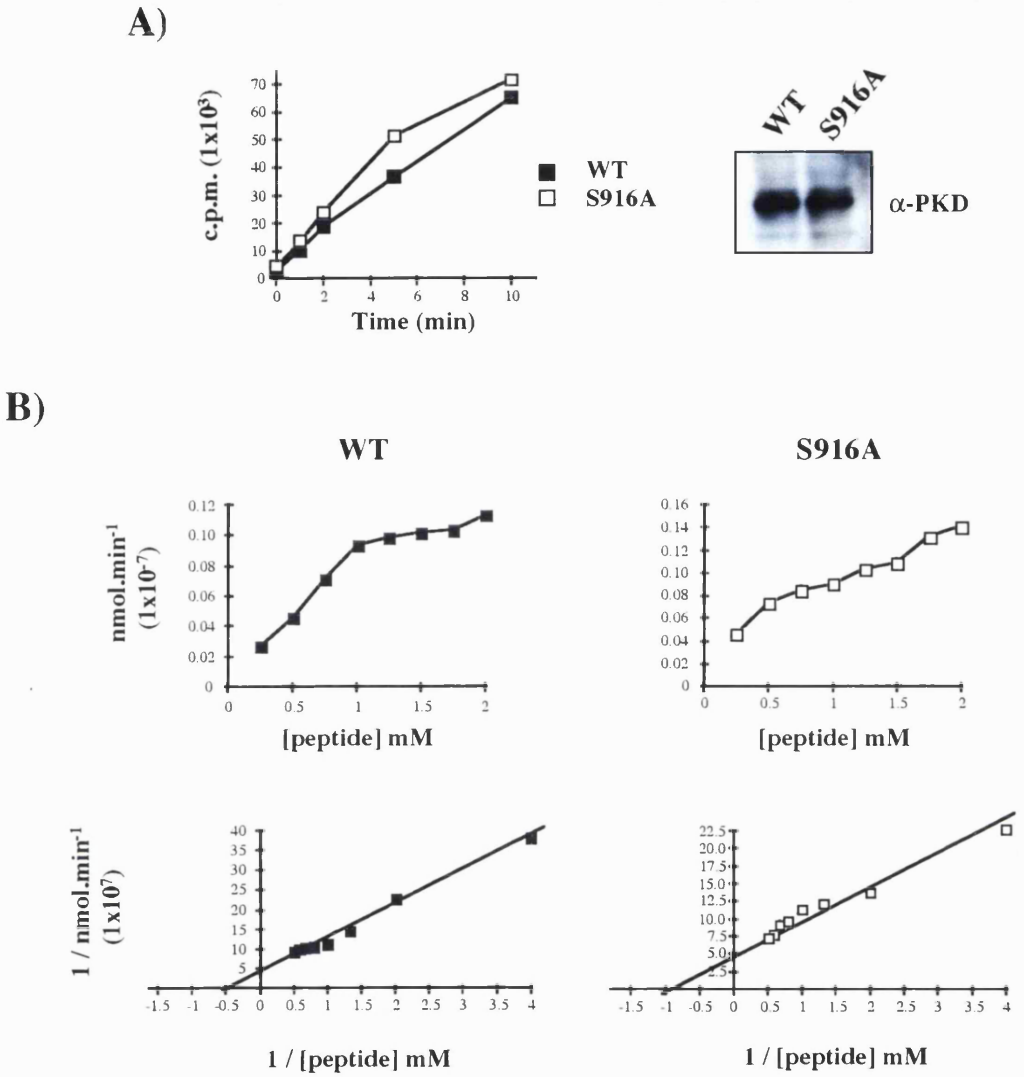


Fig. 4.10. Enzyme kinetic analysis of wild-type and S916A PKD proteins.
A20 B lymphocytes were transiently transfected with either wild-type Myc-PKD (WT) or Myc-PKD S916A. Cells were treated with 10 μg/ml F(ab)'2 anti-mouse IgG for 2 min to activate the BCR complex. Lysates were pooled and divided into individual points before Myc-tagged-PKD proteins were immunoprecipitated from whole cell lysates using 9E10-coupled Protein G-sepharose beads and analysed by *in vitro* kinase assays.
(A) Time course of phosphorylation of the the PKD C-terminal (CT) peptide by activated wild-type and S916A PKD enzymes (left). The PKD proteins were incubated for various times with a phosphorylation mixture containing 2.5 mg/ml CT peptide (final concentration), as described in Chapter 2. Linear kinetics of phosphorylation of the CT peptide up to 10 min were observed (n=2). Lysates were also analysed by SDS-PAGE and western blotting with a C-terminal PKD antibody (sc-935) to confirm similar expression levels for the WT and S916A PKD proteins (right).
(B) Concentration dependence of phosphorylation of the CT peptide by activated WT and S916A PKD. The PKD proteins were incubated for 8 min with a phosphorylation mixture containing different concentrations of CT peptide (0.25-2 mM), as described in Chapter 2. K_m values for WT and S916 PKD were 2.54±0.6 mM and 1.44±0.47 mM, respectively (n=4) and were calculated from Lineweaver Berk plots as described in Chapter 2.
All results shown are from one representative experiment out of four.

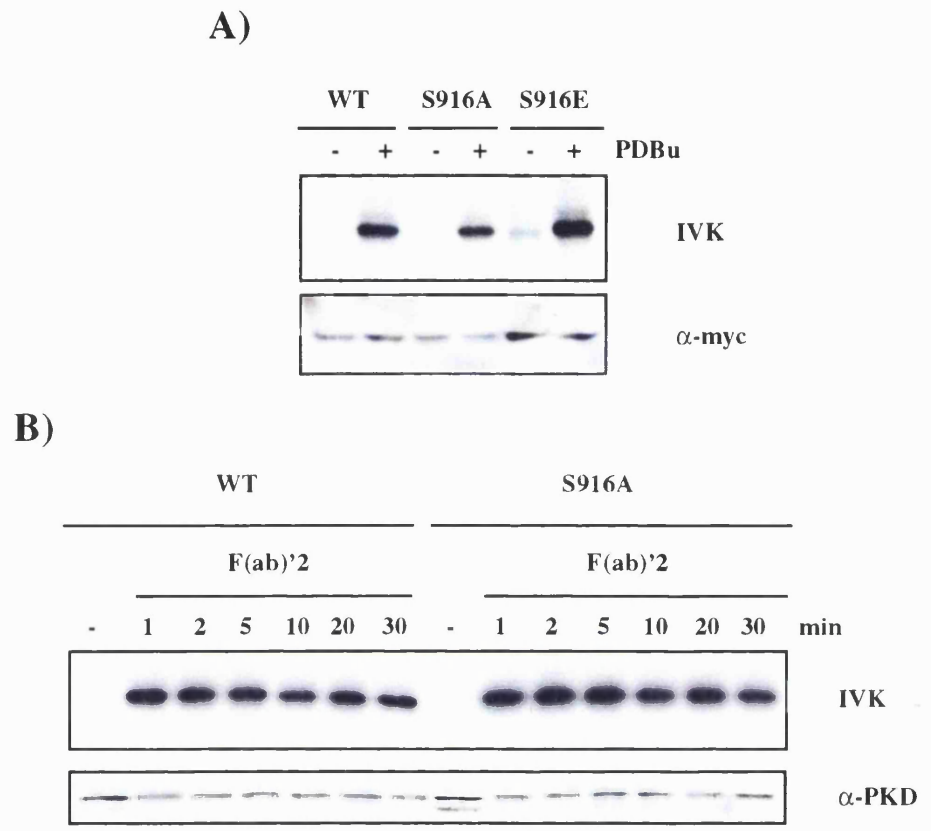


Fig. 4.11. Analysis of PKD S916 site mutants.

(A) A20 B lymphocytes were transiently transfected with either wild-type Myc-PKD (WT); Myc-PKD S916A (S916A) or Myc-PKD S916E (S916E). Cells were left unstimulated (-) or were treated with 50 ng/ml PDBu for 10 min (+). Myc-tagged-PKD proteins were immunoprecipitated from whole cell lysates using 9E10-coupled Protein G-sepharose beads and analysed by *in vitro* kinase assays (using PKD autophosphorylation as a readout for activity), (upper panel). Western blotting of lysates with a 9E10 mAb demonstrated comparable protein expression levels (lower panel). Results shown are representative of 2 individual experiments.

(B) A20 B lymphocytes were transiently transfected with either wild-type Myc-PKD (WT) or Myc-PKD S916A (S916A). Cells were left unstimulated (-) or were treated with 10 μg/ml F(ab)'2 fragments of anti-mouse IgG for different times (1-30 min). Myc-tagged-PKD proteins were immunoprecipitated from whole cell lysates using 9E10-coupled Protein G-sepharose beads and analysed by *in vitro* kinase assays (using PKD autophosphorylation as a readout for activity), (upper panel). Western blotting of lysates with the pan C-terminal PKD antibody (sc-935) showed equivalent protein expression levels (lower panel). Results shown are representative of 3 individual experiments.

4.3. DISCUSSION.

Previous work has shown that activation of PKD, both *in vitro* and *in vivo,* results in the phosphorylation of this enzyme on multiple serine residues. The data presented in this chapter describes the generation and characterisation of a site-specific phosphopeptide antibody that was used to identify serine 916 an *in vivo* phosphorylation site within the C-terminal region of PKD.

The pS916 antibody preferentially recognised active PKD in western blot analysis of whole cell lysates and mutational analysis confirmed that this phospho-antibody was specific for the C-terminal S916 site of PKD. The amino-acid sequence surrounding the serine 916 residue shows a high degree of homology to the optimal peptide substrate sequence proposed for PKD by Nishikawa and co-workers (Nishikawa *et al.*, 1997), which raised the possibility that serine 916 is an autophosphorylation site for this enzyme. *In vitro* kinase assays established that PKD could efficiently phosphorylate a synthetic peptide corresponding to the carboxy-terminal region of PKD containing the S916 residue. Importantly, the S916 residue of PKD was constitutively phosphorylated in activated PKD mutants, whilst no S916 phosphorylation could be detected in kinase-deficient PKD mutants, demonstrating that the C-terminal S916 residue of PKD is phosphorylated through an autophosphorylation mechanism *in vivo.* Whether this is an intra- or an intermolecular event remains to be determined.

Whether phosphorylation of S916 in the carboxy-tail of PKD plays an important role in the regulation and/or function of PKD is unclear at present. Such an hypothesis would be supported by previously described data regarding the functional importance of carboxy-terminal phosphorylation events for other serine/threonine kinases. Thus, the activity of PKB/Akt is directly regulated, in response to PI3-kinase, by two critical activating phosphorylation sites located within its kinase domain (T308, mediated by PDK1) and the carboxy-terminal region (S473, mediated by PDK1/PRK2), respectively (Alessi and Cohen, 1998; Balendran *et al.*, 1999; Downward, 1998). Mutation of either residue to alanine inhibits insulin-induced PKB/Akt activity, whilst single or double mutations of these sites to negatively charged aspartic acid residues results in a ~5-fold (T308) or ~20-fold (S473) increase in PKB/Akt activity (Alessi *et al.*, 1996).

Similarly, the activity of $p70^{S6}$ kinase is a regulated (again through PI3-kinase) through a complex set of multiple interdependent phosphorylation events (Pullen and Thomas, 1997). Mutational analysis indicates that phosphorylation of four serine/threonine-proline motifs (S411; S418; T421 and S424) within the carboxy-terminal

region of $p70^{S6}$ kinase are required, but are not sufficient, for the induction of full $p70^{S6}$ kinase activity (Ferrari *et al.*, 1993; Han *et al.*, 1995). Rather, these phosphorylation events appear to stabilise the C-terminal autoinhibitory domain away from the kinase domain. This then allows the subsequent phosphorylation of other sites to occur, including T389 within the linker region and T229 (mediated by PDK1) within the kinase domain, which cooperate to induce maximal $p70^{S6}$ kinase activation (Alessi *et al.*, 1997a; Pullen *et al.*, 1998; Pullen and Thomas, 1997; Weng *et al.*, 1998).

As discussed in Chapter 1, classical, novel and atypical PKC enzymes are regulated by three priming phosphorylation events, two of which are located at the carboxy-terminus of these enzymes (Karanen *et al.*, 1995; Tsutakawa *et al.*, 1995). Mutational analysis indicates that phosphorylation of these carboxy-terminal sites is not essential for PKC activity *per se*. Rather they appear to play a more subtle role, ensuring the correct processing of immature PKC for maximal catalytic competency; phosphatase resistance and catalytic stability of the mature kinase upon subsequent lipid-induced activation (Bornancin and Parker, 1996; Bornancin and Parker, 1997; Edwards *et al.*, 1999; Karanen *et al.*, 1995). Moreover, the archetypal enzyme PKA (cAMP-dependent kinase) requires carboxy-terminal phosphorylation at residue S338 to stabilise the kinase (Yonemoto *et al.*, 1997).

Carboxy-terminal phosphorylation events thus play an important role in the regulation of multiple serine/threonine kinases. Mutational analysis indicates that the PKD S916 phosphorylation site is not required for the initial activation of PKD (as was expected given that S916 is phosphorylated through an autophosphorylation mechanism *in vivo*) nor is it sufficient to increase PKD activity above basal levels. Autophosphorylation of the PKD S916 residue is also unimportant for maintaining the duration/stability of PKD activity *in vivo*. Therefore at present the precise function of the S916 phosphorylation in PKD remains unknown.

A number of alternative potential functions for the PKD S916 autophosphorylation site exist. Feng and Hannun have demonstrated a role for the two C-terminal priming autophosphorylation sites of $PKC\beta_{II}$ in controlling the subcellular redistribution of this kinase following receptor activation (Feng and Hannun, 1998b). However, the catalytically inactive PKD mutant D733A translocates to the plasma membrane of intact cells upon phorbol ester or antigen receptor stimulation in the same manner as wild-type PKD (see Chapter 6). This same PKD mutant does not autophosphorylate on S916 (this chapter) indicating that of phosphorylation S916 is not likely to regulate PKD subcellular localisation,

although this remains to be formally proven. Two recent studies have implicated roles for autophosphorylation sites within the N-terminal regulatory region of PKD in mediating protein or lipid interactions. In the first study, Hausser *et al* demonstrated that GST-14-3-3 proteins can interact with a serine-rich motif located between residues 197-242 of PKD, a response that was enhanced upon phorbol ester stimulation of PKD and which was sensitive to phosphatase treatment (Hausser *et al.*, 1999). It was suggested that this motif represents a PKD autophosphorylation site(s) since the interaction between PKD and GST-14-3-3 was not observed for a kinase-dead PKD mutant. Secondly, Nishikawa *et al* have identified a constitutive basal association between PKD and two lipid kinases (Type I PI4-Kinase and Type I PI4-Phosphate-5-Kinase) *in vivo.* Again a role for a PKD autophosphorylation event in this interaction was proposed since a kinase-dead PKD mutant failed to associate with these lipid kinases, although the phosphorylation site involved was not identified in this study (Nishikawa *et al.*, 1998). In light of these observations it is possible that the PKD S916 autophosphorylation site could mediate protein-protein or protein-lipid interactions between active PKD and as yet unidentified regulators and/or substrates of PKD. Given the importance of carboxy-terminal phosphorylation events for other serine/threonine kinases the function of the PKD serine 916 autophosphorylation site is of great interest and should be a target for future studies. The identification of downstream functions and/or substrates for PKD may aid these studies.

Regardless of the precise function of the S916 phosphorylation site, the pS916 antiserum described here has been successfully used to demonstrate that phosphorylation of the serine 916 residue of PKD occurs in response to both pharmacological (phorbol ester) and physiological (antigen receptor triggering) stimulation of B lymphocytes. Hence, the pS916 antibody provides a useful tool with which to study the regulation of PKD activity *in vivo.* Further work describing the physiological regulation of PKD activity by antigen receptors will be discussed in Chapter 5.

CHAPTER 5 : Activation of PKD by antigen receptors in T and B lymphocytes and in mast cells

5.1. INTRODUCTION.

Metabolism of inositol phospholipids is a major mechanism by which antigen receptors transmit signals from the plasma membrane into the interior of cells during immune responses. The potency of subsequent calcium and PKC-regulated signalling pathways for lymphocyte activation is underlined by the observation that pharmacological agents which elevate intracellular calcium levels and activate PKC (calcium ionophores and phorbol esters respectively) can mimic many aspects of antigen receptor triggering including T and B lymphocyte proliferation and differentiation; protection from Fas-mediated apoptosis in B cells and secretion of allergic mediators in mast cells (Buccione *et al.*, 1994; Crabtree and Clipstone, 1994; DeFranco, 1997; Ozawa *et al.*, 1993a; Rao, 1994; Su *et al.*, 1994).

The role of PKC in the regulation of immune responses by antigen receptors is complicated by the expression of multiple related (and potentially redundant) PKC isoforms, although PKCθ appears to be restricted to cells of the T lymphocyte lineage within the hematopoietic system (Baier *et al.*, 1993) and discussed in Chapter 1, PKCs are critical for antigen receptor mediated immune responses. Importantly, mice lacking $PKC\beta_{I/II}$ exhibit B lymphocyte dysfunction and show impaired humoral responses to T cell-independent antigens (Leitges *et al.*, 1996). However, direct targets for PKC enzymes in antigen receptor signalling cascades have not been identified to date.

5.1.1. Inhibitory receptor signalling.

During immune responses, activation signals from antigen receptors are modulated by 'negative' or 'inhibitory' receptors. A large number of inhibitory receptors have now been described, including the FcγRIIb, the low affinity receptor for IgG in B lymphocytes and mast cells; KIRs (Killer Inhibitory Receptors) in Natural Killer cells and T lymphocytes and CTLA-4 in T lymphocytes. Co-ligation of these inhibitory receptors together with activation receptors results in the termination of specific intracellular signals that control the duration and strength of immune responses to foreign antigen (in the case of the FcγRIIb) and prevent the lytic activity of Natural Killer cells against self (reviewed in (Coggeshall, 1998; O'Long, 1999; Scharenberg and Kinet, 1996; Scharenberg and Kinet, 1998b; Yokoyama, 1997)). The importance of negative signalling pathways in the control

of immune system homeostasis is highlighted by the phenotype of animals deficient for specific inhibitory receptors. Thus, FcγRIIb$^{-/-}$ mice display elevated IgG passive allergic responses following FcγRIII engagement; enhanced humoral immune responses upon BCR-ligation and hyper-responsiveness of hematopoietic progenitors to multiple cytokines due to the lack of negative feedback signals (Helgason *et al.*, 1998; Takai *et al.*, 1996). CTLA-4 knockout mice are characterised by hyperactive T lymphocyte responses to foreign antigen, enhanced phosphorylation of proximal TCR signalling molecules and increased incidence of lymphoproliferative disease (Marengere *et al.*, 1996; Tivol *et al.*, 1995; Waterhouse *et al.*, 1995)

Phosphorylation of a conserved tyrosine-based inhibitory motif (ITIM) within the cytoplasmic tail of inhibitory receptors by active Src kinases, following appropriate co-ligation of inhibitory and activation receptors, is both necessary and sufficient for the dominant negative actions of these inhibitory receptors through the recruitment of the protein phosphatases SHP-1/2 and SHIP (Burshtyn *et al.*, 1996; D'Ambrosio and al, 1996; D'Ambrosio *et al.*, 1995; Dearon *et al.*, 1995; Kiener *et al.*, 1997; Marengere *et al.*, 1996; Muta *et al.*, 1994; Ono *et al.*, 1996; Pani *et al.*, 1995). SHP-1 and -2 are tyrosine phosphatases that inhibit proximal tyrosine phosphorylation events downstream of antigen receptors, thus preventing the activation of signalling cascades that control cellular responses to foreign antigen (Binstadt *et al.*, 1996; Marengere *et al.*, 1996). In contrast, the inositol 5' phosphatase SHIP does not effect early tyrosine phosphorylation events but instead terminates activation signals originating from antigen receptors by dephosphorylating the lipid second messengers PI(3,4,5)P_3 and I(1,3,4,5)P_4 at the 5'-position (Damen *et al.*, 1996; Lioubin *et al.*, 1996; Scharenberg *et al.*, 1998a).

Various studies, using dominant negative or knock-out approaches, have demonstrated that SHIP, but not SHP-1/2, is critical for the inhibitory effects of the FcγRIIb in B lymphocytes and in mast cells; conversely KIRs require SHP-1 but not SHIP activity (Gupta *et al.*, 1996; Liu *et al.*, 1998a; Nadler *et al.*, 1997; Ono *et al.*, 1996; Ono *et al.*, 1997). Thus, co-engagement of the BCR with the FcγRIIb, via the action of SHIP, alters the balance of specific phosphoinositides in BCR versus BCR/FcγRIIb stimulated B cells. Consequently, BCR/FcγRIIb co-ligation rapidly inhibits the plasma membrane association and activity of signalling molecules that are dependent on the PI(3,4,5)P_3 lipid product of PI3-Kinase, including the serine kinase PKB/Akt (Aman *et al.*, 1998; Astoul *et al.*, 1999; Gupta *et al.*, 1999; Jacob *et al.*, 1999) and the Tec kinase Btk (Bolland *et al.*, 1998; Scharenberg *et al.*, 1998a). In addition, under conditions of negative signalling, B cells

show reduced activity of the Ras/MAPK pathway (Sarmay *et al.*, 1996; Tridandapani *et al.*, 1997a; Tridandapani *et al.*, 1997b) and inhibition of calcium signalling due to a block in calcium influx (Amigorena *et al.*, 1992; Choquet *et al.*, 1993; Diegel *et al.*, 1994; Muta *et al.*, 1994). However, the effect of this inhibitory receptor on DAG-regulated signalling pathways in B cells has not previously been examined.

5.1.2. PKD and antigen receptor signalling.

PKD displays a ubiquitous pattern of expression (Johannes *et al.*, 1994; Valverde *et al.*, 1994), although particularly strong expression is seen in tissues or cells of hematopoietic origin, including thymus; spleen and peripheral blood lymphocytes (Rennecke *et al.*, 1996). In B lymphocytes, PKD is expressed at all stages of development from the pre-B cell to the plasma cell stage (Sidorenko *et al.*, 1996). Moreover, it has previously been described that PKD is found complexed with, and is activated by, the BCR complex in the Burkitt's lymphoma cell line, Ramos (Sidorenko *et al.*, 1996). A detailed investigation into the regulation of PKD activity by the TCR complex has not been performed as yet. Moreover the regulation of PKD by Fc receptors in hematopoietic cells has not previously been examined.

As discussed in Chapter 3, PKD is stably activated by the phosphorylation of two serine residues its kinase domain (S744 and S748), an event which is controlled through classical and/or novel PKC enzymes in response to agonists which elevate intracellular DAG levels or following treatment of cells with phorbol esters. Avian DT40 B lymphocytes which lack the cytosolic tyrosine kinases Syk or Btk, or their downstream target PLCγ, do not activate PKD in response to BCR-ligation (Sidorenko *et al.*, 1996). Since PKD does not contain a calcium binding C2 domain and is not directly regulated by calcium (Sidorenko *et al.*, 1996; Valverde *et al.*, 1994; Zugaza *et al.*, 1997) these data are consistent with a model whereby PKD is a downstream target for DAG in B cells. However, a role for PKC enzymes as proximal upstream regulators of PKD in antigen receptor signalling cascades had not previously been examined.

5.1.3. Aims.

(1) To investigate the mechanism of activation of PKD in antigen receptor stimulated T & B lymphocytes and in mast cells.

(2) To determine the effect of the inhibitory FcγRIIb receptor on BCR-induced PKD activation.

5.2 RESULTS

5.2.1. Activation of PKD by crosslinking of the B cell antigen receptor (BCR) complex.

To confirm that the BCR induces PKD activity, and to determine whether BCR ligation also induces autophosphorylation of the PKD S916 residue, PKD activation was studied in the A20 B lymphoma cell line. The kinetics of PKD activation after crosslinking of the BCR complex, using F(ab)'2 fragments of anti-mouse IgG, was first measured by *in vitro* kinase assays. The data presented in Figure 5.1 shows that stimulation of the BCR complex induces a rapid 8-10 fold increase in PKD catalytic activity, which was comparable to that induced by phorbol ester treatment. This response was maximal within 1 minute of BCR-engagement and was sustained for over 10 minutes. Importantly, the increase in PKD catalytic activity was paralleled by the rapid autophosphorylation of the carboxy-terminal S916 residue of PKD in F(ab)'2-stimulated (but not non-stimulated) B cells, as measured by western blots analyses of whole cell lysates with the pS916 antiserum (Fig. 5.1).

5.2.2. FcεR1 aggregation induces PKD activity in the mast cell line RBL 2H3.

The antigen receptor present in mast cells is the high affinity receptor for IgE, the FcεR1. To address whether the FcεR1 also coupled to PKD, RBL 2H3 mast cells were primed with DNP-specific IgE before the cells were left unstimulated or were treated with KLH-DNP to crosslink the bound monomeric IgE thus activating the FcεR1. The data presented in Figure 5.2 indicates that, in common with the BCR complex, ligation of the FcεR1 causes a rapid ~4-fold increase in PKD catalytic activity, similar to (or greater) than that observed following phorbol ester treatment. No S916 autophosphorylation was detected for PKD in FcεR1-stimulated mast cells, perhaps reflecting a level of S916 phosphorylation that is below the detection threshold of this antiserum. Indeed, western blot analysis of the expression levels of PKD in A20 B lymphoma cells compared to RBL 2H3 mast cells indicates that these B cells express ~2-3 times higher levels of PKD than the mast cells (data not shown). Alternatively, the rat PKD protein may contain an amino-acid change at the 916 residue or RBL 2H3 mast cells may express high levels of a serine phosphatase that can dephosphorylate S916.

5.2.3. T cell antigen receptor (TCR) induces PKD activity in T lymphocytes.

Experiments were subsequently carried out to determine whether the T cell antigen receptor complex (TCR) could also stimulate PKD kinase activity. For these studies, quiescent T lymphoblasts derived from human peripheral blood were used, prepared as

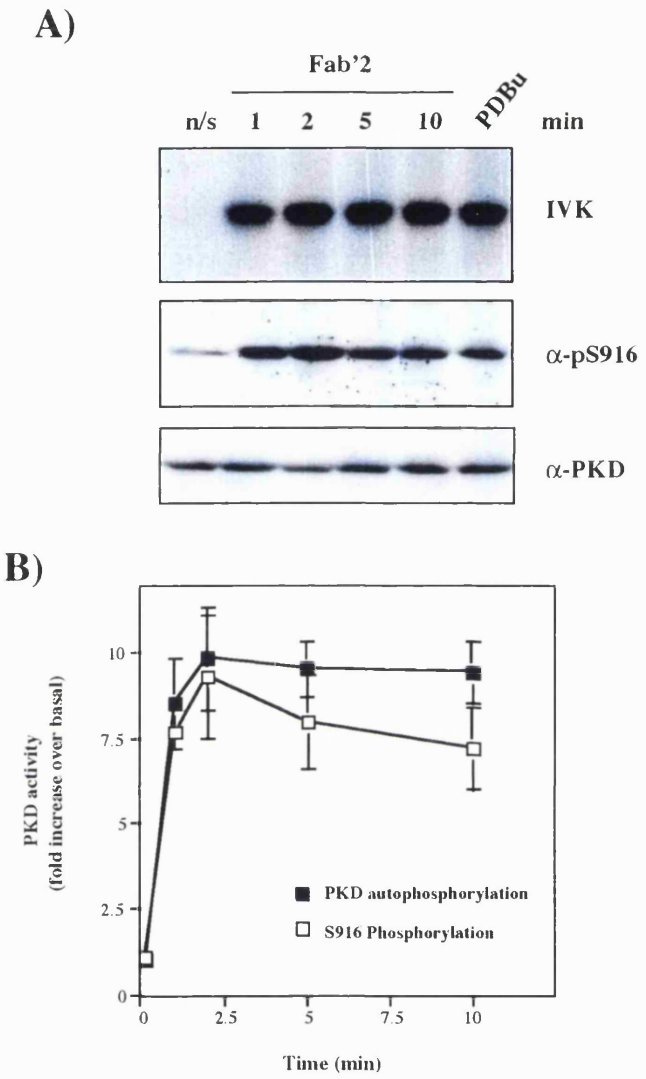


Fig. 5.1. The B cell antigen receptor (BCR) complex regulates PKD activity and S916 autophosphorylation.

(A) A20 B lymphocytes were left unstimulated (n/s) or were treated with 10 μg/ml F(ab)'2 anti-mouse IgG for various times (as indicated) to activate the BCR complex. Endogenous PKD was immunoprecipitated from whole cell lysates using the PA-1 antibody and PKD activity (autophosphorylation) was assayed by *in vitro* kinase assays (IVK, top panel). In parallel, total cell lysates were subjected to SDS-PAGE and western blot analysis with the pS916 antibody and with a pan C-terminal PKD antibody (sc-935), (middle and lower panels respectively). One representative experiment out of 4 is shown.

(B) The mean $\pm$ s.e. increase in PKD activity and S916 autophosphorylation following BCR-ligation in 4 independent experiments (as quantified by phosphoimager analysis or scanning densitometry respectively) are shown, expressed as the fold increase in PKD activity induced by F(ab)'2 over basal levels.

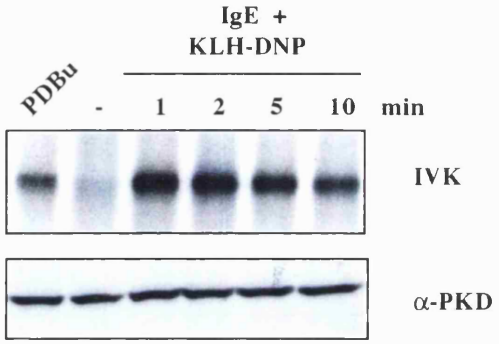


Fig. 5.2. Crosslinking of the FcεR1 activates PKD in the mast cell line RBL 2H3.
RBL 2H3 cells were primed with 1 μg/ml IgE anti-DNP for 1 h at 37°C and then either antigenic crosslinking of the bound IgE was performed, using 500 ng/ml KLH-DNP, for various times (1-10 min) or the cells were left unstimulated (-). Alternatively cells were treated with 50 ng/ml PDBu for 10 min. PKD was immunoprecipitated from cell lysates using the PA-1 antibody and PKD activity was measured by *in vitro* kinase assays (IVK). SDS-PAGE and western blot analysis of whole cell lysates shows equivalent amounts of PKD in all samples. Results are representative of 2 independent experiments.

described in Section 2.6. Activation of the TCR-complex was achieved using the UCHT1 mAb, which cross-links the CD3ε chains of the TCR complex mimicking the effect of MHC/antigen binding. As demonstrated in Figure 5.3a, PKD exhibited a low basal level of kinase activity in peripheral T lymphoblasts which was rapidly enhanced ~4-5 fold following cross-linking of the TCR complex with the UCHT1 mAb. Maximal activation of PKD was observed within ~1 minute of stimulation and was sustained for over 10 min (Fig. 5.3a). In addition to regulating the catalytic activity of PKD, ligation of the TCR complex was also found to induce autophosphorylation of the carboxy-terminal S916 residue of PKD. Western blot analysis of lysates prepared from UCHT1- or PDBu-treated T lymphoblasts showed strong immunoreactivity of the pS916 antibody for active PKD present in these cells but not with PKD isolated from non-treated cells (Fig. 5.3b).

Activation of PKD by the TCR complex was also observed in primary naive thymocytes. Freshly isolated mouse thymocytes were stimulated with either PDBu or pervanadate, or with a crosslinking CD3ε mAb (2C11) before whole cell lysates were analysed by SDS-PAGE and western blotting using the pS916 antiserum. As shown in Figure 5.3c, PKD displayed a high degree of activity (as measured by S916 autophosphorylation) in pharmacologically-stimulated thymocytes compared to non-stimulated thymocytes. Importantly, TCR-engagement also led to a significant increase in PKD S916 autophosphorylation over basal levels. This is particularly striking given that pervanadate or PDBu treatment would stimulate all thymocytes whereas only 10-15% of the thymocytes would be mature single positive T cells able to respond to crosslinking CD3ε antibodies.

5.2.4. The CD28 co-receptor activates PKD in the JH6.2 T lymphocyte cell line.

In B lymphocytes, crosslinking of the BCR co-receptor CD19 induces PKD activity (Sidorenko *et al.*, 1996). The major co-receptor expressed on T lymphocytes is CD28, which functions to enhance TCR signalling and effector functions by interacting with its physiological ligands B7-1 and B7-2, present on antigen presenting cells. Given that PKD was activated following TCR engagement it was of interest to determine whether PKD could also regulated by the CD28 co-receptor in T lymphocytes.

Cultured T lymphoblasts only poorly express surface CD28 and so it was decided to use a CD28+ T lymphocyte cell line to determine whether the CD28 co-receptor activates PKD in T lymphocytes or not. The Jurkat subclone JH6.2 is a well established model system used to study T lymphocyte signal transduction pathways and effector

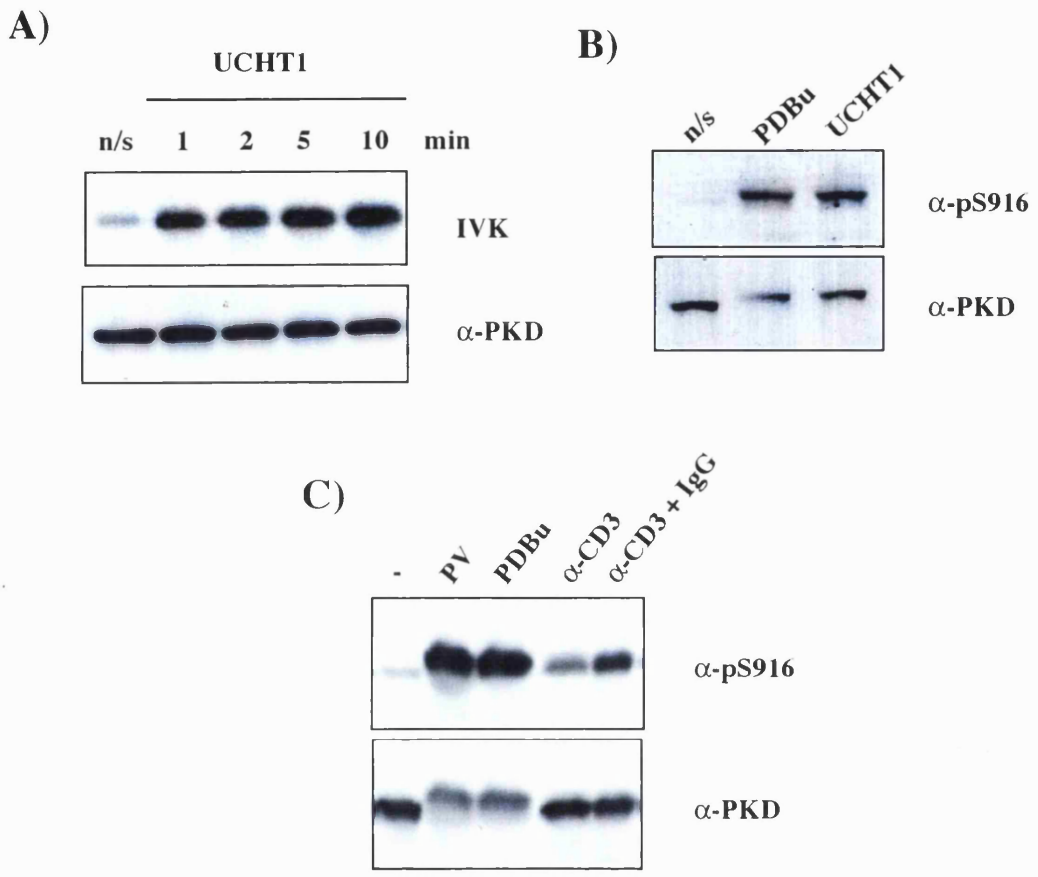


Fig. 5.3. The T cell antigen receptor (TCR) complex regulates PKD activity.

(A) T lymphoblasts were isolated and cultured from human peripheral blood as described in Chapter 2 and either left unstimulated (n/s) or treated with 10 μg/ml of the CD3 crosslinking mAb UCHT1, to activate the TCR complex. Endogenous PKD was immunoprecipitated from whole cell lysates with the PA-1 antibody and PKD activity (autophosphorylation) was assayed by *in vitro* kinase assays. The fold increase in PKD activity after TCR-ligation is shown, as measured by phosphoimager analysis. Data are representative of 4 individual experiments.

(B) T lymphoblasts were left unstimulated (n/s) or were treated with either 50 ng/ml PDBu for 10 min or with 10 μg/ml UCHT1 for 5 min to activate the TCR complex. Whole cell lysates were analysed by SDS-PAGE and western blotting with the pS916 antibody and subsequently with a C-terminal pan-PKD antibody (sc-935).

(C) Primary mouse thymocytes ($40x10^6$ cells/point) were left unstimulated (-) or were treated with either 100μM pervanadate (PV) or with 50 ng/ml PDBu for 10 min. Alternatively the cells were coated with 10μg/ml of 2C11, a hamster anti-mouse CD3 mAb, either alone (α -CD3) or with crosslinking of bound 2C11 using 100 μg/ml anti-hamster IgG for 2 min (α-CD3+IgG). Whole cell lysates were analysed by SDS-PAGE and western blotting with pS916 and pan-PKD antibodies. (Western blots are shown curtsey of Dr. P. Costello, Lymphocyte Activation Laboratory, ICRF).

functions and expresses high levels of surface CD28, as demonstrated in Figure 5.4a. To ensure that PKD was expressed in this cell line, and importantly that it was regulated by the TCR complex, JH6.2 cells were stimulated with the UCHT1 mAb and PKD activity was measured by *in vitro* kinase assays. As shown in Figure 5.4b, PKD exhibited negligible basal activity in non-stimulated JH6.2 cells and stimulation of the TCR complex with the crosslinking CD3 mAb UCHT1 led to a rapid ~4-5 fold increase in PKD activity, similar to that observed in peripheral T lymphoblasts.

Having demonstrated the presence of functional PKD in the JH6.2 T lymphocyte cell line, experiments were carried out determine whether CD28 was able to induce PKD activity. Here the CD28 crosslinking mAb 9.3, which mimics the physiological ligand for CD28, B7-1/2 was generously provided by Dr. S. Ward (University of Bath). Treatment of JH6.2 cells with the CD28 mAb induced a rapid and potent increase in PKD catalytic activity, comparable to that observed after TCR-ligation or following treatment with PDBu (Fig. 5.4c). Thus, both the TCR complex and the CD28 co-stimulatory receptor are able to regulate PKD catalytic activity in T lymphocytes.

5.2.5. IL-2 does not activate PKD in T lymphocytes.

Interleukin-2 (IL-2) is the major cytokine involved in the autocrine feedback loop that controls the proliferative expansion of MHC/antigen-activated T lymphocytes during an immune response (Smith, 1988). The heterotrimeric IL-2 receptor (composed of α, β and γ transmembrane polypeptides) is coupled to multiple signalling pathways. These include the Ras/Erk cascade (Izquierdo Pastor *et al.*, 1995); PI3-Kinase dependent signalling pathways (Brennan *et al.*, 1997; Reif *et al.*, 1997) and STAT transcription factors (Beadling *et al.*, 1994; Hou *et al.*, 1995).

To determine whether IL-2 also regulates PKD, peripheral blood derived T lymphoblasts were treated with either rIL-2, or alternatively with the phorbol ester PDBu. The data presented in Figure 5.5a shows that no inducible PKD activity over basal levels was detected in IL-2 treated T lymphoblasts, although PDBu was able to strongly activate PKD, as measured by *in vitro* kinase assays. In contrast, a phospho-erk antibody reacted strongly with active erk present in both IL-2 and PDBu-treated cells in western blot analysis of whole cell lysates (Fig. 5.5b), confirming the biological activity of the rIL-2. Even prolonged stimulation of peripheral blood derived T lymphoblasts with rIL-2 for 1 or 3 hours did not induce PKD catalytic activity, as demonstrated by the absence of S916 autophosphorylation, although PKD was fully activated following TCR ligation (Fig. 5.5b

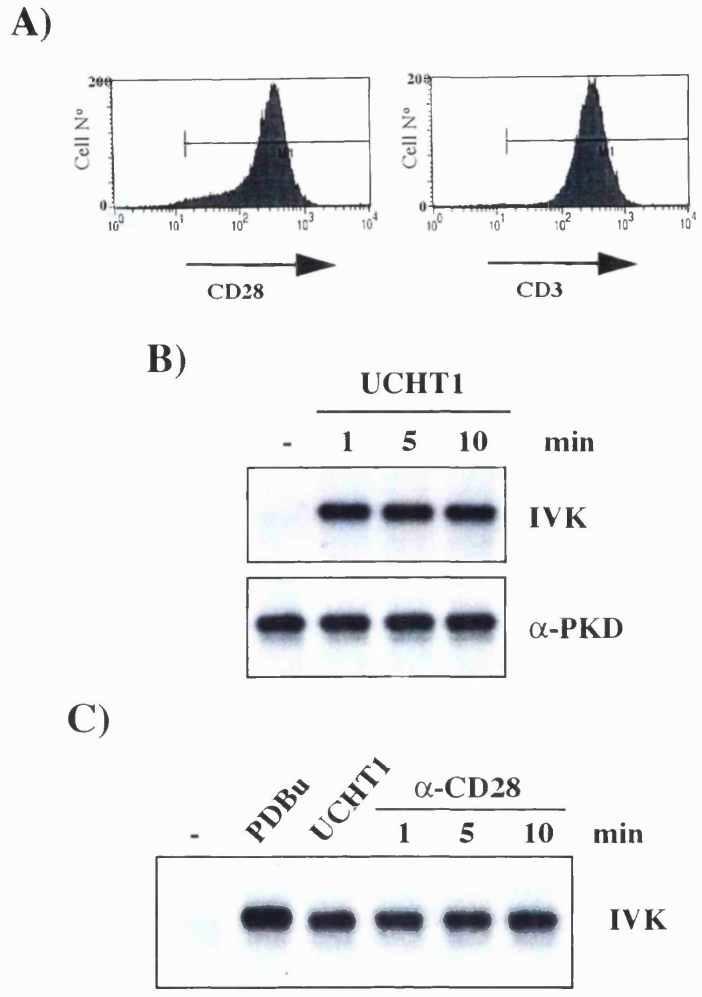


Fig. 5.4. The CD28 co-receptor couples to PKD in the JH6.2 T lymphocyte cell line.
(A) Analysis of CD28 cell surface expression on the Jurkat JH6.2 subclone. $5x10^6$ cells were stained with a mAb against CD28 (mAb 9.3) or the UCHT1 mAb against CD3ε. Staining was revealed using a secondary layer of anti-mouse IgG-FITC conjugate and analysed by flow cytometry using standard techniques.
(B) JH6.2 T lymphocytes were left unstimulated (-) or were treated with 10 μg/ml of the CD3 crosslinking mAb UCHT1, to activate the TCR complex. Endogenous PKD was immunoprecipitated from whole cell lysates with the PA-1 antibody and PKD activity (autophosphorylation) was assayed by *in vitro* kinase assays. Additionally PKD expression in cell lysates was analysed by SDS-PAGE and western blotting with a pan C-terminal PKD antibody (sc-935).
(C) JH6.2 T lymphocytes were left unstimulated (-) or were treated with 10μg/ml of the crosslinking CD28 mAb 9.3 for the times indicated, 10 μg/ml of the CD3 crosslinking mAb UCHT1 for 2 min or with 50 ng/ml PDBu for 10 min. Endogenous PKD was immunoprecipitated from whole cell lysates with the PA-1 antibody and PKD activity (autophosphorylation) was assayed by *in vitro* kinase assays.
All data are representative of at least 2 independent experiments.

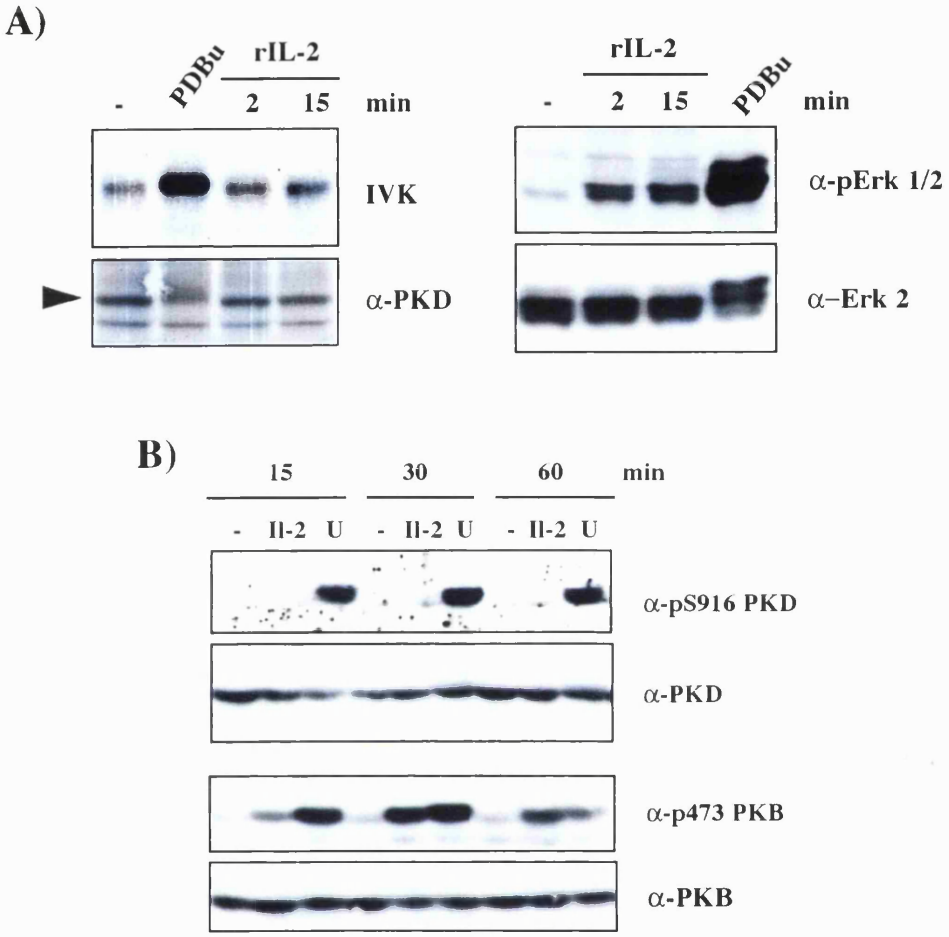


Fig. 5.5. IL-2 does not regulate PKD activity in T lymphoblasts.

(A) T lymphoblasts were left unstimulated (-) or were treated with either 50 ng/ml PDBu for 10 minutes or with 20 ng/ml rIL-2 for 5 or 15 min, as indicated. Endogenous PKD was immunoprecipitated from whole cell lysates using the PA-1 antibody and PKD activity (autophosphorylation) was assayed by *in vitro* kinase assays. Extracts from cell lysates were also analysed by SDS-PAGE and western blotting for PKD (left). The arrowhead indicates PKD. In parallel, lysates from rIL-2- or PDBu-treated T lymphoblasts were analysed by SDS-PAGE and western blotting, firstly with a phosphorylation state specific Erk1/2 antibody (which specifically recognises active erk1/2) and subsequently with a pan-erk 2 antibody (right). Data are representative of 2 individual experiments.

(B) T lymphoblasts were left unstimulated (-) or were treated with either 10μg/ml UCHT1 (U) or with 20 ng/ml rIL-2 (IL-2) for the times indicated. Whole cell lysates were analysed by SDS-PAGE and western blotting using the following antibodies. Upper panels: pS916 and pan PKD (sc-935) antibodies. Lower panels : p473 PKB and pan PKB antibodies. Data are representative of 2 individual experiments. Shown curtesy of Dr. A. Lawrence, Lymphocyte Activation Laboratory, ICRF.

and data not shown). In contrast, significant activation of the serine kinase PKB/Akt by rIL-2 treatment was apparent at these times (Fig. 5.5b).

Furthermore IL-12, a cytokine involved in the differentiation of activated T_H cells into T_{H1} cells during immune responses (Hsieh *et al.*, 1993), does not activate PKD (V. Athie-Morales, Lymphocyte Activation Laboratory, ICRF, *Personal communication*).

5.2.6. Antigen receptors regulate PKD through a PKC-dependent pathway.

The requirement for the cytosolic tyrosine kinases Syk and Btk, together with their substrate PLCγ, for PKD activation in BCR-stimulated B lymphocytes has been previously described (Sidorenko *et al.*, 1996), although the mechanism of activation of PKD in T lymphocytes and mast cells was unknown. Furthermore, recent studies have placed PKD downstream of classical and novel PKC enzymes in a hierarchical signalling cascade regulated by PLC and DAG (Rozengurt *et al.*, 1997; Van Lint *et al.*, 1998; Waldron *et al.*, 1999a). Little is known about the targets for PKC signalling in lymphocytes and the role of PKC enzymes as proximal upstream regulators of PKD in antigen receptor signalling cascades had not previously been addressed.

To examine the role of PKCs in the regulation of PKD by antigen receptors, peripheral blood derived T lymphoblasts were pre-incubated with GF 109203X, an inhibitor of classical/novel PKCs which has no direct effect on PKD kinase activity (Chapter 3 and (Zugaza *et al.*, 1996). Subsequently, the TCR complex was activated using the UCHT1 mAb and PKD activity was measured by *in vitro* kinase assays. As indicated in Figure 5.6, pre-treatment of T lymphoblasts with GF 109203X did not effect the constitutive low basal activity of PKD in these cells but severely abrogated the TCR-induced activation of PKD. Addition of this PKC-inhibitor directly to PKD during *in vitro* kinase assays did not effect the catalytic activity of PKD (Fig. 5.6a), confirming that this inhibitor has no direct action towards PKD. Similar data was observed in the JH6.2 T lymphocyte cell line where PKD activity following TCR-crosslinking was significantly reduced in GF 109203X pre-treated cells compared to control non-pre-treated cells (data not shown). Again, no inhibition of PKD activity was detected when GF 109203X was added directly to PKD during *in vitro* kinase assays in these cells.

Subsequent experiments determined whether antigen receptor mediated activation of PKD was also regulated via classical/novel PKCs in B cells and mast cells. Thus, the activity of PKD in BCR-stimulated lymphocytes that had been pre-treated with Ro 31-8220, a second inhibitor of classical and novel PKC enzymes that also has no direct inhibitory

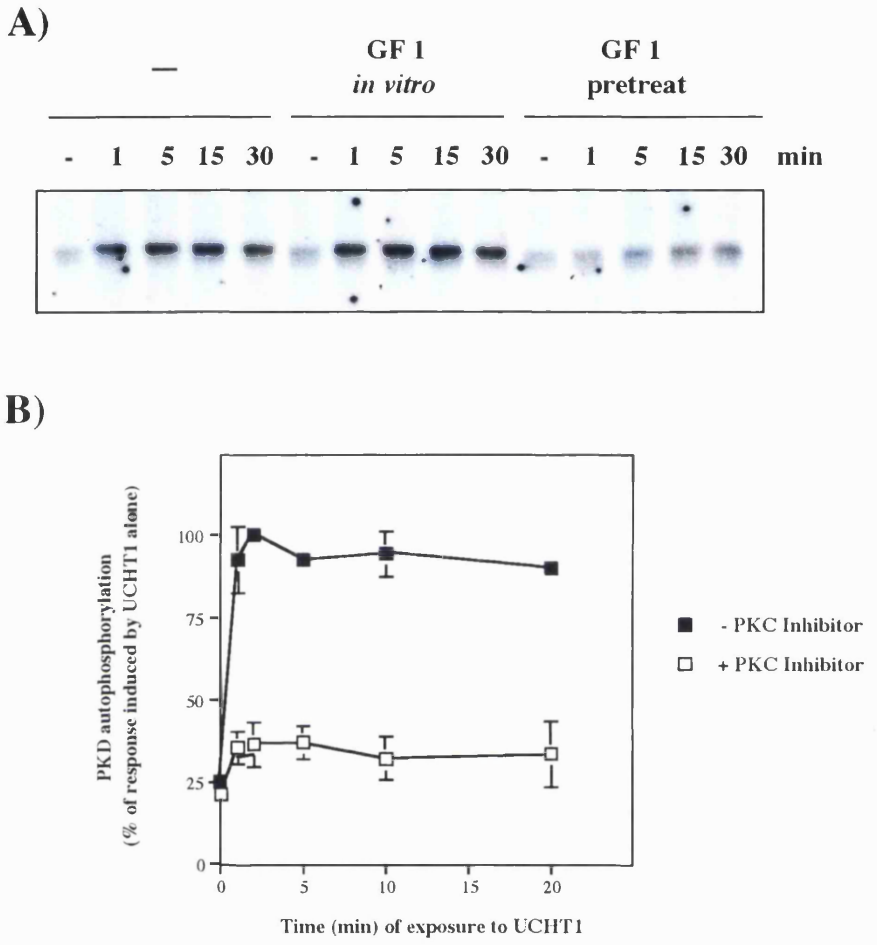


Fig. 5.6. Inhibition of classical/novel PKCs prevents activation of PKD by the TCR.
(**A**) Peripheral blood derived T lymphoblasts were pretreated with 3.5 μM GF 109203X (GF I pretreat) or an equal volume of solvent for 1 h. Cells were subsequently left unstimulated (-) or were stimulated with 10μg/ml UCHT1 mAb for the indicated times to activate the TCR complex. The cells were then lysed and PKD was immunoprecipitated using the PA-1 antiserum. PKD activity, as assessed by autophosphorylation, was determined by *in vitro* kinase assays. In some cases, PKD immunoprecipitates were incubated with of 3.5 μM GF1 during *in vitro* kinase assays (GF 1 *in vitro*). All samples were then analysed by SDS-PAGE and autoradiography. Results shown are representative of 3 independent experiments.
(**B**) The mean $\pm$ s.e. (n=3) increase in PKD activity induced by the CD3 crosslinking mAb UCHT1 in non-pretreated and in PKC-inhibitor pretreated cells. The data are expressed as a percentage of the maximum response induced by UCHT1 alone, as quantified by phosphoimager analysis of PKD autophosphorylation.

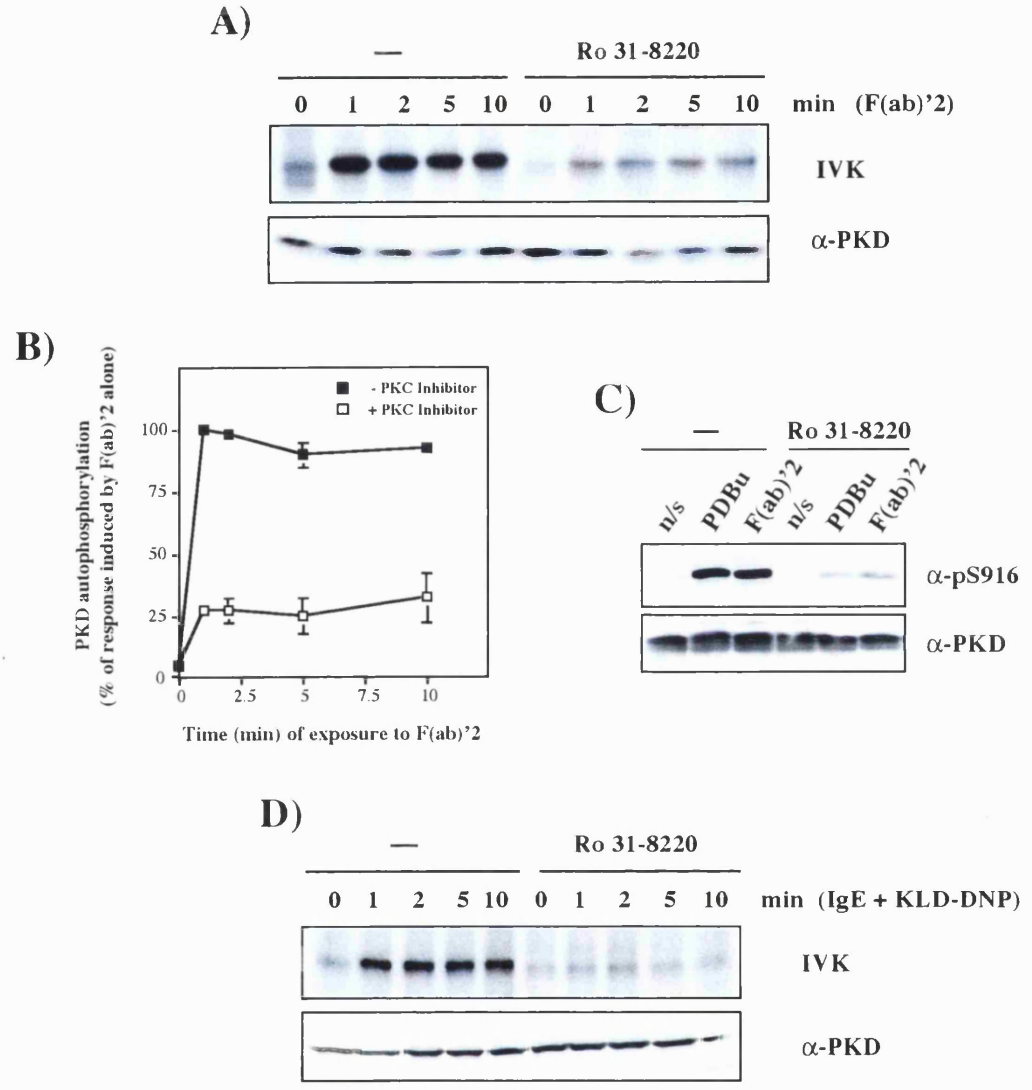


Fig. 5.7. Activation of PKD by antigen receptors is controlled by a PKC-dependent mechanism in B lymphocytes and mast cells.

(**A**) A20 B lymphocytes were preincubated with 2.5 μM Ro 31-8220 (an inhibitor of classical/novel PKCs) or with an equivalent volume of solvent (-) for 1 hour prior to activation of the BCR complex, using 10μg/ml F(ab)'2 fragments of anti-mouse IgG, for the times indicated. PKD was immunoprecipitated from cell lysates and the activity of PKD, as assessed by autophosphorylation, was measured by *in vitro* kinase assays (IVK).

(**B**) The mean ± s.e. (n=3) increase in PKD activity induced by BCR-engagement with anti-mouse F(ab)'2 in non-pretreated and in PKC-inhibitor pretreated cells. The data are expressed as a percentage of the maximum response induced by F(ab)'2 alone, as quantified by phosphoimager analysis of PKD autophosphorylation.

(**C**) A20 B lymphocytes were pre-treated for 1 h with either solvent alone (-) or with 2.5 μM Ro 31-8220. The cells were subsequently left unstimulated (n/s) or were treated with either 50 ng/ml PDBu (10 min) or 10 μg/ml F(ab)'2 (2 min). Whole cell lysates were analysed by SDS-PAGE and western blotting with the pS916 antiserum, before re-probing with a pan C-terminal PKD antibody.

(**D**) RBL 2H3 cells were pre-treated with either solvent (-) or 2.5 μM Ro 31-8220 for 1 h before the FœR1 was triggered as described in Fig. 5.2. PKD activity was measured by autophosphorylation assays, as in panel (A). Results are representative of 2 independent experiments.

effect on PKD (Zugaza *et al.*, 1996), was investigated. The data presented in Figure 5.7a/b demonstrates that BCR-induced activation of PKD was severely impaired in B cells that had been pre-treated with Ro 31-8220, compared to the marked increase in PKD activity observed in control, BCR-stimulated cells. The inhibition of BCR-induced activation of PKD by Ro 31-8220 was also detected by western blot analysis of whole cell extracts using the pS916 antibody. BCR-engagement poorly induced PKD S916 autophosphorylation in B cells that had been pre-treated with Ro 31-8220 (Fig. 5.7c). Moreover, phorbol ester-induced PKD S916 phosphorylation was also blocked by the Ro 31-8220 inhibitor (Fig. 5.7c) in agreement with previous findings implicating classical/novel PKC enzymes in the regulation of PKD by phorbol esters (Zugaza *et al.*, 1996).

The important role played by classical/novel PKCs in mediating the activation of PKD by antigen receptors was also demonstrated in mast cells. Here, pre-treatment of RBL 2H3 cells with the PKC inhibitor Ro 31-8220 abrogated the ability of the FcεR1 to induce PKD catalytic activity (Fig. 5.7d). Together, these results indicate that antigen receptor-induced activation of PKD is regulated through a PKC-dependent signalling pathway in T & B lymphocytes and in mast cells.

5.2.7. PKC activity is sufficient to induce PKD activation in lymphoid cells.

To address whether PKC was sufficient to induce PKD activation in antigen receptor systems, two different constitutively activated novel PKC enzymes were transiently expressed in RBL 2H3 mast cells and their effect on the basal catalytic activity of endogenous PKD was examined. Control RBL 2H3 cells transfected with an empty expression vector exhibited a low basal level of PKD activity (as measured by *in vitro* kinase assays) that was markedly enhanced upon crosslinking of the FcεR1 (Fig. 5.8a). In contrast, ectopic expression of activated mutants of PKCη or PKCε in RBL 2H3 mast cells (both of which activate PKD when expressed in COS-7 fibroblasts (Zugaza *et al.*, 1996)) was found to induce near maximal PKD activity in the absence of FcεR1 engagement (Fig. 5.8a), as measured by *in vitro* kinase assays.

Similar data was observed when A20 B lymphoma cells were transiently transfected with a constitutively active PKCη mutant. Here, a high basal level of PKD activity was detected in B cells that was only slightly further enhanced by stimulation of the BCR complex (Fig. 5.8b), as measured by western blot analysis of whole cell extracts using the pS916 antiserum to detect active PKD. Thus a PKC-dependent signalling pathway is required and is sufficient to activate PKD in hematopoietic cells.

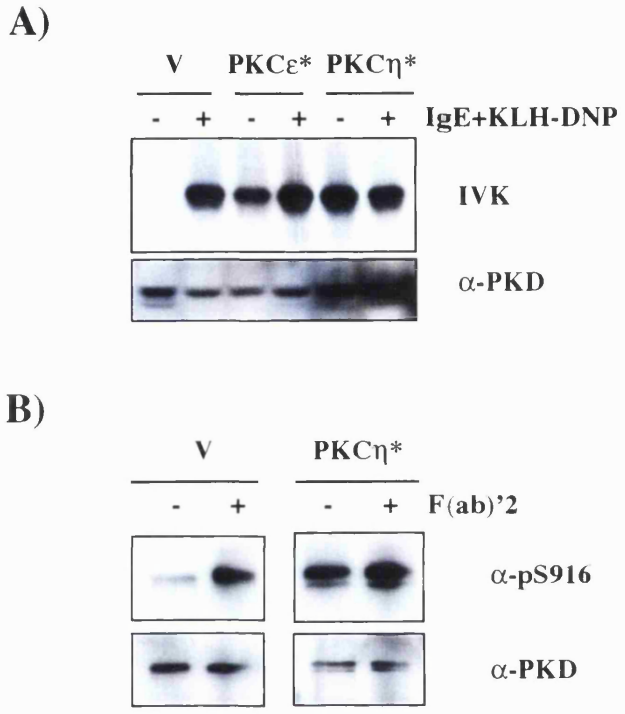


Fig. 5.8. Expression of constitutively active PKC enzymes in B lymphocytes and mast cells is sufficient to activate PKD.

(A) RBL 2H3 cells were transfected with 20 μg of vector control (V), or activated PKCη (PKCη*) or PKCε (PKCε*) cDNA constructs. After 16 h incubation at 37°C, cells were left unstimulated (-) or stimulated with IgE + KLH-DNP for 2 min (+), as described in Fig. 5.2. Endogenous PKD was immunoprecipitated from whole cell lysates using the PA-1 antibody and PKD activity (autophosphorylation) was assayed by *in vitro* kinase assays, SDS-PAGE and autoradiography. Data are representative of 2 independent experiments.

(B) A20 B lymphocytes were transiently transfected with 20 μg of either vector control (V) or activated PKCη (PKCη*) cDNA constructs. After 16 h incubation at 37°C, cells were left unstimulated (-) or were stimulated with F(ab)'2 fragments of anti-mouse IgG for 2 min. Whole cell lysates were analysed by SDS-PAGE and western blotting with the pS916 antiserum, before re-probing with a pan C-terminal PKD antibody. Data are representative of 2 individual experiments.

5.2.8. Mutation of the C1 domain of PKD does not prevent its activation *in vivo*.

The two cysteine-rich motifs (C1A and C1B) within the C1 domain of PKD are not functionally equivalent, with C1B acting as the major phorbol ester/DAG binding site both *in vitro* and *in vivo* (Iglesias *et al.*, 1998a). A highly conserved proline residue, present in all DAG-binding C1 domains described to date, is essential for high affinity binding of DAG or phorbol esters to these domains (Hurley *et al.*, 1997). Mutation of this critical residue within the C1B motif of PKD to glycine (P287G) severely abrogates phorbol ester, and thus also DAG, binding to PKD (Iglesias *et al.*, 1998a). Using this PKD mutant, experiments were carried out to determine whether direct high affinity DAG/phorbol ester binding to the PKD C1 domain was required for the activation of PKD in intact cells or whether PKD activity was solely induced through PKC-regulated phosphorylation events. Phorbol ester-induced activation of both wild-type and P287G PKD (transiently expressed in COS-7 cells) was measured in *in vitro* kinase assays. In parallel, phorbol ester binding curves for these PKD proteins was measured. The dose-response curves for the activation of wild-type PKD and PKD-P287G by the phorbol ester PDBu were identical, as shown by PKD autophosphorylation (Fig. 5.9a) or by phosphorylation of an exogenous Syntide-2 peptide (data not shown). In striking contrast, the dose-response curves for binding of [^{3}H]PDBu to wild type PKD or P287G PKD expressed in COS-7 cells were clearly distinct (Fig. 5.9b).

Furthermore the PKD P287G mutant, when transiently expressed in A20 B cells, displayed identical kinetics and magnitude of activation as wild-type PKD following stimulation of the BCR complex (Fig. 5.9c). As shown in Figure 5.9d, the PKC inhibitor GF 109303X (which has no direct action on PKD) dramatically inhibited PDBu-induced activation of either wild type PKD or PKD-P287G in transfected COS-7 cells indicating that PKD-P287G, like wild-type PKD, requires functional classical/novel PKC isoforms for its activation. Indeed, co-transfection of wild-type PKD or PKD-P287G together with a constitutively activated PKCη mutant was sufficient to induce maximal activation of both PKD forms (Fig. 5.9e). No such increases in the basal activity of wild-type or P287G mutant PKD were detected when they were co-transfected with vector alone, although they could be activated by PDBu (Fig. 5.9e). Thus, activation of PKD-P287G, like that of wild-type PKD, occurs through a PKC-dependent signalling pathway. From these data it is apparent that direct high affinity binding of DAG/phorbol esters to the PKD C1 domain is not essential for PKD activity *in vivo*, consistent with a role for classical/novel PKCs as upstream regulators of PKD in antigen receptor signalling, as demonstrated above.

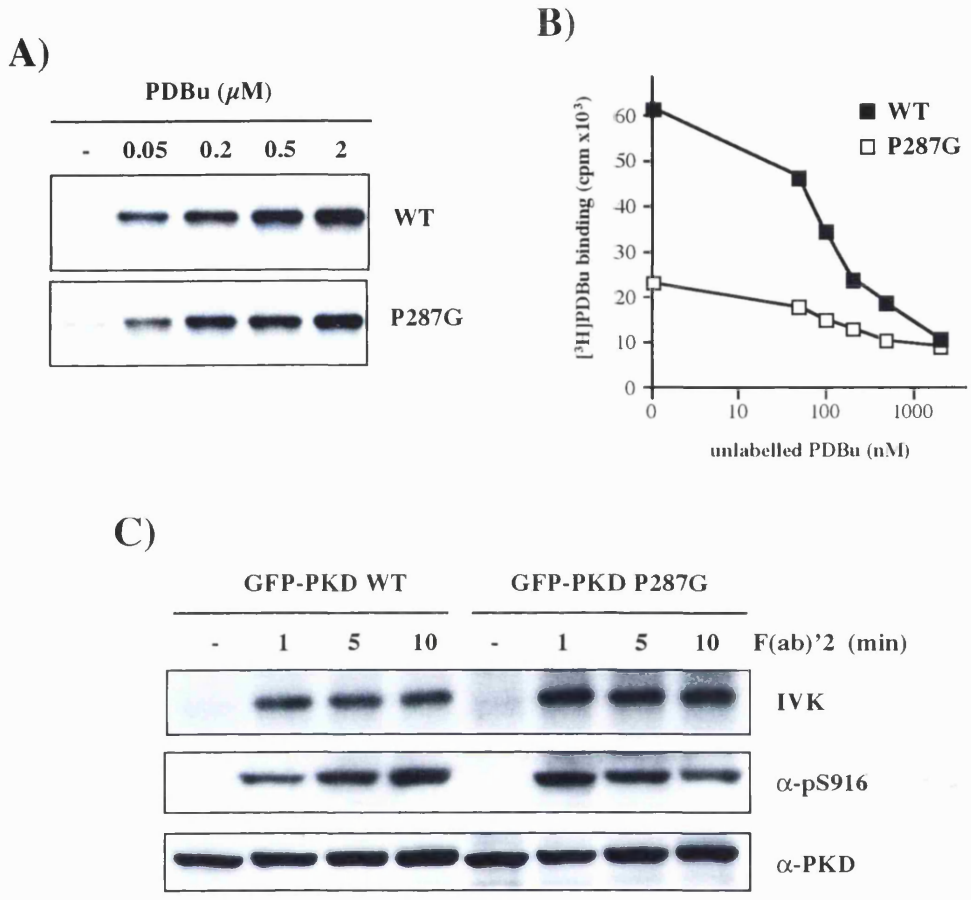


Fig. 5.9. High affinity DAG/phorbol ester binding to PKD is not essential for PKD activity in intact cells..

(A) COS-7 cells were transfected with 20 μg of wild-type or P287G PKD cDNA constructs. After 72 h incubation at 37°C, cells were left unstimulated (-) or stimulated with increasing concentrations of PDBu (0.05-2μM) for 10 min. PKD was immunoprecipitated from whole cell lysates using the PA-1 antibody and PKD activity was assayed by *in vitro* kinase assays, measuring PKD autophosphorylation. Data are representative of 2 independent experiments.

(B) Binding curve of 20 nM [^{3}H]PDBu to wild-type or P287G PKD transiently expressed in COS-7 fibroblasts - competition with increasing concentrations non-isotopic PDBu. Results are the mean of duplicates, where non-specific binding, measured in the presence of excess (10μM) unlabelled PDBu was subtracted. Similar results were obtained in 3 independent experiments.

(C) A20 B lymphocytes were transiently transfected with 20 μg of GFP-tagged wild-type or P287G PKD cDNA constructs. After 16 h incubation at 37°C, cells were left unstimulated (-) or were stimulated with F(ab)'2 fragments of anti-mouse IgG for different times, as indicated. PKD was immunoprecipitated from whole cell lysates using a GFP mAb and PKD autophosphorylation was assayed by *in vitro* kinase assays, SDS-PAGE and autoradiography. In addition, lysates were analysed by SDs-PAGE and western blotting with the pS916 antiserum and subsequently a pan PKD antibody (sc-935). Data are representative of 2 individual experiments.

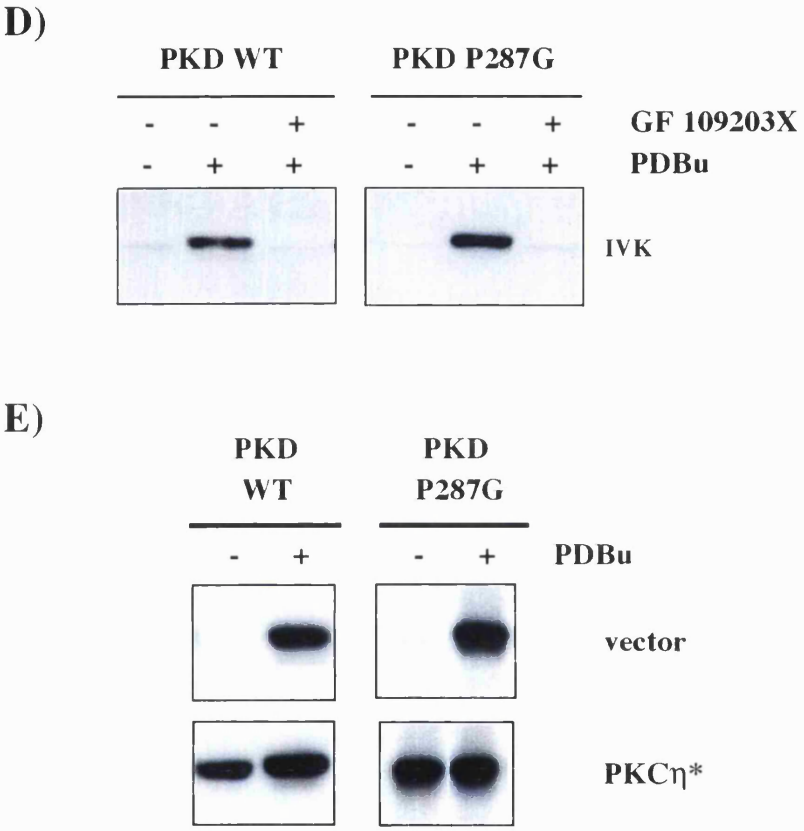


Fig. 5.9.

(D) COS-7 cells expressing PKD WT or PKD-P287 were left untreated (-) or were pretreated (+) with the PKC-specific inhibitor GF 109203X (3.5 mM) for 1 h, as indicated. The cells were subsequently left unstimulated (-) or were treated with 50 ng/ml PDBu for 15 min (+) before the cells were lysed and PKD immunoprecipitated with the PA-1 antiserum. PKD activity was measured using *in vitro* kinase assays.

(E) COS-7 cells were co-transfected with either vector control or PKC η* constructs in the presence of either PKD WT or PKD-P287G, as indicated. Cells were then left unstimulated (-) or were treated with 50 ng/ml PDBu for 15 min (+). PKD activity was determined by *in vitro* kinase assays. Data are representative of 2 independent experiments.

5.2.9. A role for PI3-Kinase in the regulation of PKD by the BCR complex.

The presence of a PH domain within the N-terminal regulatory region of PKD suggested a second potential mechanism for the modulation of PKD activity following antigen receptor engagement. The lipid products of PI3-Kinase have been previously shown to bind to the PH domains of various signalling intermediates promoting their activation, including the serine kinase PKB/Akt (reviewed in (Alessi and Cohen, 1998; Downward, 1998)) and the Tec family of tyrosine kinases (Rameh *et al.*, 1997; Salim *et al.*, 1996). Antigen receptors are known to regulate the lipid-kinase activity of PI3-Kinase leading to the production of these phosphoinositides (see Chapter 1). Thus, a potential role for PI3-Kinase in the regulation PKD activation was investigated.

A20 B cells were pre-treated with LY 294002 (a well characterised inhibitor of PI3-Kinase) prior to stimulation of the BCR complex with F(ab)'2 fragments of anti-mouse IgG. As shown in Figure 5.10a/b, BCR-mediated activation of PKD was reduced by ~50-70% in LY 294002 pre-treated cells compared to control non-pretreated lymphocytes. In contrast the activity of the PI3-Kinase-dependent serine kinase PKB/Akt was completely abrogated under these conditions (Fig. 5.10a). Moreover, a 5-fold higher concentration of LY 294002 did not inhibit BCR-induced PKD activation to any greater extent (Fig. 5.10c).

To determine whether PI3-Kinase directly regulates PKD, a constitutively active PI3-Kinase mutant was transiently expressed in B cells and the activity of endogenous PKD was subsequently measured under basal and BCR-stimulated conditions. As indicated in Figure 5.11, transient expression of an active PI3-Kinase did not increase PKD activity above basal levels, although subsequent BCR-ligation did induce significant PKD activity in these cells. In contrast the activity of the serine kinase PKB/Akt was maximally induced by expression of active PI3-Kinase in non-stimulated B cells (Fig. 5.11).

5.2.10. The inhibitory co-receptor FcγRIIb attenuates BCR-induced PKD activity.

Negative regulatory signalling cascades are vital for a balanced immune response and to ensure homeostasis of the immune system. One important negative feedback mechanism that operates in B lymphocytes is mediated by the FcγRIIb inhibitory co-receptor so that simultaneous occupancy of the FcγRIIb, together with the BCR complex, attenuates specific BCR-induced activation signals, including PKB/Akt; the Ras/MAPK signalling pathway and calcium mobilisation (Coggeshall, 1998; O'Long, 1999; Scharenberg and Kinet, 1996; Scharenberg and Kinet, 1998b; Yokoyama, 1997). A role for the

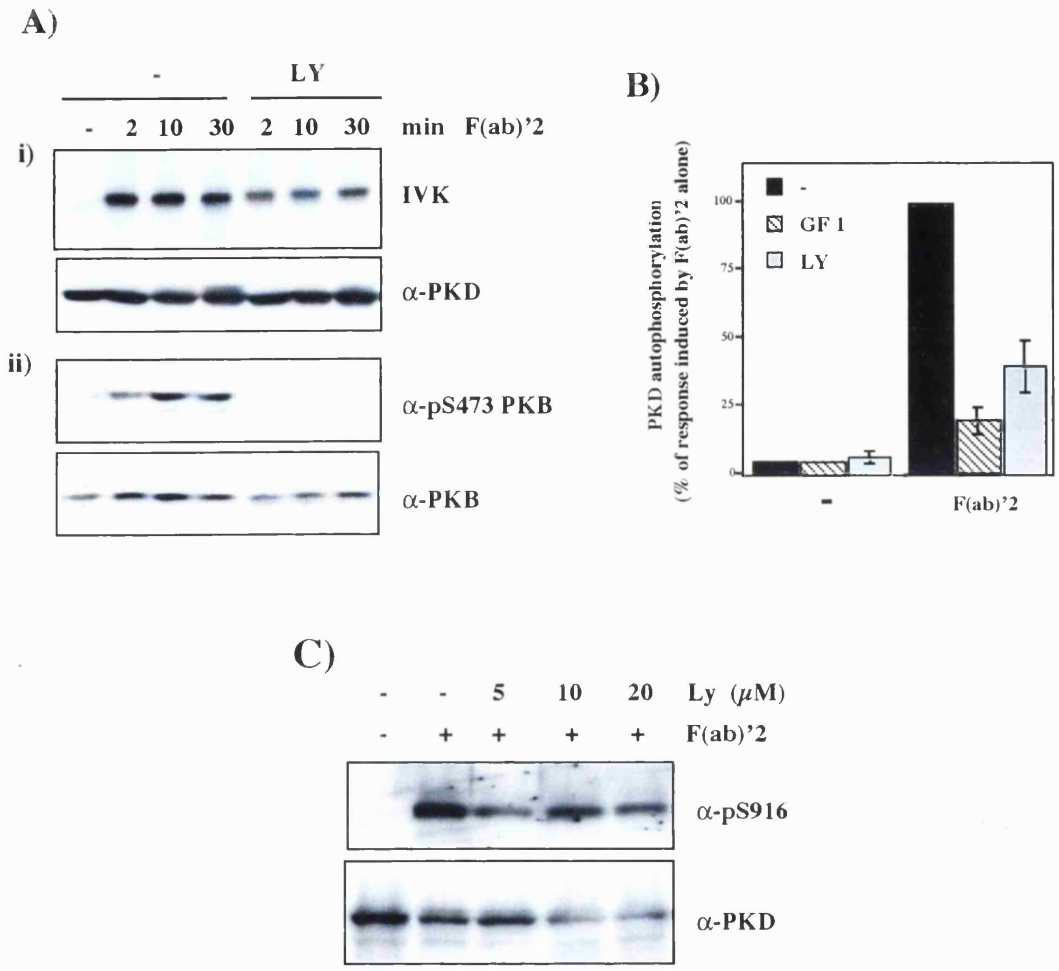


Fig. 5.10. A role for P13-Kinase in the regulation of PKD activity in B lymphocytes.
(A) Inhibition of PI3-Kinase reduces BCR-activation of PKD. A20 B lymphocytes were preincubated with 5 μM LY 294002 or with an equivalent volume of solvent (-) for 30 min prior to activation of the BCR complex using 10μg/ml F(ab)'2 fragments of anti-mouse IgG for 2-30 min, as indicated. PKD was immunoprecipitated from cell lysates and the activity of PKD, as assessed by autophosphorylation, was measured by *in vitro* kinase assays (Panel i, IVK). Lysates were also analysed by SDS-PAGE and western blotting with a C-terminal PKD antibody (sc-935), Panel i). The blot was stripped and reprobed with a pS473 PKB/Akt antibody and subsequently with a pan-PKB antibody, Panel ii). Data are representative of 3 independent experiments.
(B) The mean $\pm$ s.e. (n=3) increase in PKD activity in A20 B lymphocytes stimulated with 10μg/ml anti-mouse F(ab)'2 anti-mouse IgG for 2min. The cells were either left non-pretreated or were pretreated with either GF 109203X (GF 1, 3.5 μM) or LY 294002 (LY, 5 μM) for 1 h. The data are expressed as a percentage of the maximum response induced by F(ab)'2 alone, as quantified by phosphoimager analysis of PKD autophosphorylation.
(C) A20 B lymphocytes were preincubated with increasing concentrations of LY 294002 (5-20 μM) or with an equivalent volume of solvent (-) for 30 min prior to activation of the BCR complex with 10 μg/ml F(ab)'2 fragments of anti-mouse IgG for 2 min (+), as indicated. Total cell lysates were subjected to SDS-PAGE and western blot analysis using the pS916 antiserum and a pan C-terminal PKD antibody (sc-935).

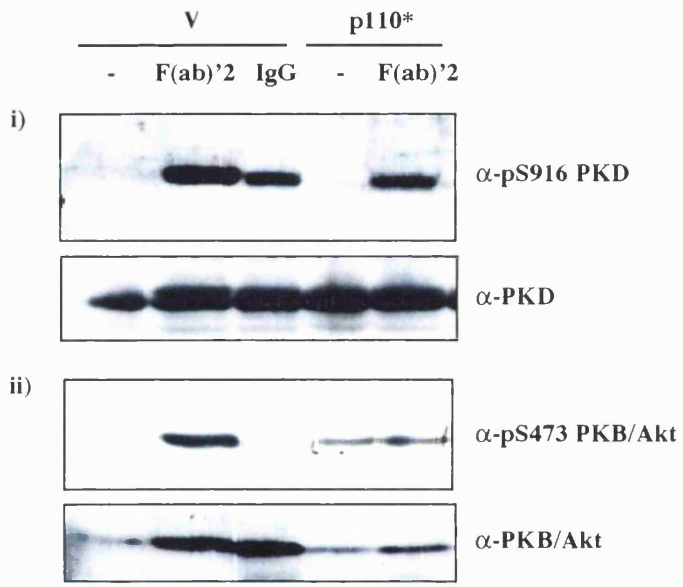


Fig. 5.11. A constitutively active PI3-Kinase mutant is not sufficient to activate PKD.
A20 B lymphocytes were transiently transfected with 20 μg of either vector control (V) or activated PI3-Kinase (p110*) cDNA constructs. After 16 h incubation at 37°C, cells were left unstimulated (-) or were stimulated with 10 μg/ml F(ab)'2 fragments of anti-mouse IgG for 2 min to stimulate the BCR complex. Alternatively, cells were treated with 7.5 μg/ml intact anti-mouse IgG to co-crosslink the inhibitory FcγRIIb receptor into the BCR complex. Whole cell lysates were analysed by SDS-PAGE and western blotting, either with the pS916 antiserum and a pan C-terminal PKD antibody (i) or with pS473 PKB and pan PKB antibodies (ii).
Identical results were seen in 2 independent experiments.

inhibitory FcγRIIb receptor in DAG-PKC signalling cascades has not previously been explored.

To determine whether PKD was regulated by the inhibitory FcγRIIb co-receptor, B cells were stimulated with equimolar amounts of either F(ab)'2 fragments of anti-mouse IgG (to activate the BCR complex) or with Intact anti-mouse IgG (to co-crosslink the inhibitory FcγRIIb into the BCR complex). The data in Figure 5.11 shows that stimulation of B cells with Intact Ig completely abolished BCR-induced PKB/Akt activity within <2 min of stimulation, in agreement with previous observations (Dr. E. Astoul, Lymphocyte Activation Laboratory, ICRF, *Personal communication*). However, at this time point PKD activity (as measured by autophosphorylation of S916) was only slightly reduced.

When analysis of PKD activity in BCR versus BCR/FcγRIIb stimulated B cells was extended over longer time period a striking, delayed inhibition of PKD activity by the FcγRIIb was observed. Thus, stimulation of the BCR with F(ab)'2 induced a rapid and sustained activation of PKD, as observed by *in vitro* kinase assays measuring PKD autophosphorylation and also by western blot analysis of cell lysates with the pS916 antiserum, which specifically recognises active PKD (Fig. 5.12). In contrast, stimulation of B cells with Intact IgG had a marked inhibitory effect on BCR-induced PKD activation, with a rapid but now transient increase in PKD catalytic activity observed (Fig. 5.12). Co-ligation of FcγRIIb with the BCR caused PKD activity to decrease to basal levels within <20 min of stimulation at times when PKD activity in BCR-stimulated lymphocytes was still maximal. This pattern of PKD activation, as revealed by *in vitro* kinase assays (Fig. 5.12), was exactly paralleled by transient autophosphorylation of the C-terminal S916 residue of PKD, as detected by western blot analysis of total cell lysates using the pS916 antiserum (Fig. 5.12). In contrast, PKD activity induced by the pharmacological agent PDBu was not abrogated by the FcγRIIb (Fig. 5.13a).

Conformation that the inhibitory action of Intact IgG did indeed arise from the specific recruitment of the FcγRIIb into the BCR complex came from studies using an anti-FcγRIIb antibody. This antibody blocks the interaction of Intact IgG with the FcγRIIb co-receptor but not with the BCR complex, thus allowing Intact IgG to act as a positive stimulatory agonist (i.e. similar to F(ab)'2). As shown in Figure 5.13b, pre-incubation of B cells with the anti-FcγRIIb blocking antibody prevented the abolition of BCR-induced PKD activity by the FcγRIIb in B cells stimulated with Intact IgG. Thus, PKD is regulated both by both positive and negative signalling cascades in B lymphocytes.

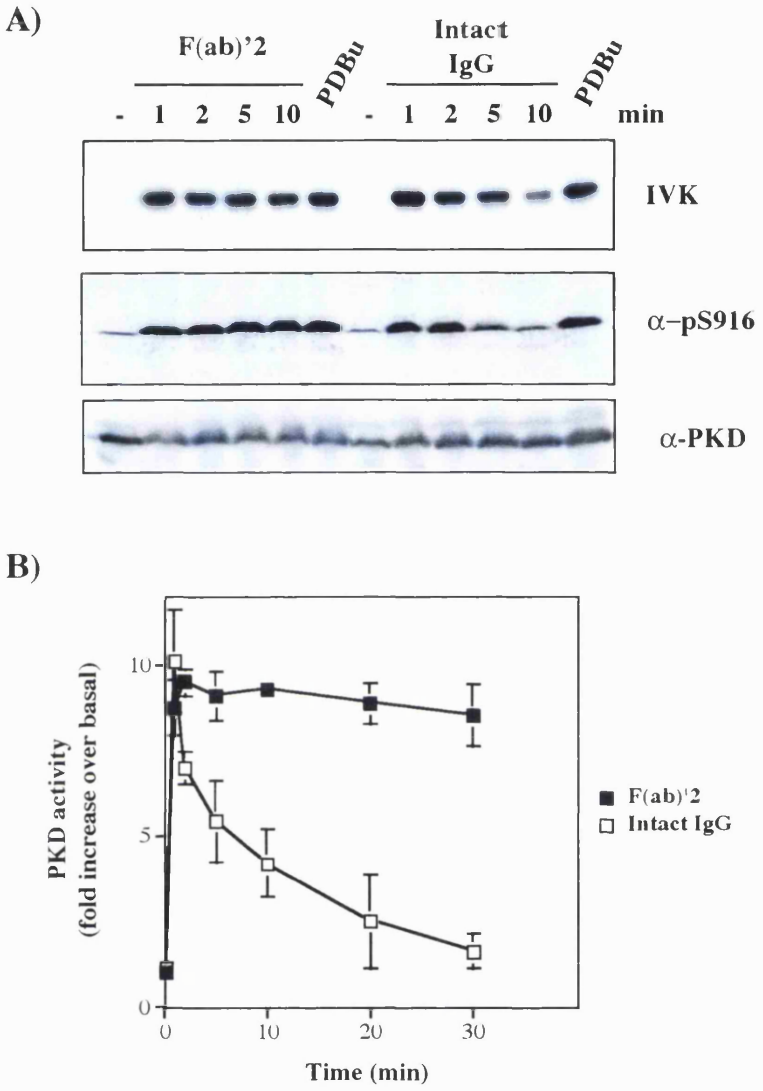


Fig. 5.12. The inhibitory FcγRIIb co-receptor supresses BCR-induced PKD activity.
(A) A20 B lymphocytes were left unstimulated (-) or were treated with 50 ng/ml PDBu for 10 min. Alternatively, the cells were stimulated with either 10 μg/ml F(ab)'2 anti-mouse IgG or 7.5 μg/ml Intact anti-mouse IgG for various times (as indicated) in order to activate the BCR and BCR/FcγRIIb complexes, respectively. Endogenous PKD was immunoprecipitated from whole cell lysates using the PA-1 antibody and was assayed by *in vitro* kinase assays, using PKD autophosphorylation as a readout for activity (IVK). In parallel the lysates were also analysed by SDS-PAGE and western blotting with the pS916 antibody and with a pan C-terminal PKD antibody (sc-935). Results are representative of three independent experiments.
(B) Graphical presentation of the effects of BCR and BCR/FcγRIIb co-ligation on PKD activity (autophosphorylation) in A20 B lymphocytes, as measured by *in vitro* kinase assays and quantitation by phosphoimager analysis. The data are expressed as the mean ± s.e. fold-increase in activity induced by F(ab)'2 or Intact IgG above basal PKD activity in non-stimulated cells in 3 independent experiments.

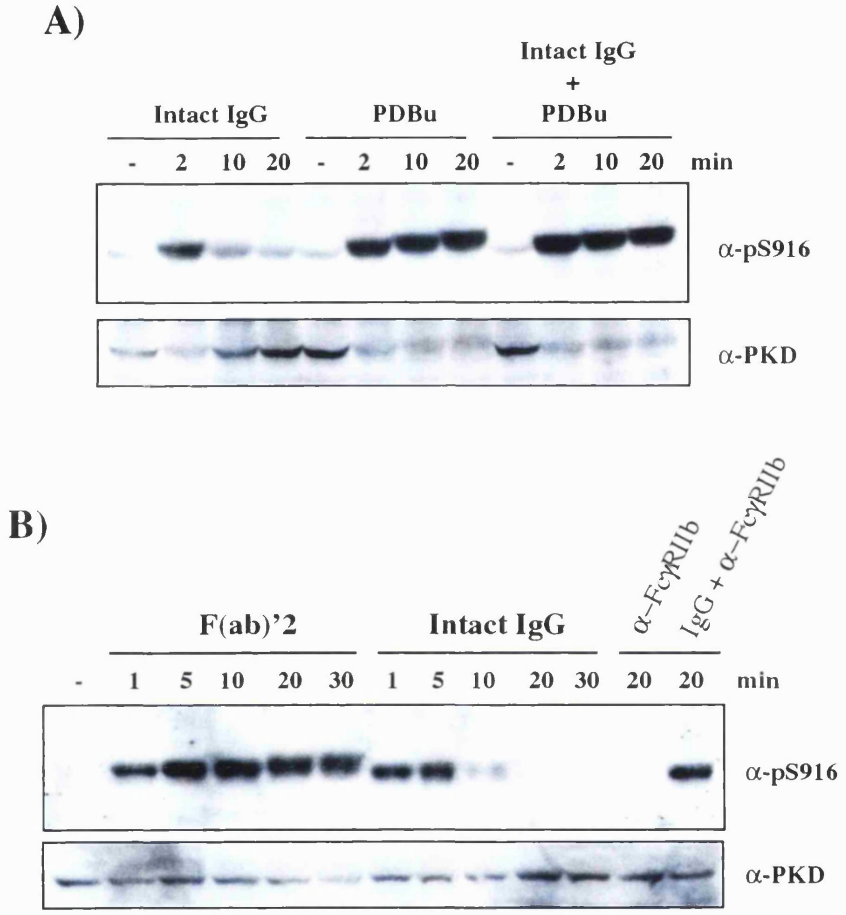


Fig. 5.13. The FcγRIIB does not prevent activation of PKD by phorbol esters.
(A) A20 B lymphocytes were left unstimulated (-) or were treated with 50 ng/ml PDBu for 2, 10 or 20 min in the presence or absence of 7.5 μg/ml Intact anti-mouse IgG. Whole cell lysates were analysed by SDS-PAGE and western blotting with the pS916 antibody (which specifically recognises active PKD) and with a pan C-terminal PKD antibody (sc-935).
(B) A20 B lymphocytes were pretreated with 2.5μg/ml α-FcγRIIb for 30 min before stimulation with either 10 μg/ml F(ab)'2 or 7.5 μg/ml Intact anti-mouse IgG for various times (as indicated). Whole cell lysates were analysed by SDS-PAGE and western blotting with the pS916 antibody and with a pan C-terminal PKD antibody (sc-935). Results are representative of two independent experiments.

5.3. DISCUSSION.

The data presented in this chapter identifies the PKC-related serine kinase PKD as a target for antigen receptor-mediated activation signals in B and T lymphocytes and in mast cells. Triggering of antigen receptors in B and T lymphocytes was found to induce PKD catalytic activity. A similar increase in PKD activity was also observed in antigen receptor-stimulated mast cells. These responses were extremely rapid, with maximal PKD activity detectable within less than one minute, indicating that activation of PKD is an early signalling event downstream of antigen receptors. The level of PKD activity stimulated by antigen receptor ligation was comparable to that observed in phorbol ester-treated cells. Phosphorylation of the carboxy-terminal S916 residue of PKD was found to correlate strikingly well with the activity status of PKD in antigen receptor regulated lymphocytes, confirming that the pS916 antibody is a novel, sensitive tool which can be used to study PKD regulation *in vivo*.

5.3.1. Antigen receptors regulate a PKC-PKD signalling cascade in T & B lymphocytes and in mast cells.

Historically, DAG/phorbol ester sensitive PKC enzymes were positioned in the earliest models of antigen receptor signal transduction but there has been little real progress in identifying the targets for PKC signalling in lymphocytes. Although dependent on PLCγ in avian DT40 B lymphocytes (Sidorenko *et al.*, 1996), a critical aspect of the data presented in this chapter demonstrates that PKD activity in hematopoietic cells is not directly regulated by DAG but is instead indirectly regulated by DAG via classical and/or novel PKC enzymes. Specifically, inhibitors of classical and novel PKCs (that do not directly effect PKD activity) were able to block the activation of PKD following BCR, TCR or FcεR1 ligation. In addition, expression of constitutively active mutants of novel PKC enzymes (PKCη or ε) was sufficient to maximally stimulate PKD catalytic activity in B cells and in mast cells. These data firmly establish PKD as a PKC-regulated serine kinase in leukocytes.

Further evidence that PKD is not directly regulated by DAG in hematopoietic cells came from studies using a PKD mutant containing a proline to glycine point mutation within its C1B motif (P287G). The PKD P287G mutant exhibits severely reduced phorbol ester binding affinity *in vivo* (Iglesias *et al.*, 1998a) but strikingly its activation by PDBu or following BCR-ligation was normal when compared to wild-type PKD. Thus the regulation of PKD activity in intact cells can clearly be dissociated from direct high affinity binding of exogenous phorbol esters or endogenous DAG to

the PKD C1 domain. It should be noted that a minor contributory role for low affinity phorbol ester to binding to the PKD C1 domain in the activation process cannot be completely excluded since a PKD deletion mutant lacking the entire C1 domain shows constitutive high basal activity (Iglesias and Rozengurt, 1999). However, the observation that this PKD-P287G mutant does not translocate to cellular membranes in response to physiological or indeed pharamcological agonists (see Chapter 6), indicating that this mutant is unable to bind endogenous DAG, would argue against this possibility. Thus the data described in this chapter demonstrates that the major mechanism by which PKD is activated by antigen receptors *in vivo* is via a DAG-regulated, PKC-dependent signalling pathway.

Exactly which PKC isoforms activate PKD *in vivo* within antigen receptor signalling cascades is unclear at present and will be the subject of future research. Of particular interest would be PKCθ and PKCβ, since these PKC isoforms have been specifically implicated in TCR and BCR signalling cascades, respectively. Expression of PKCθ is primarily restricted in the immune system to cells of the T lymphocyte lineage (Baier *et al.*, 1993) and confocal microscopy studies have revealed the selective recruitment of PKCθ to the plasma membrane at the contact zone formed between T lymphocytes and antigen presenting cells (Monks *et al.*, 1998; Monks *et al.*, 1997). PKCθ has also been implicated in the regulation of JNK activity and IL-2 transcription, in synergy with calcineurin (Werlen *et al.*, 1998). In contrast, $PKC\beta_{I/II}$ knockout mice show a B lymphocyte specific phenotype, such that humoral responses to T cell-independent antigens are lost (Leitges *et al.*, 1996). Interestingly, a constitutively active $PKC\beta_I$ mutant has been shown to substantially increase the basal activity of wild-type PKD when the two are co-expressed in COS-7 fibroblasts (Zugaza *et al.*, 1996). Thus, it will be important to test the contribution of PKCθ and PKCβ signalling pathways for PKD activity in T and B lymphocytes, respectively.

The identification of PKD as a proximal downstream target for DAG-regulated PKC kinases advances our understanding of this signalling cascade in T & B cells and in mast cells. However, the exact mechanism by which PKCs regulate successful immune responses, and importantly whether PKD is implicated in these events or not, remains unclear.

5.3.2. CD28, but not the IL-2 receptor, activates PKD in T lymphocytes.

Crosslinking of cell surface CD28 molecules using the 9.3 mAb antibody induced significant PKD activity in the JH6.2 T cells, in agreement with previous data demonstrating

that high levels of CD28 aggregation (induced by crosslinking mAbs) is associated with PLCγ activation and subsequent calcium mobilisation in T lymphocytes (Ledbetter *et al.*, 1988; Nunes *et al.*, 1993; Weiss *et al.*, 1986). However, other studies have established that crosslinking CD28 mAbs regulate distinct intracellular signalling cascades from those induced by the interaction of CD28 with its physiological ligand, B7 (Nunes *et al.*, 1994; Vandenberghe *et al.*, 1992). Thus, although PLCγ is activated by CD28 crosslinking antibodies, stimulation of CD28 by B7 is not classically associated with the activation of PLCγ-regulated signalling cascades (Nunes *et al.*, 1994; Ward *et al.*, 1993). Whether the T cell CD28 co-stimulatory receptor can regulate PKD under physiological conditions remains to be determined. In addition, it will be of interest to investigate whether CD28 co-stimulation would enhance a TCR-regulated PLCγ/PKC/PKD signalling cascade *in vivo*.

Stimulation of T lymphocytes with the regulatory cytokine IL-2 does not induce $PI(4,5)P_2$ hydrolysis, calcium mobilisation or PKC translocation, indicating that PLCγ is not regulated by the IL-2 receptor in T lymphocytes (Farrar and Anderson, 1985; Mills *et al.*, 1985; Mills *et al.*, 1986; Sawami *et al.*, 1992). Generation of mono-unsaturated/saturated DAGs has been detected in IL-2-treated T lymphocytes however, indicative of PC-PLC and/or PLD activity (Lu *et al.*, 1999; Merida *et al.*, 1990), although other reports have dispute this (Jones *et al.*, 1998; Jones *et al.*, 1999). Dukin & co-workers have proposed a model whereby IL-2 activates membrane-localised PKC through these PC-PLC/PLD-derived DAG species in T cells (Lu and Durkin, 1997b; Lu *et al.*, 1999). Moreover, in the early 1980's PKC membrane translocation in T cells induced by IL-2 was reported (Farrar and Anderson, 1985). However, subsequent studies have not reproduced these findings. Furthermore, it is generally thought that mono-unsaturated/saturated DAGs are biologically inactive *in vivo* (Hodgkin *et al.*, 1998). The data presented in this chapter demonstrating that IL-2 treatment of peripheral T lymphoblasts does not activate PKD would provide additional evidence to support the hypothesis that IL-2 does not regulate PKC enzymes, given that PKD is activated via classical/novel PKC enzymes in these cells.

5.3.3. A role for PI3-Kinase in the activation of PKD by the B cell antigen receptor.

The multidomain structure of PKD indicates that other signalling inputs to PKD could potentially regulate PKD, in addition to the PKC-dependent pathway described above. In this context, PKD contains a PH domain, a regulatory module that controls the localisation and activity of many intracellular signalling molecules through binding the D3-

phosphoinositide lipid products of PI3-Kinase (Gibson *et al.*, 1994; Lemmon *et al.*, 1996). Indeed, a specific inhibitor of PI3-Kinase, LY 294002, was shown to reduce antigen receptor-induced PKD activity in B cells. PI3-Kinase dependent signals themselves were not sufficient to stimulate PKD however, since transient expression of a constitutively active PI3-Kinase in B cells did not enhance PKD activity above basal levels. These data suggest that PI3-Kinase is important, but is not sufficient, for maximal PKD activity in B cells.

As discussed in Chapter 1, the critical activation loop priming sites of PKC enzymes are regulated by the phosphoinositide-dependent kinase PDK1, which could potentially account for the partial requirement for PI3-Kinase in the regulation of PKD activity by the BCR complex. However, this hypothesis would not explain the PI3-Kinase independent regulation of PKD activity by various G proteins coupled receptors and tyrosine kinase receptors in fibroblast cell lines. For example, PKD catalytic activity mediated by bombesin or PDGF receptors is not blocked by inhibitors of PI3-Kinase and a PDGF receptor mutant lacking a PI3-Kinase docking site is able to activate PKD as efficiently as a wild-type PDGF receptor (Van Lint *et al.*, 1998; Zugaza *et al.*, 1997).

An alternative explanation may arise from the observation that in T and B lymphocytes and in mast cells membrane localised PLCγ is co-ordinately phosphorylated and activated by Src tyrosine kinases in partnership with Tec family tyrosine kinases. In contrast, expression of Tec family kinases has not been detected outside of the hematopoietic system. Here, the activation of Tec kinases is PI3-Kinase dependent: $PI(3,4,5)P_3$ interacts with the PH domain of Tec family members resulting in their membrane targeting and subsequent activation. $PI(3,4,5)P_3$-dependent Tec kinases are not absolutely required for PLCγ activity however, since inhibition of PI3-Kinase only partially blocks IP_3 production or calcium mobilisation (Scharenberg and Kinet, 1998), rather Tec kinases function to maximise Src kinase-induced PLCγ activity. A role for PI3-Kinase-dependent Tec kinases in the regulation of PLCγ in B cells, and thus in the control of a DAG/PKC/PKD signalling cascade, could therefore account for the observed reduction of PKD activity in LY 294002-pretreated B cells.

This hypothesis is supported by data demonstrating that activation of PKD by the BCR complex is reduced in B cells which lack the Tec kinase Btk but is completely abrogated when either PLCγ or Syk (an upstream tyrosine kinase that regulates both Btk and PLCγ) are absent (Sidorenko *et al.*, 1996). One major Tec kinase expressed in T lymphocytes is Rlk, which lacks a PH domain and is activated independently of PI3-Kinase upon TCR-ligation (Debnath *et al.*, 1999). Interestingly, preliminary observations indicate that PKD activity in TCR-stimulated T lymphocytes appears to be independent of PI3-Kinase, perhaps through regulation of PLCγ by this $PI(3,4,5)P_3$-independent Tec kinase (Dr A. Lawrence, Lymphocyte Activation laboratory, ICRF, *Personal communication*).

Taken together, these data would support a model whereby PI3-Kinase dependent Tec kinases control a regulatory input to a PLCγ/DAG/PKC signalling cascade that regulates PKD activity in response to antigen receptor ligation in B cells, but not in other cell types. A similar mechanism may also regulate FcεR1-mediated activation of PKD in mast cells since a PI3-Kinase/Tec kinase link to PLCγ has been described here and inhibitors of PI3-Kinase block calcium mobilisation following FcεR1 ligation (Kinet, 1999).

5.3.4. Inhibition of PKD catalytic activity by the inhibitory FcγRIIb receptor.

The FcγRIIb co-receptor mediates vital homeostatic control of B lymphocyte activation and effector functions through dominant inhibition of specific BCR-regulated signalling pathways, via the inositol 5' phosphatase SHIP (Coggeshall, 1998; Scharenberg and Kinet, 1998b). FcγRIIb/SHIP inhibitory signals have long been known to inhibit calcium responses from the BCR. However, the FcγRIIb does not abolish all BCR-induced calcium signals but instead specifically blocks the sustained phase of calcium mobilisation that results from the influx of extracellular calcium (Amigorena *et al.*, 1992; Bijsterbosch and Klaus, 1985; Choquet *et al.*, 1993; Diegel *et al.*, 1994; Muta *et al.*, 1994; Ono *et al.*, 1996; Ono *et al.*, 1997). In contrast, BCR-induced PLCγ tyrosine phosphorylation; IP_3 generation and calcium release from intracellular stores are unaffected, or are only partially inhibited, upon co-engagement of the FcγRIIb (Bijsterbosch and Klaus, 1985; Hippen *et al.*, 1997; Kiener *et al.*, 1997; Muta *et al.*, 1994; Scharenberg *et al.*, 1998a).

Importantly, PI3-Kinase-dependent Tec kinases have recently been implicated in the regulation of calcium influx (Bolland *et al.*, 1998; Debnath *et al.*, 1999; Fluckiger *et al.*, 1998; Liu *et al.*, 1998b). Thus, FcγRIIb/SHIP-mediated dephosphorylation of $PI(3,4,5)P_3$ inhibits calcium influxes by inhibiting the activity of PI3-Kinase-dependent Tec kinases. From studies involving Tec-deficient mice or B cells ectopically expressing different Tec kinases two distinct models have been proposed for how Tec kinases may regulate calcium influxes. In the first model, proposed by Scharenberg and Kinet, Tec kinases in combination with Syk would fully activate PLCγ resulting in high levels of IP_3, continued depletion of intracellular calcium stores and efficient coupling to plasma membrane store-operated channels. Inhibition of Tec kinases by the FcγRIIb would result in only a partial activation of PLCγ and thus reduced IP_3 generation. This would prevent calcium stores from being fully depleted, leading to inefficient coupling to store-operated channels (Scharenberg and Kinet, 1998b). Alternatively, Tec kinases may function independently of PLCγ to control calcium influxes, either by acting directly on plasma membrane store-

operated channels or on the store-operated pathway that controls the opening of these channels.

The data presented in this chapter now demonstrates that the FcγRIIb inhibitory co-receptor also suppresses a DAG-regulated signalling pathway in B cells. Co-recruitment of the FcγRIIb into the BCR complex did not prevent the initial activation of PKD, but was found to terminate PKD activity (and autophosphorylation of its C-terminal S916 residue) within <20 minutes of receptor co-engagement. Although activation of PKD by the BCR is regulated by PI3-Kinase, probably through PI-(3,4,5)P_3-dependent Tec kinase regulation of PLCγ, several lines of evidence indicate that inhibition of Btk by SHIP-mediated dephosphorylation of PI(3,4,5)P_3 may not be the primary mechanism by which the FcγRIIb terminates PKD activity in B cells. First, the initial phase of PKD activity is identical in BCR versus BCR/FcγRIIb activated B cells, indicating that PLCγ is functionally active under these conditions and is capable stimulating a DAG-PKC-PKD signalling pathway, consistent with only a partial role for Tec kinases in the regulation of PLCγ. In contrast the activity of the PI3-Kinase dependent kinase PKB/Akt is extremely rapidly terminated (within <2 min) upon co-ligation of the FcγRIIb with the BCR in A20 B cells (Dr. E. Astoul, Lymphocyte Activation Laboratory, ICRF, *Personal communication*). Second, the PI3-Kinase inhibitor LY 294002 only partially abrogates BCR-induced PKD activity, whereas co-engagement of the FcγRIIb completely suppresses BCR-induced PKD activation.

At present the exact mechanism by which the FcγRIIb inhibits PKD activity in B cells is unknown and represents an intriguing challenge for future investigation. It is possible that the FcγRIIb inhibits PKD through a novel mechanism since not all of the inhibitory actions of this receptor can be explained through the 3'phosphoinositide phosphatase activity of SHIP. For example, PI3-Kinase inhibitors do not prevent BCR-induced activation of ERK1/2 but FcγRIIb/BCR co-stimulation does inhibit ERK activity in B lymphocytes (Dr. E Astoul, Lymphocyte Activation Laboratory, ICRF, *Personal communication*). Moreover, it has been proposed that FcγRIIb-mediated inhibition of the Ras/ERK pathway in B cells results from competition between the SH_2 domains of SHIP and Shc for Grb2 binding, thus preventing the initiation of a Shc/Grb2/Ras signalling cascade (Sarmay *et al.*, 1996; Tridandapani *et al.*, 1997a; Tridandapani *et al.*, 1997b). However, recent work by the Kurosaki laboratory indicates that ERK can be activated normally in Shc-deficient DT40 B cells, at least with respect to EGF receptor signalling (Hashimoto *et al.*, 1999) suggesting that this model may not account for the inhibitory actions of the FcγRIIb towards ERK.

Thus it will be important to test whether the phosphatase or SH_2 domains of SHIP are required to inhibit PKD activity, and indeed whether SHIP mediates the inhibitory effect of the FcγRIIb towards PKD or not. Two clear possibilities exist: (1) the FcγRIIb could recruit (either directly or indirectly) a serine phosphatase that dephosphorylates and inactivates PKD or (2) PKD activity requires sustained upstream signals for activity that are terminated upon FγRIIb and BCR co-engagement. Both of these hypotheses require further investigation.

CHAPTER 6 : Spatial and Temporal regulation of PKD by antigen receptors

6.1. INTRODUCTION.

Protein targeting to specific cellular locations as the result of increases in lipid second messengers or the activation of adaptor molecules is a key mechanism for regulating complex intracellular signalling cascades (reviewed in (Lemmon, 1999; Mochly-Rosen, 1995; Pawson and Scott, 1997)). The integration of spatial localisation with activity is crucial in determining the sites of action of different signalling intermediates, which is important as it affords insight into potential functions and downstream targets for these molecules.

6.1.1. C1 domains as membrane targeting modules.

Early studies revealed that PKC enzymes are translocated to cellular membranes, and in particular to the plasma membrane, upon $PI(4,5)P_2$ hydrolysis or phorbol ester stimulation (Nishizuka, 1986). C1 domains function as important structural determinants for the recruitment of classical and novel PKC enzymes to the plasma membrane of various epithelial/fibroblast cell lines in response to physiological and pharmacological agonists (Feng *et al.*, 1998a; Oancea *et al.*, 1998a; Ohamori *et al.*, 1998; Sakai *et al.*, 1997; Shirai *et al.*, 1998). C1 domains may additionally target PKCs to other intracellular membranes, including the nuclear envelope (Oancea and Meyer, 1998b; Oancea *et al.*, 1998a; Ohamori *et al.*, 1998). Moreover, DAG/phorbol ester binding C1 domains also regulates membrane association of other cellular proteins including Munc-13, a neuronal specific protein involved in exocytosis (Betz *et al.*, 1998) and RasGFP, a novel Ras-specific GEF (Tognon *et al.*, 1998).

Structural studies have demonstrated that the highly conserved cysteine-rich sequence present within C1 domains form a globular structure with two β-sheets lying either side of a hydrophilic binding cavity. Phorbol ester (and thus also DAG) binding to this cavity results in the formation of a continuous hydrophobic surface that promotes the association of C1 domains with cellular membranes (Zhang *et al.*, 1995). Recently, Oancea and Meyer have defined the molecular basis of the plasma membrane recruitment of a classical PKC isoform, PKCγ (Oancea and Meyer, 1998b). A PKCγ deletion mutant lacking its pseudosubstrate motif translocates to the plasma membrane more rapidly than the wild-type enzyme upon FcεR1-engagement, indicating that the pseudosubstrate motif

may mask the C1 domain. Calcium binding to the C2 domain was sufficient to induce a rapid on/off phase association of PKCγ with membrane phospholipids, however stable interaction of PKCγ with the plasma membrane required DAG binding to the C1 domain. Based on these results, they proposed that membrane recruitment of PKCγ in activated cells is mediated by the sequential binding of calcium to the C2 domain, displacement of the pseudosubstrate motif and finally DAG binding to the C1 domain (Oancea and Meyer, 1998b).

6.1.2. PH domains as membrane targeting modules.

PH domains are key regulatory motifs that were first described in pleckstrin (Haslam *et al.*, 1993; Mayer *et al.*, 1993) but have since been identified in a variety of other proteins that possess signalling or cyoskeletal functions, as reviewed in (Gibson *et al.*, 1994; Lemmon, 1999; Shaw, 1995). Although the different PH domains possess limited primary sequence identity they share a common 3-dimensional structure that consists of two β-sheets and a C-terminal α-helix joined by charged variable loops that are often divergent.

A number of PH domains function to control the membrane localisation of a cellular signalling proteins, either through binding to lipid second messengers or via protein/protein interactions. For example, the $PI(3,4,5)_3$ lipid product of PI3-Kinase binds with high affinity to the PH domains of both Btk and PKB/Akt, an event which is required for the plasma membrane targeting and subsequent activation of these kinases (Alessi and Cohen, 1998; Bolland *et al.*, 1998; Downward, 1998; Leevers *et al.*, 1999; Lemmon, 1999; Varnai *et al.*, 1999). The importance of the Btk PH domain for the activation/function of this kinase is illustrated by the observation that mutations in this domain (which lower the affinity for $PI(3,4,5)P_3$ and prevent plasma membrane recruitment) compromise BCR-induced cellular responses resulting in X-linked immunodefficiency diseases (Hyvonen and Saraste, 1997; Salim *et al.*, 1996; Tsukada *et al.*, 1994; Varnai *et al.*, 1999). In contrast, gain of function Btk PH domain mutants exhibit constitutive membrane localisation due to increased affinity for other inositol phospholipids (Varnai *et al.*, 1999). Similar $PI(3,4,5)P_3$-dependent membrane translocations have now been observed for the PH domains of various Ras-GAP proteins and for the ARNO and GRP1 proteins, which regulate the small GTPase Arf-1 (Leevers *et al.*, 1999; Lemmon, 1999). In addition, $PI(3,4,5)P_3$ may enhance PLCγ signalling through binding to the PH and/or SH_2 -domains of PLCγ, potentially stabilising the

membrane association of this enzyme (Bae *et al.*, 1998; Falasca *et al.*, 1998; Rameh *et al.*, 1998).

Membrane targeting roles have also been described for the PH domains of proteins which only weakly (and non-specifically) bind to phosphoinositide lipids, here an additional domain is often required for efficient membrane association of these proteins. For example, agonist-dependent translocation of the β adrenergic receptor kinase (βARK) to the plasma membrane, where it phosphorylates the β_2-adrenergic receptor, requires the co-operative binding of βγ subunits of heterotrimeric G proteins and $PI(4,5)P_2$ to the βARK PH domain and to an adjacent C-terminal region (Pitcher *et al.*, 1995; Touhara *et al.*, 1994). Neither of these interactions alone is sufficient to drive βARK membrane recruitment. Similarly, both the PH and the PTB domains of insulin receptor substrate-1 (IRS1) are required for efficient membrane targeting and subsequent tyrosine phosphorylation of IRS1 by active insulin receptors (Yenush *et al.*, 1996) and membrane targeting of Tiam1 (a Rac-1 GEF) requires both its PH domain and an adjacent putative coiled-coil motif (Stam *et al.*, 1997).

6.1.3. PKD cellular localisation.

PKD is highly expressed in hematopoietic cells (Rennecke *et al.*, 1996) and antigen receptor engagement in T and B cells rapidly stimulates PKD activity (see Chapter 5). However, despite the clear functional link between PKD and antigen receptors in lymphocytes there has been no analysis of PKD localisation in resting or in antigen receptor-activated lymphocytes. In this regard it has been previously described that PKD is partially localised with the Golgi compartment in a human hepatocellular carcinoma cell line and in human antral gastrin cells (Moore *et al.*, 1999; Prestle *et al.*, 1996). It has also been reported that PKD functions to regulate Golgi organisation and transport processes (Jamora *et al.*, 1999; Prestle *et al.*, 1996). Therefore a possibility existed that PKD would also be localised to the Golgi complex in hematopoietic cells.

One feature of PKD that might determine its subcellular localisation is the presence of a C1 domain within its N-terminal regulatory region that has previously been demonstrated to bind DAG and phorbol esters (Dieterich *et al.*, 1996; Valverde *et al.*, 1994; Van Lint *et al.*, 1995). The current consensus view is that C1 and C2 domains cooperate to bring classical PKC enzymes to the plasma membrane for activation. In this context, the regulatory domain of PKD does not possess a calcium binding C2 domain but does contain a PH domain that has been shown to mediate interactions with PKCη (Waldron *et al.*,

1999b) and is also regulated by Gβγ subunits (Jamora *et al.*, 1999). Thus the PKD PH domain could also potentially regulate the subcellular localisation of PKD *in vivo*.

6.1.4. Aims.

(1) Establish a system to study the subcellular localisation of PKD *in vivo*.

(2) Examine the regulation of PKD localisation by phorbol esters and antigen receptors.

(3) To correlate spatial and temporal aspects of PKD localisation with catalytic activity.

6.2. RESULTS.

6.2.1. Generation of a biologically active GFP-PKD chimeric fusion protein.

An assay was required to follow the subcellular localisation of PKD in hematopoietic cells. As addressed in Chapter 4, antibodies directed against the C-terminus of PKD weakly recognise activated PKD, whilst a commercial N-terminal PKD antiserum only poorly recognises murine PKD in western blotting experiments (data not shown). Thus conventional immunofluorescence or biochemical fractionation techniques to study PKD translocation were not practical.

Fluorescent molecules such as GFP (originally isolated from *Aequorea victoria*) and its derivatives have proved valuable as reporter molecules for the study of protein localisation and movement *in vivo* (Heim and Tsien, 1996; Lippincott-Schwartz, 1998; Tsien, 1998). The GFP chromophore forms an independent ~30 kDa domain with inherent fluorescence due to the cyclisation of three residues (Ser65, Tyr66 and Gly67) and dehyrogenation of Tyr67. GFP has two absorbance peaks (395nm and 475nm) and excitation at 395nm leads to emission at 508nm. As GFP fluorescence can be visualised in intact living cells this provides the opportunity to study the dynamics of protein translocation under physiological conditions in real time. This technique has been used successfully to study the translocation of a number of serine kinases *in vivo*, including PKB/Akt (Astoul *et al.*, 1999) and DAG-regulated classical/novel PKC enzymes (Feng *et al.*, 1998a; Oancea *et al.*, 1998a; Shirai *et al.*, 1998).

In order to explore the spatial-temporal dynamics of PKD regulation under conditions of pharmacological and physiological stimulation a chimeric GFP-PKD molecule was generated. This molecule, in which the GFP domain was fused to the N-terminus of PKD (as described in section 2.18) is shown schematically in Figure 6.1.

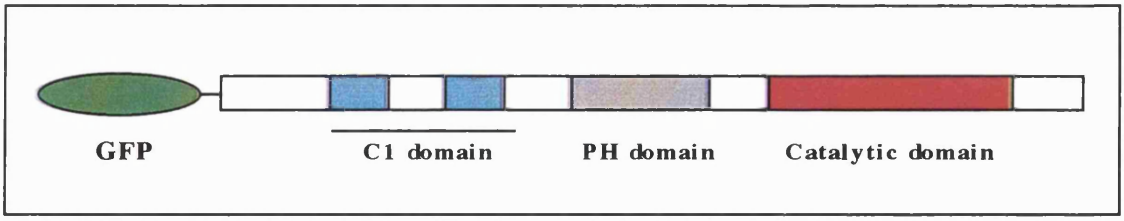


Fig. 6.1. GFP-PKD. Full length PKD was fused to the C-terminus of GFP by subcloning into a pEFplink-EGFP$_{C3}$ mammalian expression vector (provided by S. Cleverley, Lymphocyte Activation Laboratory, ICRF).

Initial experiments confirmed that the GFP-PKD molecule, when transiently expressed in A20 B cells, possessed the expected molecular mass (140 kDa) and could

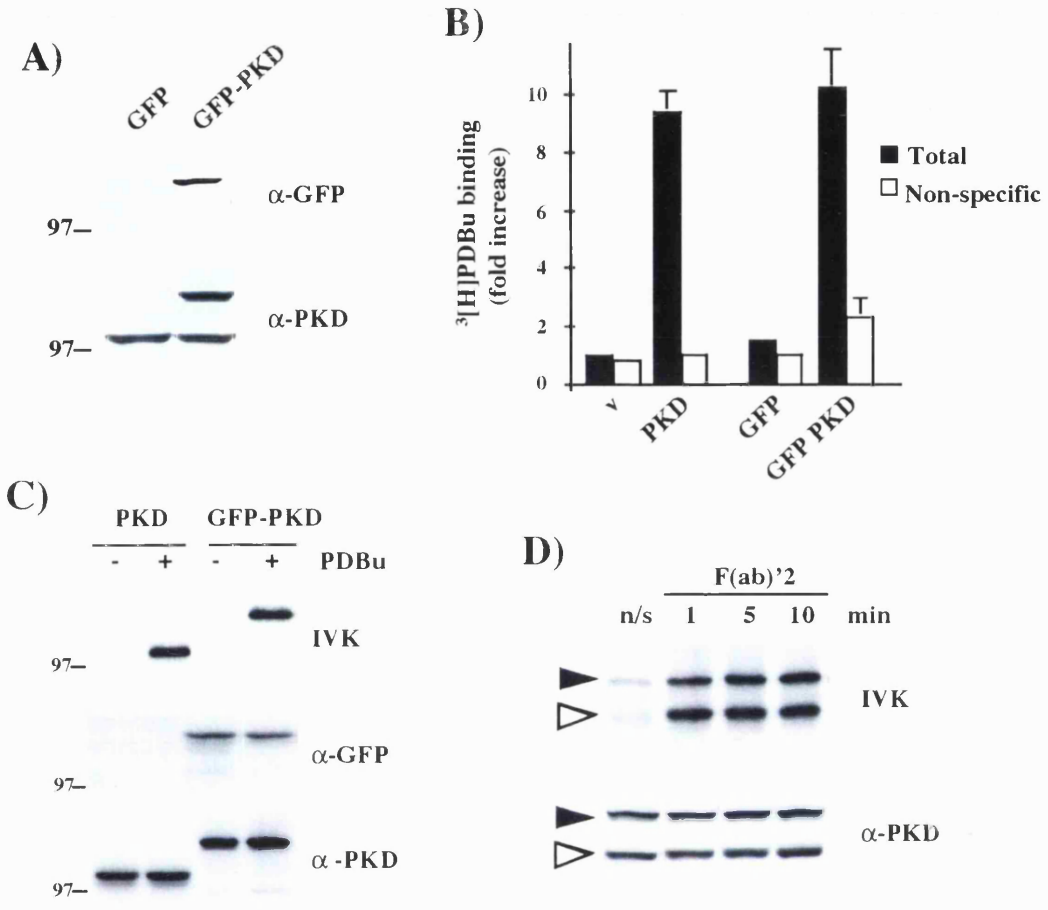


Fig. 6.2. Generation and characterisation of GFP-PKD.

(A) A20 B lymphocytes were transiently transfected with 20 μg of GFP-PKD cDNA. Whole cells lysates were fractionated by SDS-PAGE and analysed by western blot using a GFP mAb and a pan C-terminal PKD antibody (sc-935).

(B) Total (black columns) and non-specific (open columns) binding of 20 nM [^{3}H]PDBu to COS-7 cells transiently transfected with pcDNA3 vector alone (v), pcDNA3-PKD (PKD), GFP alone (GFP) or GFP-PKD cDNA constructs. Data are the mean $\pm$ s.e. of 2 independent experiments, each carried out in duplicate, and are expressed as the fold-increase in binding over vector control background levels.

(C) Wild-type PKD and GFP-PKD were transiently expressed in COS-7 cells and subsequently either left unstimulated (-) or stimulated with 50 ng/ml PDBu for 15 min (+). PKD was immunoprecipitated from whole cell lysates using the PA-1 antiserum and PKD activity (autophosphorylation) was measured by *in vitro* kinase assays. In parallel, western blot analysis of whole cell extracts was performed using a GFP mAb and a pan C-terminal PKD antibody (sc-935).

(D) A20 B lymphocytes were transiently transfected with wild-type GFP-PKD and left to recover overnight. Cells were left untreated (n/s) or were stimulated with 10 μg/ml F(ab)'2 fragments of anti-mouse IgG for various times (1-10 min). The cells were subsequently lysed before PKD (endogenous) and GFP-PKD were immunoprecipitated using the PA-1 antiserum and *in vitro* kinase assays performed. PKD activity was measured by autophosphorylation (IVK). In parallel western blot analysis of whole cell lysates with a pan C-terminal PKD antibody (sc-935) revealed PKD expression levels. Black arrowhead, GFP-PKD; white arrowhead, endogenous PKD. Similar results were obtained in 2 independent experiments.

easily be distinguished from endogenous PKD (110 kDa), as shown by western blot analysis of whole cell lysates using either PKD or GFP antibodies (Fig. 6.2a).

Fusion to the GFP molecule is a major modification of the PKD protein. It was therefore important to verify that the GFP-PKD molecule displayed biological properties similar to that of native PKD. As shown in Figure 6.2b, COS-7 cells expressing either PKD or GFP-PKD displayed an ~9-fold increase in specific [^{3}H]PDBu binding as compared to the level of [^{3}H]PDBu binding observed in COS-7 cells transfected with vector alone. In addition, fusion of the GFP-tag to the N-terminus of PKD did not effect the low basal catalytic activity of PKD, nor did it prevent PDBu-induced activation of PKD in transiently transfected COS-7 cells (Fig. 6.2c). Importantly, the kinetics of PKD activation (when ectopically expressed in B cells) following antigen receptor engagement were also uneffected by the N-terminal GFP-tag (Fig. 6.2d). These results indicate that the GFP-PKD molecule is likely to closely model the behaviour of endogenous PKD.

6.2.2. Subcellular localisation of GFP-PKD in hematopoietic cells.

To determine the subcellular distribution of PKD within hematopoietic cells, GFP-PKD was transiently expressed in either A20 B lymphoma cells or in RBL 2H3 mast cells. The data presented in Figure 6.3a shows mid-section confocal images of RBL 2H3 cells or A20 B cells that had been transiently transfected with GFP-PKD. These images demonstrate that in resting cells GFP-PKD was evenly distributed throughout the cytosol of these with no apparent association to specific intracellular compartments. In the RBL 2H3 mast cells GFP-PKD appeared to excluded from cytoplasmic vesicle-type structures (Fig. 6.3a, right) which may represent the secretory granules present in these cells. In contrast, GFP-PKD was excluded from the nuclei of both B cells and the mast cells (Fig. 6.3a), an observation that was further confirmed when the nucleus was identified by staining genomic DNA with propidium iodide. As shown in Figure 6.5b, no overlap between GFP-PKD and propidium iodide fluorescence staining was observed in resting RBL 2H3 cells. Importantly, immunofluorescence staining using a specific PKD antibody confirmed that the endogenous PKD present in B cells (as well as that of ectopically expressed PKD in transfected COS-7 cells) was distributed evenly throughout the cytosol of these cells and was excluded from the nucleus (Figure 6.3b and c).

The cytosolic distribution of GFP-PKD in B cells and mast cells described here contrasts with the proposed Golgi-localisation of PKD reported in a hepatocellular carcinoma cell line and in human antral gastrin cells (Moore *et al.*, 1999; Prestle *et al.*,

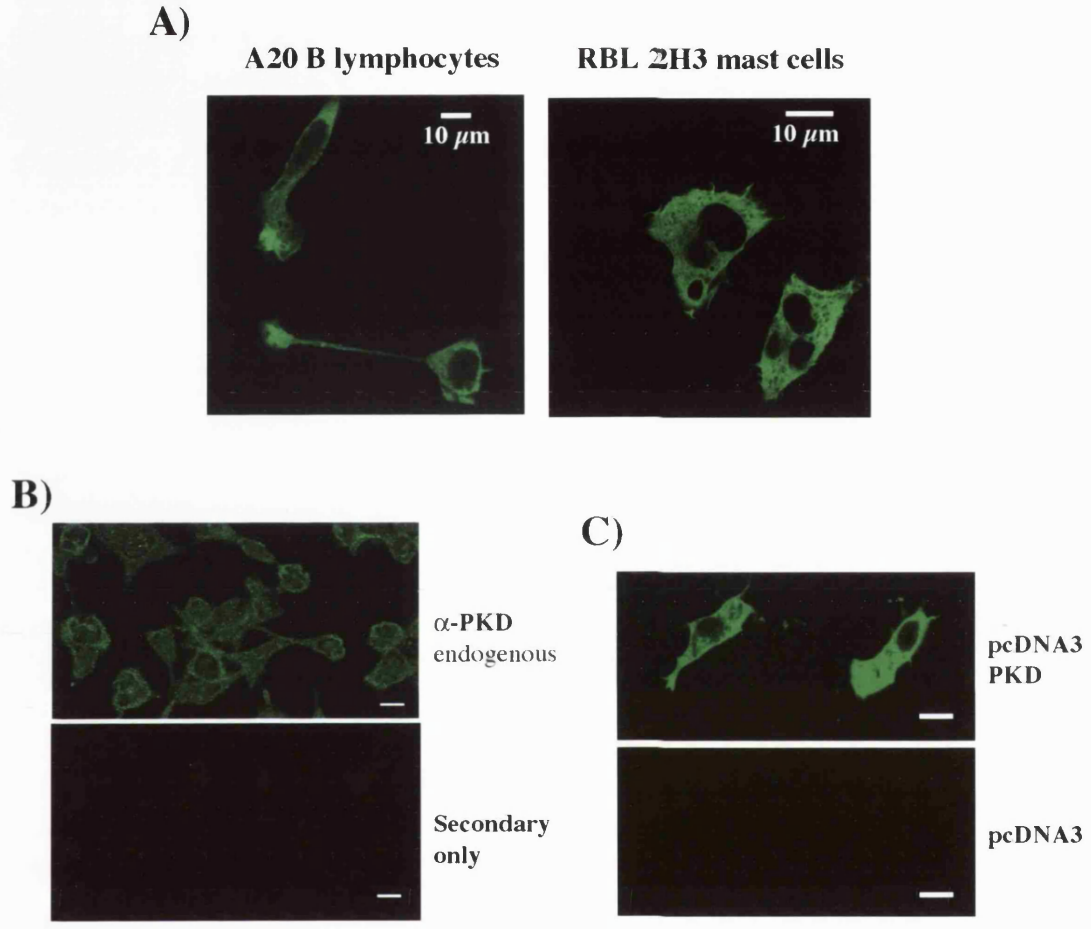


Fig. 6.3. Subcellular localisation of PKD in A20 B lymphocytes and in RBL 2H3 mast cells.
(A) A20 B lymphocytes or RBL 2H3 mast cells were transiently transfected overnight with 10 μg of GFP-PKD cDNA and subsequently analysed by confocal microscopy. Mid-section confocal images are shown demonstrating the cytosolic distribution of GFP-PKD in these cells.
(B) Subcellular distribution of endogenous PKD in A20 B lymphocytes. Immunofluorescence staining using a polyclonal anti-PKD (sc-935, 1μg/ml) was carried out as described in Chapter 2. The secondary layer was anti-rabbit Alexa^{488} (Molecular Probes, 5μg/ml). Top, confocal images of cells stained with the PKD antibody. Bottom, cells stained with secondary layer only. Bar, 10μm.
(C) COS-7 cells were transiently transfected with either pcDNA3 or pcDNA3-PKD constructs. After 24 h the cells were processed for immunofluorescence staining and confocal imaging of ectopically expressed PKD as described in (B). Bar, 10μm.

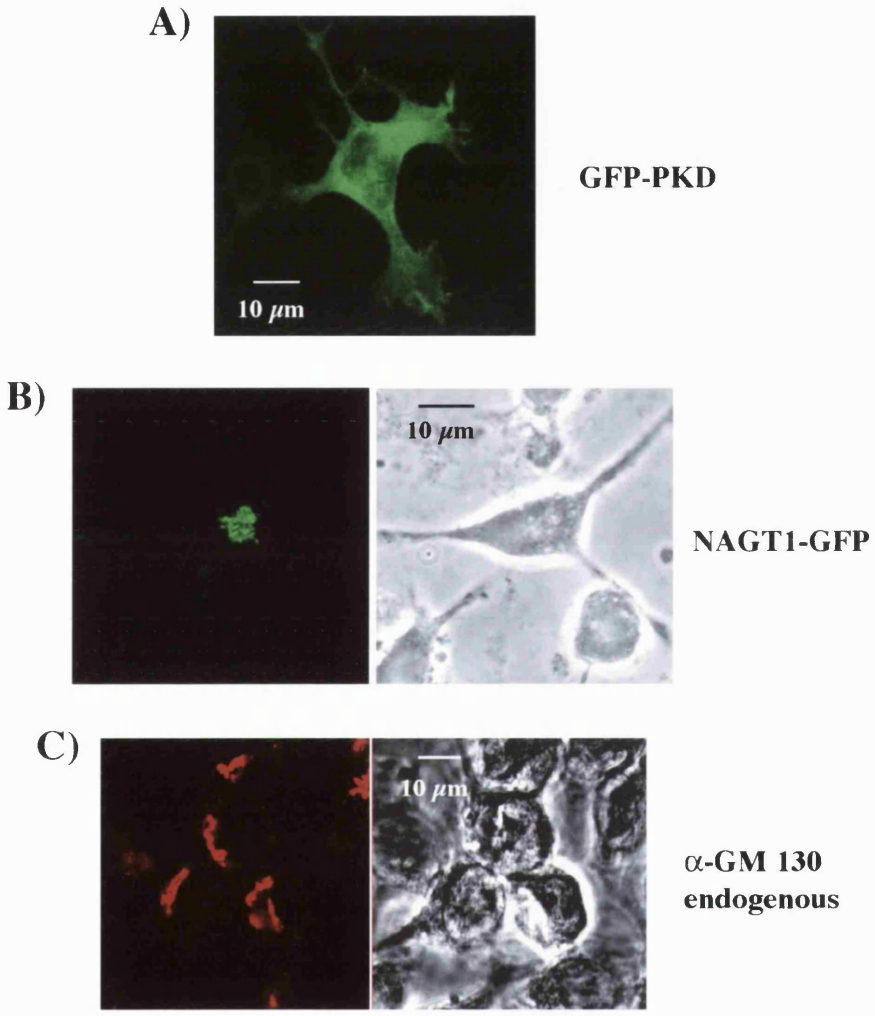


Fig. 6.4. GFP-PKD is not prefentially localised to the golgi complex in A20 B lymphocytes.
Comparision of the subcellular distribution of exogenously expressed GFP-PKD (**A**) or NAGT1-GFP (**B**) in A20 B lymphocytes. Confocal images show 2-dimensional projections of sections in Z-series taken across the depth of the cells at 0.3 μm intervals. In B) the image on the right shows a phase-contrast image of the A20 B lymphocytes.
(**C**) Subcellular distribution of the endogenous GM130 present in A20 B lymphocytes. Immunofluorescence staining was carried out using a polyclonal GM130 antiserum (ICRF, 1:200 dilution of serum) as described in Chapter 2. The secondary layer was anti-rabbit Alexa^{568} (Molecular Probes, 5μg/ml). Left, midsection confocal image of cells stained with the GM130 antibody. Right, phase-contrast image of the same cells.

1996). In further experiments it was noted that the pattern of GFP-PKD localisation in A20 B cells was clearly distinct from that of endogenous GM 130 or ectopically expressed NAGT1-GFP (Fig. 6.4), two well characterised Golgi-linked proteins (Nakamura *et al.*, 1995; Shima *et al.*, 1997). A detailed comparison of the subcellular distribution of GFP-PKD or NAGT1-GFP in A20 B cells is shown in Figure 6.4 (compare panel A and panel b). Here two-dimensional projections of sections in Z-series taken across the depth of the cells at 0.3 μm intervals clearly reveals the distinct pattern of PKD localisation compared to this Golgi marker. Thus GFP-PKD is not preferentially localised to the Golgi complex in B cells or in mast cells.

6.2.3. Phorbol esters induce plasma membrane translocation of GFP-PKD.

Translocation of classical and novel PKC enzymes to cellular membranes upon phorbol ester treatment is well established. As the N-terminal regulatory region of PKD contains a C1 domain initial experiments were carried out to determine whether the subcellular localisation of PKD was also altered upon exposure to phorbol esters. Cytosolic and membrane fractions were prepared from non-stimulated or PDBu-stimulated RBL 2H3 cells (that had been transiently transfected with GFP-PKD). Western blot analysis of the resulting fractions with a GFP mAb demonstrated the presence of GFP-PKD within both the cytosolic and also the membrane fractions of these cells (Fig. 6.5a), in agreement with previous observations for endogenous PKD (Johannes *et al.*, 1995; Valverde *et al.*, 1994). Following phorbol ester-treatment a dramatic redistribution of GFP-PKD from the cytosol to cellular membranes was observed (Fig. 6.5a).

To determine which cellular membrane(s) GFP-PKD was translocating to, RBL 2H3 mast cells expressing GFP-PKD were left unstimulated or were stimulated with PDBu before fixation and analysis by confocal microscopy. Treatment with phorbol ester resulted in a striking translocation of GFP-PKD from the cytosol to the plasma membrane of these cells (Fig. 6.5b). After a 15 minute exposure to PDBu 85±5% of the cells showed predominant plasma membrane localisation of GFP-PKD. In contrast, GFP-PKD remained localised within the cytoplasm in non-stimulated cells (Fig. 6.5b) and in RBL 2H3 cells treated with a biologically inactive phorbol ester, 4-α-phorbol (data not shown).

To study the effect of phorbol esters on the subcellular localisation of PKD in greater detail living RBL-2H3 mast cells, expressing GFP-PKD, were stimulated with PDBu and confocal images of the cells were taken at various times using an inverted LSM 510 confocal microscope. This technique allows the kinetics of PDBu-induced PKD

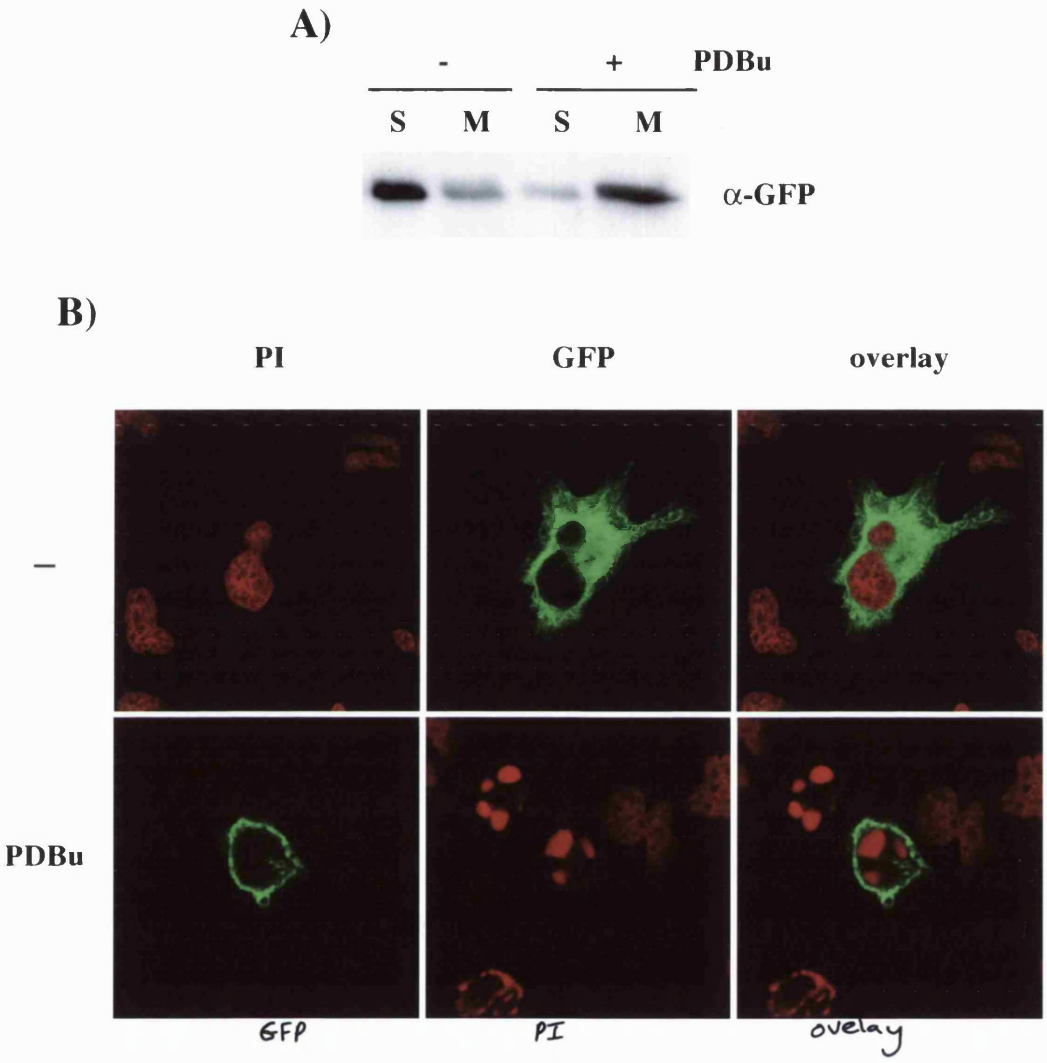


Fig. 6.5. Phorbol esters induce the plasma membrane translocation of GFP-PKD.
(A) Biochemical fractionation of RBL 2H3 cells, transiently expressing GFP-PKD, stimulated with (+) or without (-) 50 ng/ml PDBu for 15 min. Soluble (S) and membrane (M) fractions were prepared as described in Chapter 2 and analysed by SDS-PAGE and western blotting with a GFP mAb (ICRF). Identical results were obtained in 4 independent experiments.
(B) Confocal images of GFP-PKD expressing RBL 2H3 cells that were left unstimulated (upper panels) or were stimulated with PDBu for 15 min (lower panels) prior to fixation in 4% paraformaldehyde/PBS. GFP is shown in green with the nuclei counterstained with propidium iodide (red).

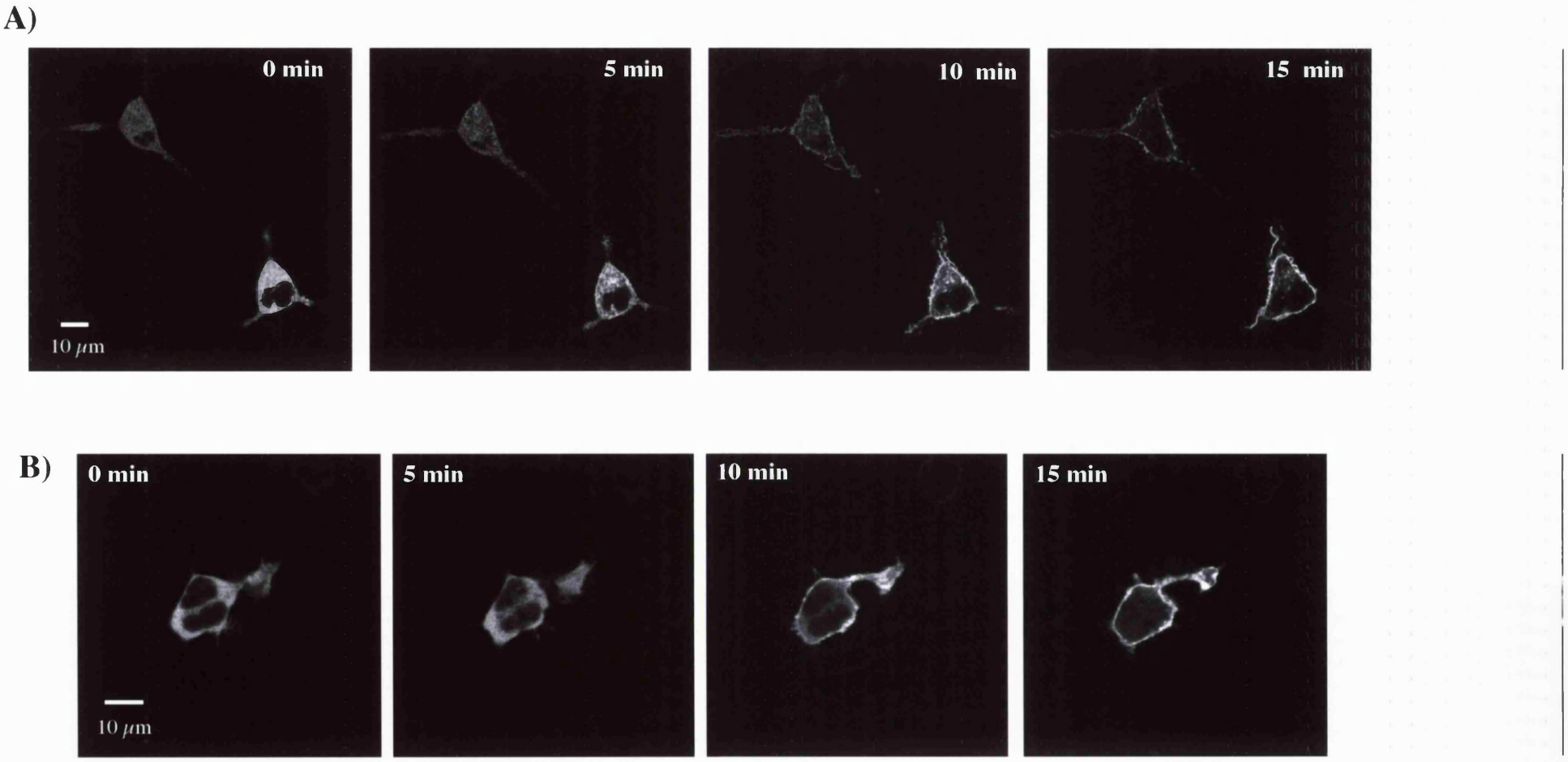


Fig. 6.6. Kinetics of phorbol ester-induced subcellular redistribution of GFP-PKD.
RBL 2H3 cells (**A**) or A20 B lymphocytes (**B**), transiently expressing GFP-PKD, were imaged in real time before and after the addition of 50 ng/ml PDBu, using an inverted confocal microscope, at 37°C. Midsection confocal images, taken at the indicated times are shown, with treatment carried out after the zero time point image had been acquired. Similar kinetics of translocation were observed in 4 independent experiments, where at least 4-5 individual cells were analysed per experiment.

translocation to be monitored in living cells in real time. The data presented in Figure 6.6a demonstrates that membrane targeting of GFP-PKD was detectable within ~6-8 minutes of PDBu treatment and was maximal within ~10-15 minutes. The effect of phorbol esters on the subcellular localisation of GFP-PKD was also investigated in B lymphocytes. Stimulation of A20 B lymphoma cells with PDBu induced the complete translocation of GFP-PKD from the cytosol to the plasma membrane of these cells within ~15 minutes (Fig. 6.6b). Analysis of these cells for longer time periods indicated that the association of GFP-PKD with the plasma membrane was maintained for over 3 hours in the continued presence of PDBu, with no evidence for redistribution of PKD back into the cytosol (data not shown).

6.2.4. Antigen receptors induce a transient subcellular redistribution of GFP-PKD.

To explore the spatial dynamics of PKD regulation following antigen receptor engagement, RBL-2H3 cells expressing GFP-PKD were activated via the FcεR1 and confocal images of the living cells were taken different times, as described above. The data presented in Figure 6.7a demonstrates that activation of the FcεR1 induced a rapid redistribution of GFP-PKD from the cytosol to the plasma membrane of mast cells. Membrane targeting of GFP-PKD was detectable within less than 1 minute after FcεR1 ligation of RBL 2H3 cells and was maximal within ~1-2 minutes. A similar response was also observed in B lymphocytes. Thus, stimulation of the BCR complex using F(ab)'2 fragments of anti-mouse IgG, induced a rapid translocation of GFP-PKD from the cytosol to the plasma membrane of these cells within ~1-2 minutes of BCR-activation (Fig. 6.7b).

Strikingly, GFP-PKD did not stably associate with the plasma membrane in antigen receptor-stimulated cells, instead this was a transient response. Consistently, GFP-PKD dissociated from the plasma membrane of BCR-stimulated lymphocytes and FcεR1-activated mast cells, returning to the cytosolic compartment of these cells within ~8-10 minutes of antigen receptor engagement (Fig. 6.7). Subsequently, GFP-PKD remained diffusely localised within the cytosol of these cells for up to 2 hours post-receptor triggering, with no evidence for significant association to specific intracellular compartments (data not shown). This was in marked contrast to the stable association of GFP-PKD at the plasma membrane of PDBu-stimulated mast cells and B cells, which was maintained over a period of hours.

As discussed in Chapter 5, the catalytic activity of PKD is negatively regulated by the FcγRIIb in B lymphocyes. To study the effect of this inhibitory co-receptor on GFP-PKD

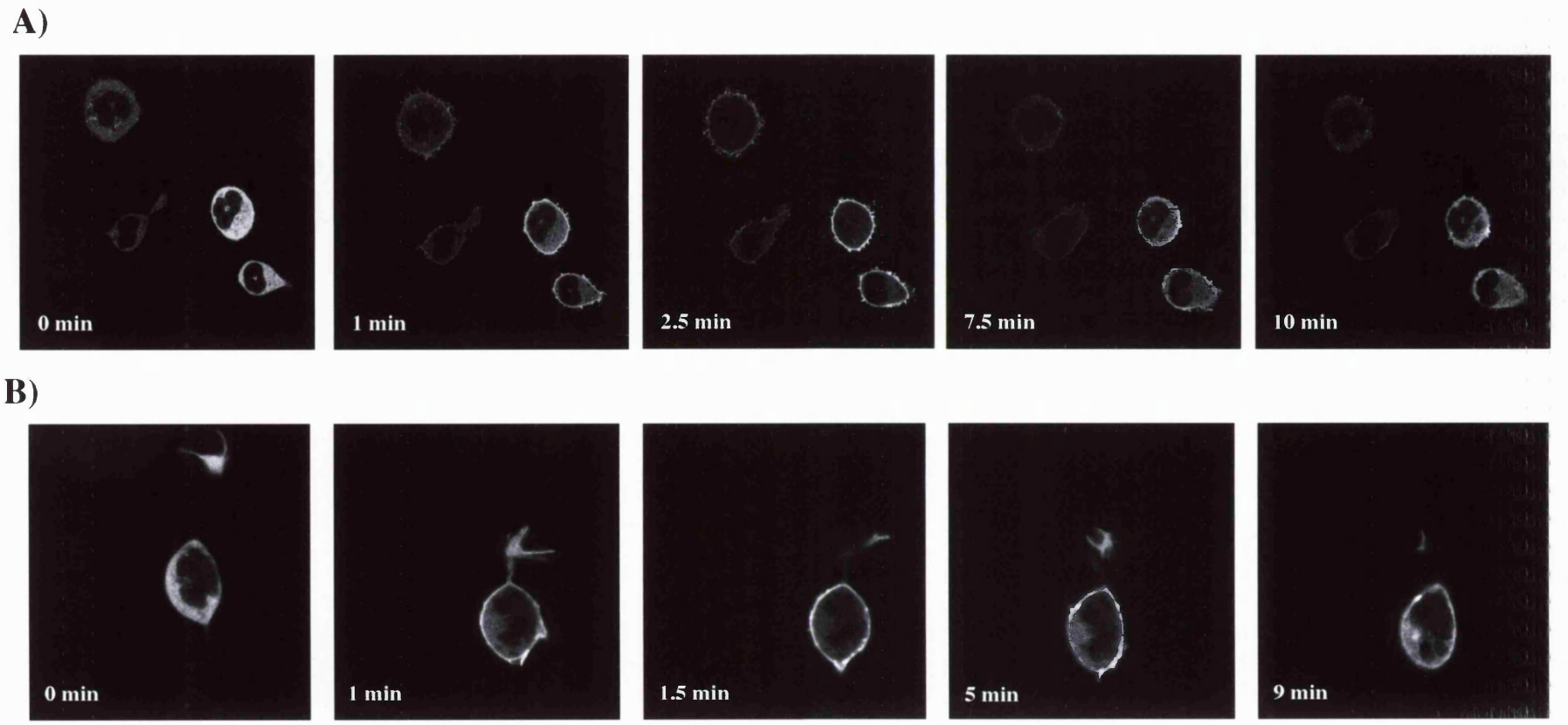


Fig. 6.7. Antigen receptor ligation induces a rapid but transient association of GFP-PKD with the plasma membrane.
RBL 2H3 cells **(A)** or A20 B lymphocytes **(B),** transiently expressing GFP-PKD, were imaged in real time before and after BCR or FcεR1 ligation respectively. GFP-PKD rapidly translocated to the plasma membrane of antigen receptor activated cells after antigen receptor engagement. The precise kinetics of GFP-PKD translocation varied subtly from cell to cell, but consistently maximal plasma membrane localisation of GFP-PKD was seen within 1-2 min of antigen receptor stimulation. GFP-PKD subsequently dissociated from the membrane, returning to the cytosol completely after ~8-10 min of antigen receptor activation. Similar results were observed in the majority of B lymphocytes (>95%) or of the mast cells (>90%) analysed. The results shown are representative of at least 4 independent experiments where each time 6-8 individual cells were imaged.

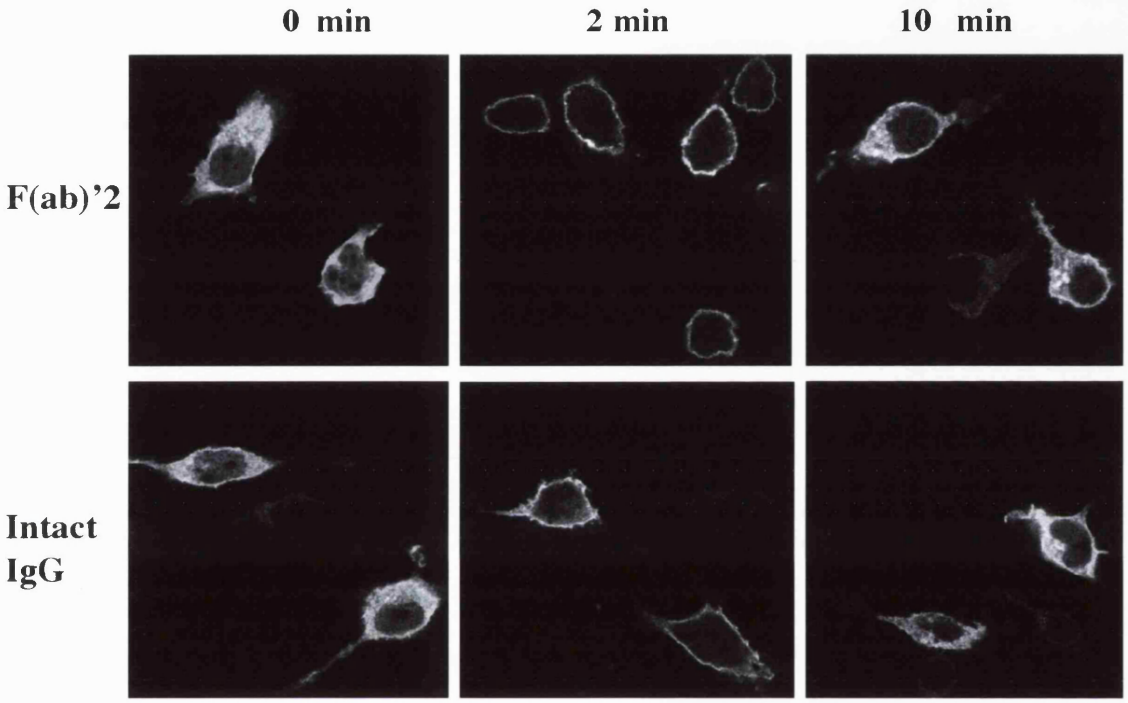


Fig. 6.8. The inhibitory FcγRIIb co-receptor does not block BCR-induced GFP-PKD translocation.

A20 B lymphocytes, transiently expressing GFP-PKD, were stimulated for different times with either 10 μg/ml F(ab)'2 to activate the BCR complex, (Upper panels) or with 7.5 μg/ml Intact IgG to co-crosslink the FcγRIIb into the BCR complex (Lower panels). The cells were fixed in 4% paraformaldehyde and processed for confocal imaging. BCR-ligation and BCR/FcγRIIb co-engagement induced a transient plasma membrane association of GFP-PKD in >95% and >90% of the cells analysed respectively. The images shown are representative of 3 independent experiments in which 100 cells/coverslip were analysed for each experiment.

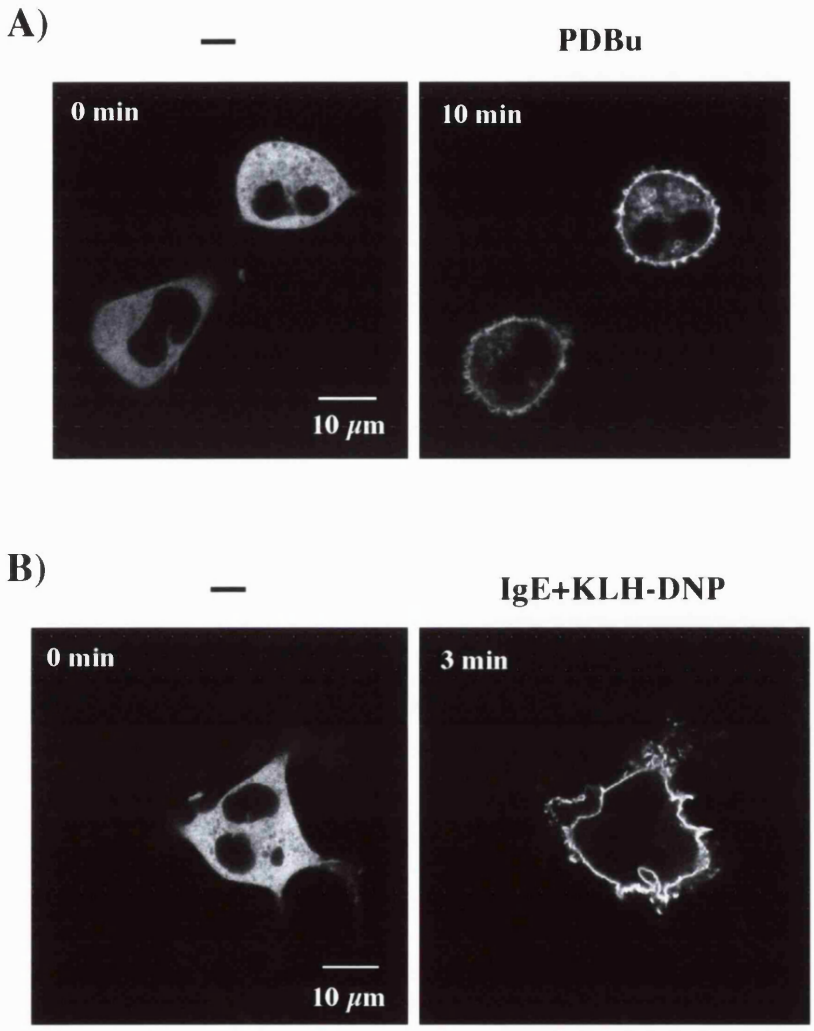


Fig. 6.9. Association of GFP-PKD with intracellular granules in phorbol ester but not FcεR1-stimulated RBL 2H3 cells.

RBL 2H3 mast cells, transiently expressing GFP-PKD, were imaged in real time before and after treatment with PDBu (**A**) or following FcεR1 ligation (**B**). PDBu induces association of GFP-PKD with both the plasma membrane and with the membranes of cytosolic granules. In contrast, FcεR1-ligation specifically induces association of GFP-PKD with the plasma membrane. The images in (A) show mid-section confocal images of RBL 2H3 mast cells before and after 10 min exposure to 50 ng/ml PDBu. The images in (B) show basal sections of RBL 2H3 mast cells before and after 3 min of FcεR1 engagement.

translocation, B cells were stimulated with either F(ab)'2 fragments of anti-mouse IgG (to stimulate the BCR alone) or with Intact anti-mouse IgG (to co-crosslink the FcγRIIb co-receptor into the BCR complex). The cells were subsequently fixed at different time periods after the addition of the stimulus and the localisation of GFP-PKD was analysed by confocal microscopy. The results presented in Figure 6.8 demonstrate that the transient plasma membrane association of GFP-PKD induced by BCR-engagement was not blocked by co-ligation of the inhibitory FcγRIIb receptor with the BCR. Significant plasma membrane association of GFP-PKD was observed in 93±6.5% of B cells after 2 min of BCR/FcγRIIb stimulation. This was followed by the redistribution of GFP-PKD back into the cytosol of the majority (>90%) of these cells within ~8-12 minutes of receptor co-stimulation (Fig. 6.8).

On close examination a subtle difference in the subcellular localisation of GFP-PKD in PDBu-treated versus antigen receptor-activated RBL-2H3 mast cells (but not A20 B cells) was apparent. PDBu stimulation caused the majority of GFP-PKD to translocate to the plasma membrane. However, redistribution of GFP-PKD to the membranes of cytoplasmic vesicle-type structures was also observed (Fig. 6.9). In contrast, the total cellular pool of GFP-PKD was translocated to the plasma membrane in FcεR1-stimulated RBL 2H3 cells, with no detectable association to cytoplasmic granules (Fig. 6.9).

6.2.5. GFP-PKD does not localise to specialised plasma membrane microdomains.

Recently it has been shown that phorbol esters can induce the relocalisation of PKC enzymes (and in particular PKCα) into detergent insoluble plasma membrane fractions in peripheral T lymphocytes (Parolini *et al.*, 1999) thought to correspond to glycosphinogolipid-enriched microdomains (GEMs, also referred to as Rafts). Phorbol esters have also been reported to induce recruitment of specific PKC enzymes into specialised membrane microdomains in other cells types, including cardiomyocytes (Rybin *et al.*, 1999) and rat fibroblasts (Kim *et al.*, 1999b).

GEMs have been detected within the plasma membrane of a wide variety of cell types (Dietrich and Jacobson, 1999; Simons and Ikonen, 1997) and have been postulated to regulate intracellular signal transduction pathways by controlling the spatial organisation of membrane-bound and cytosolic signalling molecules (Sheets *et al.*, 1999; Stulnig *et al.*, 1998; Xavier *et al.*, 1998). Importantly, a number of molecules involved in antigen receptor signalling are found concentrated within GEMs in leukocytes, including Src family kinases

and adapter molecules (Field *et al.*, 1997; Sheets *et al.*, 1999; Stauffer and Meyer, 1997; Xavier *et al.*, 1998; Zhang *et al.*, 1998b). It has also been described that antigen receptors can be targeted to GEMs during their activation (Field *et al.*, 1997; Montixi *et al.*, 1998; Stauffer and Meyer, 1997; Xavier *et al.*, 1998). The location of GEMs within plasma membranes can be visualised by staining cells with a fluorescently labelled cholera toxin B subunit, which selectively binds to the GM_1 glycolipid found associated with these membrane microdomains (Stauffer and Meyer, 1997). As indicated above, GFP-PKD transiently redistributes to the plasma membrane in antigen receptor-activated cells. GFP-PKD was generally uniformly distributed around the membrane of these cells, although occasionally a few spots of higher intensity GFP-PKD staining at the membrane was apparent. To determine whether GFP-PKD was associating with GEMs following its redistribution to the plasma membrane in response to antigen receptor ligation the localisation of GFP-PKD was compared with that of these membrane microdomains, as identified by cholera toxin B staining.

B cells, expressing GFP-PKD, were left unstimulated or were briefly stimulated with F(ab)'2 before they were fixed and stained with biotinylated cholera toxin (CT) and Streptavidin-Alexa^{568} conjugate. Confocal images of both the GFP and the CT signals were then obtained. As indicated in Figure 6.10 (upper panels) quiescent B cells showed a uniform distribution of CT staining (red) at the plasma membrane, whilst GFP-PKD (green) was present within the cytosol of these cells. When the GFP and CT signals were combined, no co-localisation of GFP-PKD with the CT was observed (as indicated by the absence of yellow pseudocolour). Following activation of the BCR complex, the distribution of CT staining at the plasma membrane was altered, with numerous punctate spots of staining apparent (Fig. 6.10, lower panels). These images also show the plasma membrane association of GFP-PKD following BCR-ligation. When these confocal images were merged remarkably little overlap (yellow) in the GFP-PKD (green) and the CT (red) staining at the plasma membrane was observed. These results indicate that GFP-PKD does not predominantly localise to lipid-enriched membrane microdomains at the plasma membrane following BCR engagement.

6.2.6. Phorbol ester-induced membrane targeting of GFP-PKD is regulated by the C1 domain.

Activation of classical and novel PKCs by phorbol esters is accompanied by their recruitment to cellular membranes. Here, DAG/phorbol ester binding to the C1 domain of

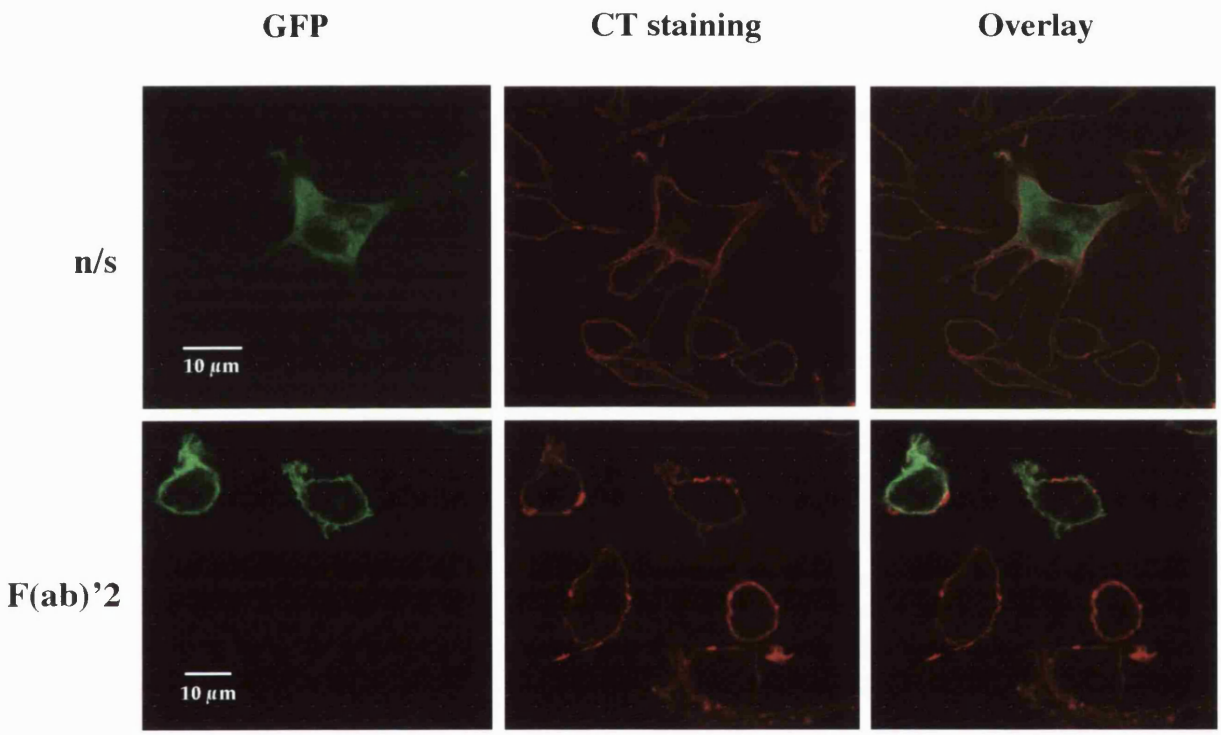


Fig. 6.10. GFP-PKD does not associate with lipid-rich membrane microdomains following BCR-ligation.

A20 B lymphocytes, transiently expressing GFP-PKD, were left untreated (n/s) or were treated with 10μg/ml F(ab)'2 fragments of anti-mouse IgG for 2 min before the cells were fixed in 4% paraformaldehyde/PBS for 20 min at RT. Lipid-rich membrane microdomains (GEMs) were visualised by staining for the microdomain-associated glycolipid GM1, using cholera toxin B subunit-biotin/streptavidin-Alexa^{488}, as detailed in Chapter 2. Cholera toxin (CT) staining is shown in red, GFP-PKD in green. Areas of co-localisation are indicated in yellow pseudocolour. Results are representative of 3 independent experiments.

these enzymes promotes their insertion into membranes through hydrophobic interactions. Mutations within the C1 domains of various classical and novel PKCs, which abolish or severely reduce the binding capacity of these enzymes, ablates these membrane interactions (Bogi *et al.*, 1999; Oancea *et al.*, 1998a; Sakai *et al.*, 1997; Szallasi *et al.*, 1996b). Similarly, mutations within the C1 domains of Munc13 and RasGRP, that prevent DAG/phorbol ester-binding, also block the inducible association of these proteins with cellular membranes (Betz *et al.*, 1998; Tognon *et al.*, 1998).

Since PKD binds DAG and phorbol esters with high affinity (Dieterich *et al.*, 1996; Van Lint *et al.*, 1995) the hypothesis that the C1 domain of PKD regulates the translocation of PKD to the plasma membrane was tested. As described in section 2.18, GFP-tagged versions of two different PKD C1 domain mutants were generated. The GFP-PKD ΔC1 mutant contains a deletion of the entire C1 domain, whilst the GFP-PKD P287G mutant contains a proline to glycine substitution within the PKD C1B motif. In both cases the integrity of the C1 domain of PKD is destroyed and phorbol ester (and thus DAG) binding is either completely abolished (ΔC1) or severely abrogated (P287G), (Iglesias *et al.*, 1998a).

In initial experiments the effect of these C1 domain mutations on phorbol ester-induced translocation of GFP-PKD was examined. Living RBL 2H3 cells, transiently expressing either wild-type GFP-PKD, GFP-PKD ΔC1 or GFP-PKD P287G, were imaged before and during stimulation with PDBu in order to visualize the redistribution of PKD in real time. As shown in Figure 6.11a, GFP-PKD ΔC1 and GFP-PKD P287G were evenly distributed throughout the cytosol of quiescent RBL 2H3 cells and excluded from the nucleus, in a manner indistinguishable from wild-type GFP-PKD. As demonstrated earlier, translocation of wild-type GFP-PKD from the cytosol to the plasma membrane (and to granular-like structures within the cytosol) of RBL 2H3 cells was evident ~6-8 minutes after the addition of PDBu, an effect that was maximal after ~15 minutes (Fig. 6.11a). In contrast, addition of PDBu did not induce any significant changes in the subcellular localization of either GFP-PKD ΔC1 or GFP-PKD P287G (Fig. 6.11a).

These results were also confirmed by quantitative analysis of the subcellular distribution of the different GFP-PKD proteins in resting and in phorbol ester-stimulated RBL 2H3 cells. These data revealed that PDBu-treatment led to plasma membrane translocation of wild-type GFP-PKD in 85±5% of the cells analysed (Fig. 6.11b). In contrast, phorbol ester treatment did not alter the basal cytosolic localisation of GFP-PKD ΔC1 in 95±1.5% of the cells analysed (Fig. 6.11b), even after 1 hour of continuous exposure to PDBu (data not shown). Similarly, phorbol ester-induced membrane targeting of PKD was severely

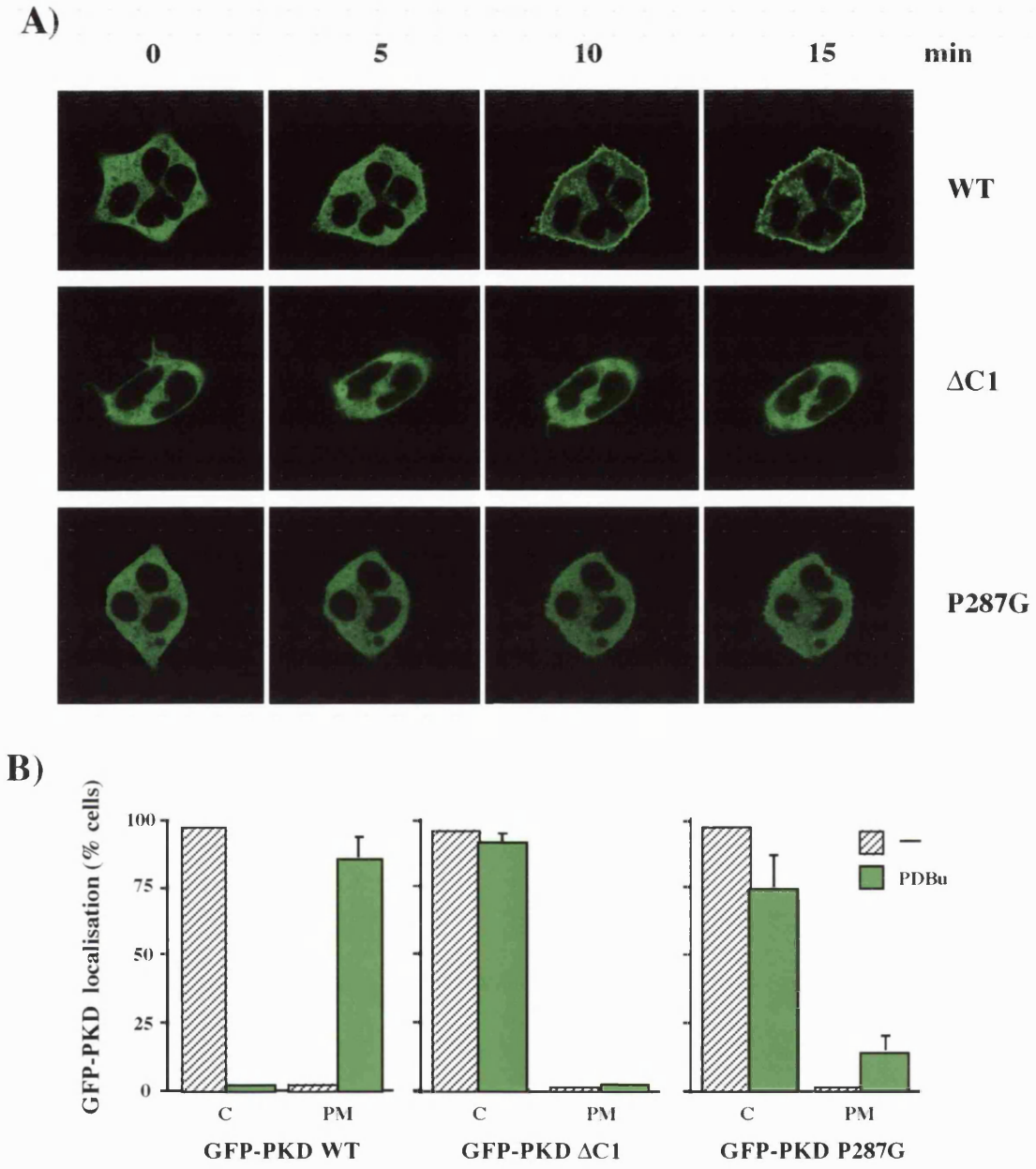


Fig. 6.11. Phorbol ester-induced GFP-PKD translocation is mediated by the C1 domain.
(A) RBL 2H3 mast cells, transiently expressing either wild-type GFP-PKD, GFP-PKD ΔC1 or GFP-PKD P287G were imaged at 37°C in real time before and after the addition of 50 ng/ml PDBu, using an inverted confocal microscope. Midsection confocal images, taken at the indicated times are shown with treatment carried out after the zero time point image had been acquired. Similar kinetics of translocation were observed in 5 independent experiments, where at least 4 individual cells were analysed per experiment.
(B) Quantation of GFP-PKD translocation induced by PDBu. RBL 2H3 cells, transiently transfected with either wild-type, ΔC1 or P287G GFP-PKD cDNA constructs, were left unstimulated (striped bars) or were stimulated with 50 ng/ml PDBu for 15 mins (green bars). The cells were then fixed, mounted on coverslips and analysed by fluorescence microscopy. The predominant localisation of GFP-PKD in these cells, cytosolic (C) or plasma membrane (PM), (expressed as a percentage of the total cells counted) is shown presented as the mean ± s.e. from 3 individual experiments where 100 cells were counted in each experiment (in a blinded fashion).

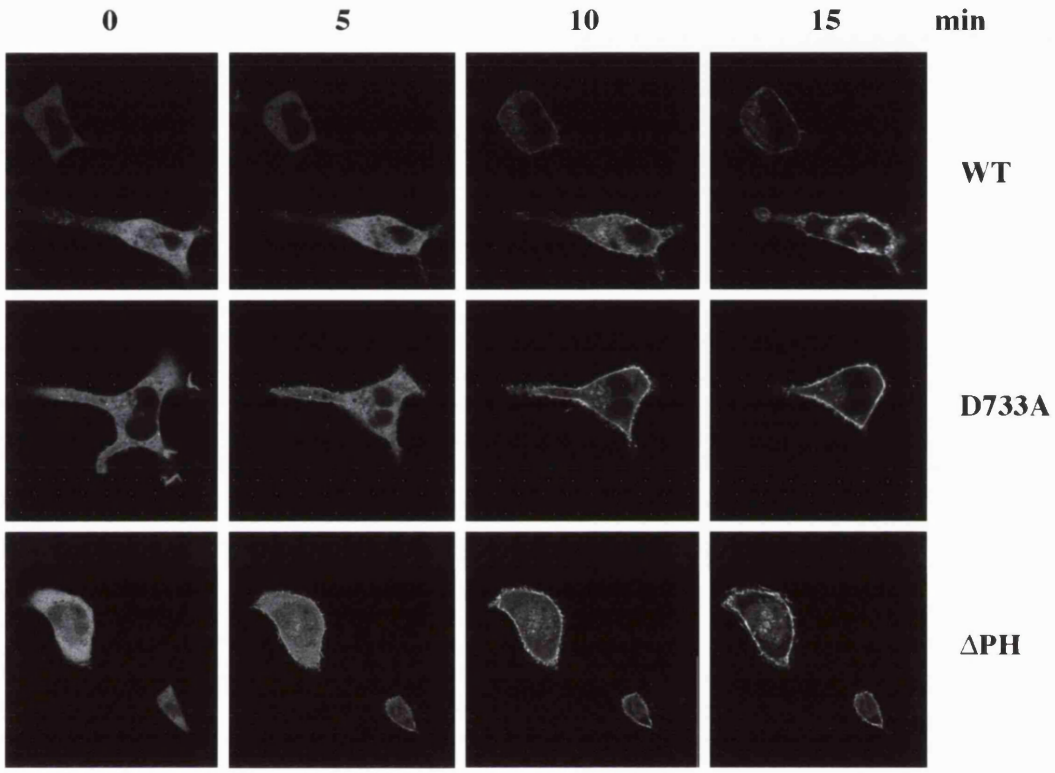


Fig. 6.11.
(C) Phorbol ester-induced membrane translocation of GFP-PKD is independent of the PKD PH domain and of PKD catalytic activity. RBL 2H3 mast cells, transiently expressing either wild-type GFP-PKD, GFP-PKD D733A or GFP-PKD ΔPH were imaged at 37°C in real time before and after the addition of 50 ng/ml PDBu, using an inverted confocal microscope. Midsection confocal images, taken at the indicated times are shown with treatment carried out after the zero time point image had been acquired. Similar kinetics of translocation were observed in 4 independent experiments, where at least 3 individual cells were analysed.

abrogated by the P287G mutation, with GFP-PKD P287G remaining within the cytosol of 75±8% of the cells analysed (Fig. 6.11b). Comparable results were also observed in transfected A20 B lymphocytes where wild-type GFP-PKD, but not GFP-tagged ΔC1 or P287G mutants of PKD, translocated to the plasma membrane of these cells in response to phorbol ester stimulation (data not shown). In contrast two other PKD mutants containing a deletion of the PH domain (GFP-PKD PH) and a kinase-dead PKD mutant (GFP-PKD D733A) were able to translocate to the plasma membrane and to the membranes of cytoplasmic granules in RBL 2H3 cells in response to PDBu, demonstrating the specificity of the PKD C1 domain for regulating phorbol ester-induced membrane targeting of PKD (Fig. 6.11c).

6.2.7. Plasma membrane targeting of GFP-PKD by the BCR is regulated by the C1 domain but not the PH domain.

Subsequently, the role of the C1 domain in regulating the transient association of GFP-PKD with the plasma membrane in antigen receptor-stimulated cells was examined. Importantly, the presence of a DAG binding C1 domain is not necessarily predictive of the targeting potential of a protein. For example, it has been reported that PKCθ, rather than other C1 domain-containing PKC isoforms, is specifically recruited to the plasma membrane at contact zone formed between T cells and antigen presenting cells during TCR activation (Monks *et al.*, 1998; Monks *et al.*, 1997). Moreover, the N terminal regulatory region of PKD also contains a PH domain (Valverde *et al.*, 1994) a signalling domain which, as discussed in section 6.1.2., controls the association of multiple proteins with cellular membranes through regulating protein/protein or protein/lipid interactions. Thus the PKD PH domain could potentially regulate the subcellular localisation of PKD following antigen receptor engagement.

To determine the relative contributions of the PH domain and the C1 domain to antigen receptor-induced PKD translocation, GFP-tagged versions of different PKD mutants were transiently expressed in B cells. A comparison of the distribution of these molecules in quiescent and in BCR-stimulated cells was then made by real time confocal imaging of living cells. Following BCR ligation, wild-type GFP-PKD redistributed from the cytosol to the plasma membrane in the same transient manner as observed previously (Fig. 6.12). In contrast, real time confocal imaging studies of cells showed that BCR ligation was repeatedly unable to induce the plasma membrane redistribution of either GFP-PKD ΔC1 or GFP-PKD P287G (Fig. 6.12). Hence BCR-induced translocation of PKD to the

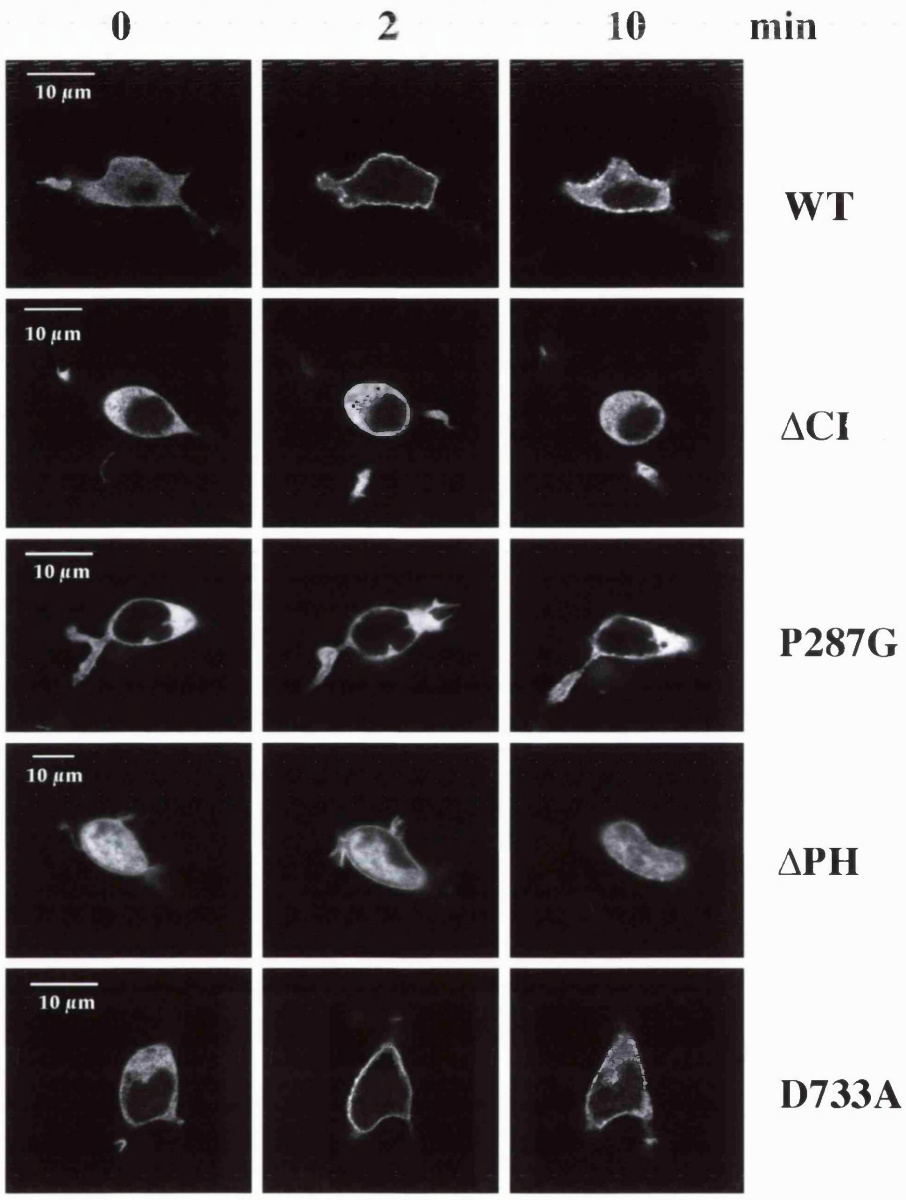


Fig. 6.12. Plasma membrane targeting of GFP-PKD upon BCR-ligation is mediated by the C1 domain.

A20 B lymphocytes were transiently transfected with different GFP-PKD mutations - a deletion of the DAG-binding C1 domain (ΔC1); a point mutation within C1 domain (P287G); a deletion of the PH domain (ΔPH) and a kinase-deficient PKD mutant (D733A). Midsection confocal images of representative living cells are shown at different time points before and after BCR ligation, as indicated. Transient plasma membrane association of the kinase-dead GFP-PKD D733A and the GFP-PKD ΔPH mutants was observed following BCR-ligation, comparable to wild-type GFP-PKD. The kinetics of subcellular redistribution of the ΔPH and the D733A GFP-PKD mutants was similar to that of wild-type GFP-PKD, with maximal membrane association observed at ~1-2 min, before returning to the cytosol within ~7-10 min of receptor engagement in >80% of the cells analysed. In contrast the cytosolic localisation of GFP-PKD ΔC1 and GFP-PKD P287G was not altered upon BCR ligation in >95% or >75% of the BCR-stimulated cells imaged, respectively. The translocation of each GFP-PKD mutant was visualised in least 6 independent experiments where 4-6 individual cells were analysed during BCR activation.

plasma membrane was dependent on the integrity of the DAG-binding C1 domain of PKD.

To ascertain whether the PH domain of PKD was involved in modulating its subcellular localisation, B cells were transiently transfected with a PKD mutant containing a deletion of the entire PH domain (GFP-PKD ΔPH). The confocal images presented in Figure 6.12 show a homogenous distribution of GFP-PKD ΔPH throughout the cytosol and the nuclei of resting B cells, unlike that of wild-type PKD and indeed of the PKD C1 mutants whose localisation was restricted to the cytosolic compartment. The distinct subcellular localisation of GFP-PKD ΔPH compared to that of wild-type GFP-PKD was also observed in RBL 2H3 cells (see Fig. 6.11c). In response to BCR stimulation, a significant accumulation of GFP-PKD ΔPH at the plasma membrane of these cells was observed with an accompanying depletion of the cytosolic pool of GFP-PKD ΔPH (Fig. 6.12). This plasma membrane targeting of GFP-PKD ΔPH was transient, similar to that observed for wild-type GFP-PKD. However, no apparent change in the level of the nuclear-localised GFP-PKD ΔPH was seen following BCR-stimulation, or indeed following PDBu-stimulation (see Fig. 6.11c). These results indicate that the PH domain of PKD is not necessary for the redistribution of PKD to or from the plasma membrane during antigen receptor triggering. However, the PKD PH domain may regulate the basal cytosolic localisation of this kinase *in vivo*, through specific protein or lipid interactions.

To determine whether PKD catalytic activity was required for membrane targeting of PKD in response to antigen receptor ligation, a GFP-tagged version of a kinase-deficient PKD mutant (D733A) was expressed in B cells. As shown in Figure 6.12, GFP-PKD D733A localised exclusively to the cytosol of resting B cells and translocated to the plasma membrane following BCR-ligation in the same transient manner as that observed for wild-type PKD. Thus, PKD activity is not required for PKD redistribution following BCR-engagement or in response to phorbol esters(Fig. 6.11c).

6.2.8. Sustained activation of PKD following antigen receptor engagement.

The data presented so far in this chapter demonstrates that PKD transiently localises to the plasma membrane of B cells and mast cells in response to antigen receptor ligation before subsequently returning to the cytosol, within 10 minutes of the initiation of antigen receptor signalling. One question that was important to address was whether dissociation of PKD from the plasma membrane was accompanied by a reduction in its kinase activity. The data presented in Chapter 5 indicated that substantial PKD activity following antigen receptor engagement in both B cells and in mast cells could be detected

at times when PKD dissociation from the plasma membrane was essentially complete (this chapter).

To investigate the long-term kinetics of PKD activation by antigen receptors, A20 B cells were stimulated with F(ab)'2 fragments of anti-mouse IgG, to stimulate the BCR, for a period of up to 2 hours. PKD activity was then measured at the indicated time points by *in vitro* kinase assays and also by western blot analysis of total cell lysates using the pS916 antibody (which selectively recognises active PKD). As demonstrated in Figure 6.13, BCR-induced activation of PKD in B cells was an extremely sustained response: maximal activity was observed within 1 minute of antigen receptor ligation and this was maintained at high levels throughout the 10 minute period that PKD was found associated with the plasma membrane in confocal microscopy experiments. Importantly, both PKD catalytic activity and S916 phosphorylation remained high for at least two hours post-receptor triggering, at times when PKD had fully dissociated from the plasma membrane (compare Fig. 6.7 and Fig. 6.13).

The confocal images presented in Figure 6.7 revealed that PKD does not appear to localise to specific intracellular structures following its dissociation from the plasma membrane of antigen receptor-stimulated cells. To explore the subcellular localisation of active PKD further, cytosolic and total membrane fractions were prepared from BCR-stimulated A20 B cells. Western blot analysis of these fractions, using the pS916 antibody to identify activated PKD, revealed that active PKD could be found localised to the particulate (membrane) fraction of these cells within 2 minutes of BCR-stimulation but within ~15 minutes PKD activity within this compartment had diminished (Fig. 6.14a). In contrast, active PKD was detectable within the soluble (cytosolic) compartment of B cells up to 30 min after BCR engagement (Fig. 6.14a), indicating that active PKD does not strongly associate with intracellular membranes following its dissociation from the plasma membrane. Importantly, only PKD isolated from membrane fractions of PDBu-treated B cells showed immunoreactivity with the pS916 antiserum. Moreover, prolonged stimulation of PKD with phorbol esters (>1 h) resulted a sustained association of active PKD with cellular membranes (Fig. 6.14b). This was in agreement with the confocal data presented in this chapter demonstrating that phorbol esters induce the sustained plasma membrane localisation of GFP-PKD in intact cells. Accordingly, these confocal images and biochemical data indicate that there are two phases of PKD regulation following antigen receptor stimulation in B cells and in mast cells: an initial activation/recruitment to the plasma membrane followed by a sustained period of PKD activity within the cytosol.

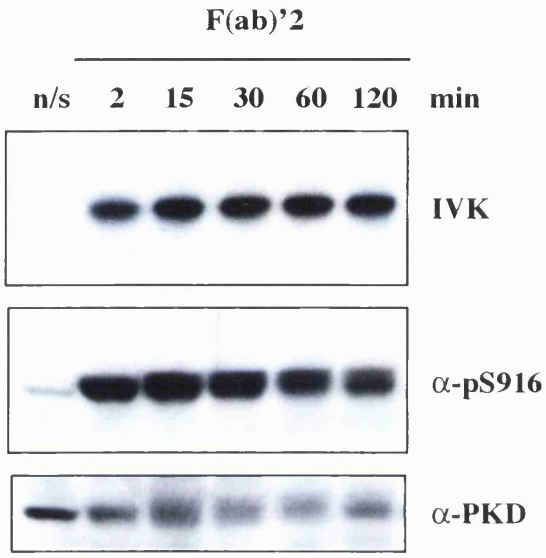


Fig. 6.13. Sustained PKD activity following BCR ligation - I.
A20 B lymphocytes were plated in complete medium at a density of $1.5x10^7$ cells/ml in a 24 well plate and left to recover at 37°C for 1 h before stimulation. Subsequently the cells were left untreated (n/s) or were stimulated with 10 μg/ml F(ab)'2 fragments of anti-mouse IgG for various times (2-120 min). The cells were lysed and PKD immunoprecipitated using the PA-1 antiserum. *In vitro* kinase assays (IVK) were the performed using PKD autophosphorylation as the measure of catalytic activity. Alternatively, proteins in the lysates were precipitated with cold acetone, separated by SDS-PAGE and subjected to western blot analysis using the pS916 antiserum and a pan C-terminal PKD antibody (sc-935). Results are representative of 3 independent experiments.

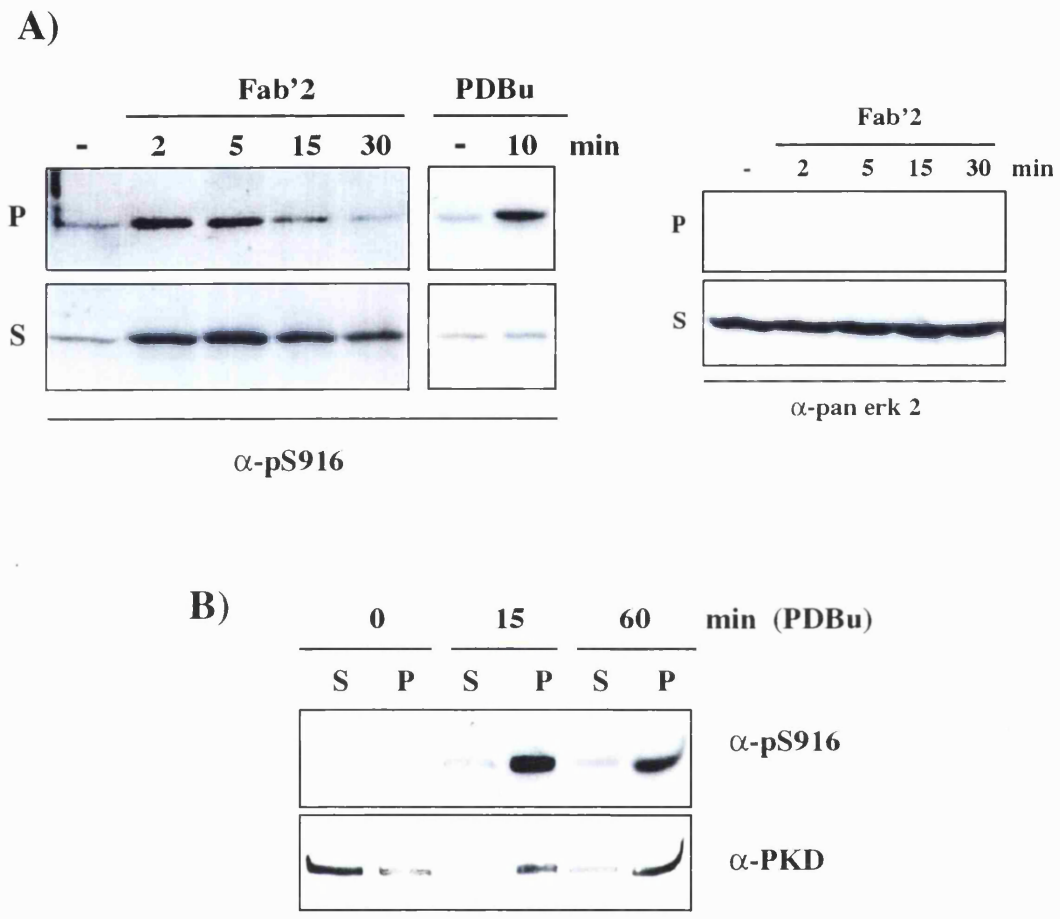


Fig. 6.14. Sustained PKD activity following BCR ligation - II.

(A) A20 B lymphocytes were left untreated (-), treated with 50 ng/ml PDBu for 10 min or were stimulated with 10 μg/ml F(ab)'2 fragments of anti-mouse IgG for various times (2-30 min). Particulate (P; membrane) and soluble (S; cytosolic) fractions were prepared from the cells, as described in Chapter 2. Total proteins in the resulting fractions were precipitated with cold acetone, separated by SDS-PAGE and subjected to western blot analysis using the pS916 antiserum to detect active PKD (left panel) before reprobing with a pan erk 2 antibody (right panel). Results are representative of 2 independent experiments.

(B) PKD activity is associated with cellular membranes in a sustained manner in phorbol ester-stimulated cells. COS-7 cells expressing wild-type PKD were left unstimulated (0) or were treated with 50 ng/ml PDBu for 15 or 60 min. Cells were fractionated as described in Chapter 2 and the fractions analysed by SDS-PAGE and western blotting with the pS916 antiserum to detect active PKD followed by a pan C-terminal PKD antibody (sc-935). Data are representative of 3 independent experiments.

6.3. DISCUSSION.

The data presented in this chapter uses a combination of biochemical and confocal microscopy techniques to reveal spatial and temporal aspects of PKD regulation. There has been no previous analysis of the localisation of PKD during a physiological response in lymphocytes and in particular no analysis of the subcellular distribution of active PKD *in vivo*. The data presented in this chapter has explored PKD activity and subcellular localisation in response to both pharmacological (phorbol esters) and physiological (antigen receptors) stimuli.

6.3.1. Subcellular localisation of PKD in B cells and mast cells.

Previously it has been reported that PKD is localised to the Golgi compartment in a human hepatocellular carcinoma cell line and in human gastral cells (Moore *et al.*, 1999; Prestle *et al.*, 1996). However the reported association of active PKD with the BCR complex in antigen receptor-stimulated B cells indicates that this may not be the case for all cell types (Sidorenko *et al.*, 1996). Indeed, as demonstrated in this chapter PKD shows a diffuse cytosolic pattern of expression in quiescent mast cells and B cells. It should be noted that Prestle and co-workers observed only a partial co-localisation of endogenous PKD with the Golgi-specific enzyme β1,4-galactosyltransferase (Prestle *et al.*, 1996). Moreover, no subcellular redistribution of PKD upon phorbol ester treatment was observed in this study, despite the presence of a functional phorbol ester-binding C1 domain in PKD and in contrast to the data described here. A second study has also shown a Golgi-specific localisation for PKD which was uneffected by phorbol esters (Moore *et al.*, 1999). The discrepancy between these two reports and the data presented in this chapter is puzzling. It is possible that PKD is localised to distinct subcellular sites in different cell types, thus implying that PKD has different functions in different cells. Alternatively, the immunofluorescence data shown in these two reports may not specific for PKD. For example, the N-terminal monoclonal antibody from Transduction Laboratories used in the study by Moore *et al* is now known to crossreact with several unknown cellular proteins, one of which could potentially be the recently described PKCυ serine kinase which displays a high degree of homology to PKD within the N-terminal region (Hayashi *et al.*, 1999).

The data presented in this chapter has used two distinct approaches to identify PKD as a cytosolic kinase in B cells and mast cells: firstly, by immunofluorescence staining of endogenous PKD (using a specific C-terminal PKD antibody directed against an epitope

not present in the closely related PKCυ kinase) and secondly, by visualisation of ectopically expressed GFP-tagged PKD molecules. Thus, PKD is not selectively localised to the Golgi complex in B cells or mast cells indicating that it may be difficult to extrapolate data regarding PKD localisation (and thus potential downstream functions for PKD) between different cells types.

Strikingly, in addition to inducing PKD catalytic activity the data presented in this chapter demonstrates that antigen receptors and phorbol esters also regulate the membrane targeting of PKD. Thus, PDBu-treatment induced a dramatic, sustained redistribution of GFP-PKD away from the cytosol to the plasma membrane of B cells and mast cells, and also to vesicle-type structures within the cytosol of mast cells. In contrast, BCR or FcεR1-mediated regulation of PKD was a dynamic process with an initial rapid but transient recruitment of PKD to the plasma membrane of B cells and mast cells. Redistribution of PKD back to the cytosol of these cells occurred within ~8-10 minutes after the initiation of antigen receptor signalling. Significantly the total cellular pool of PKD moves to the plasma membrane in response to antigen receptor ligation, indicating the strength of this translocation signal.

Recently, plasma membrane glycosphinogolipid-enriched microdomains (GEMs, also referred to as lipid rafts) have been proposed to function as platforms for signal transduction through controlling the spatial organisation of key signalling molecules. Antigen receptors are targeted to these lipid microdomains during an activation response and it is suggested that this is where interactions with tyrosine kinases and adapter molecules occur (Field *et al.*, 1997; Stauffer and Meyer, 1997; Xavier *et al.*, 1998). GEMs have also been implicated as the site of inositol lipid metabolism since both PLCγ and PIP_2 have been found concentrated within GEMs (Hope and Pike, 1996; Stauffer and Meyer, 1997; Xavier *et al.*, 1998; Zhang *et al.*, 1998b). This hypothesis is supported by observations showing that PIP_2 is preferentially hydrolysed within specific lipid-enriched membrane microdomains (Pike and Casey, 1996). Whether DAG subsequently remains in GEMs or more uniformly diffuses throughout the plasma membrane is not known. Importantly, membrane associated PKD did not co-localise with lipid-rich membrane microdomains (GEMs), as visualised by staining with fluorescently labelled cholera toxin B, a marker for the GEM associated GM_1 glycosphingolipid. Given that PKD membrane association is mediated through its DAG-binding C1 motif, the observed uniform distribution of PKD at the plasma membrane of antigen receptor- or PDBu-stimulated cells would be consistent with a general dispersion of DAG throughout the plasma membrane of activated

B cells and mast cells. Thus, antigen receptor signalling may initiate within GEMs but the present data, showing active PKD uniformly distributed at the plasma membrane within ~1 minute of antigen receptor ligation, demonstrates how rapidly biochemical signals can propagate away from specialised membrane microdomains.

6.3.2. The molecular basis for PKD translocation.

To identify the regulatory domain within PKD that was mediating its transient association to the plasma membrane of antigen receptor activated cells, GFP-tagged versions of different PKD mutants were generated and their response to BCR ligation was assessed. Phorbol ester-induced PKD translocation was completely prevented by deletion of the PKD C1 domain and severely impaired by a point mutation within the second cysteine-rich motif of the C1 domain that severely abrogates PDBu-binding affinity. Furthermore, C1 domain mutations also completely abolished the transient plasma membrane translocation of PKD induced by stimulation of antigen receptors. These data clearly establish a role for the DAG-binding C1 domain in regulating the rapid redistribution of PKD from the cytosol to the plasma membrane in antigen receptor-activated B cells and mast cells.

The 5'inositol phosphatase SHIP dephosphorylates $PI(3,4,5)P_3$ to $PI(3,4)P_2$ (Damen *et al.*, 1996; Lioubin *et al.*, 1996; Scharenberg *et al.*, 1998a). Consistent with this, co-ligation of the BCR together with the inhibitory FcγRIIb receptor decreases plasma membrane $PI(3,4,5)P_3$ levels through the recruitment of SHIP (Scharenberg *et al.*, 1998a) and consequently inhibits the PH domain-mediated membrane recruitment and activation of Btk and PKB/Akt (Astoul *et al.*, 1999; Bolland *et al.*, 1998; Scharenberg *et al.*, 1998a). In striking contrast, co-ligation of the inhibitory FcγRIIb receptor with the BCR did not prevent PKD translocation, indicating that antigen receptor-induced subcellular redistribution of PKD is not dependent on the binding of $PI(3,4,5)P_3$ to the PKD PH domain. This correlates with preliminary observations showing that an isolated PKD PH domain does not bind $PI(3,4,5)P_3$ or $PI(4,5)P_2$-containing vesicles with high affinity *in vitro* (Dr. T. Iglesias, ICRF, *Personal communication*). Moreover, a GFP-tagged PKD mutant lacking the entire PH domain retained the capacity to transiently redistribute to the plasma membrane of B cells following BCR-activation, indicating that the PH domain of PKD is not essential for PKD membrane targeting.

Wild-type GFP-PKD was consistently observed to be excluded from the nucleus of B cells and mutation of the PKD C1 domain did not alter this pattern of nuclear exclusion. In

contrast the GFP-PKD ΔPH mutant was evenly distributed throughout the cytosolic and nuclear compartments of quiescent B cells (see Fig. 6.11c/6.12). The functional significance of this altered distribution remains unclear but it is apparent that although not required for antigen receptor-induced membrane targeting, the PKD PH domain is important for regulating the basal cytosolic distribution of this kinase, either through mediating protein and/or lipid interactions.

The molecular mechanism by which PKD translocates to the plasma membrane of antigen receptor-stimulated cells is not known. Given that PKD translocation is mediated through its DAG-binding C1 domain, similar to that of classical and novels PKCs, it may be possible to draw parallels between these kinases. For example, although interactions between PKCs and cytoskeletal components have been reported in phorbol ester-stimulated cells (Goodnight *et al.*, 1995; Jaken *et al.*, 1989; Kiley and Jaken, 1990) inhibitors of actin or microtubule polymerisation (cytochalasin D and colchine, respectively) do not effect PKC translocation (Almholt *et al.*, 1999; Oancea *et al.*, 1998a; Sakai *et al.*, 1997). Thus PKC translocation is not regulated through cytoskeletal reorganisation. Whether PKCs (and potentially also PKD) are targeted to cellular membranes through simple diffusion or whether an energy-dependent mechanism is involved is not known at present.

It is clear however that the intrinsic catalytic activity of PKCs is not required for membrane translocation, as shown by the use of PKC inhibitors or by kinase-deficient PKC mutants (Feng *et al.*, 1998a; Ohamori *et al.*, 1998; Sakai *et al.*, 1997). Similarly, PKD membrane translocation is independent of PKD catalytic activity, as demonstrated by the use of a kinase-dead PKD mutant (GFP-PKD D733A). Feng and Hannun have however demonstrated a requirement for kinase activity in the dissociation of $PKC\beta_{II}$ from the plasma membrane of HEK 293 fibroblasts following the activation of Gq receptors (Feng and Hannun, 1998b). Whether this is true for all classical/novel PKD isoforms is not known. Thus, although a kinase dead PKD mutant was able to dissociate from the plasma membrane of BCR-stimulated A20 B cells in a manner comparable to that of wild-type PKD a role for a distinct kinase in the regulation of this dissociation step cannot be excluded at present.

The transient nature of the plasma membrane association of PKD in antigen receptor-activated cells may be explained by the rapid turnover of PLC-derived DAG within the plasma membrane (Nishizuka, 1992). In this context, lymphocytes typically express high levels of DAG kinases which metabolise DAG to phosphatidic acid and

rapidly terminate DAG signalling pathways. The actions of these DAG kinases in clearing DAG would ensure that the C1 domain-mediated association of PKD with the plasma membrane of these cells would be transient. This hypothesis is supported by the transient association of classical or novel PKC enzymes with the plasma membrane of cells stimulated via G protein-coupled receptors (Feng *et al.*, 1998a; Oancea *et al.*, 1998a; Ohamori *et al.*, 1998; Sakai *et al.*, 1997). Moreover, an isolated DAG-binding C1 domain (from PKCγ) only transiently associates with the plasma membrane of FcεR1-stimulated mast cells (Oancea *et al.*, 1998a). In contrast, the sustained plasma membrane association of GFP-PKD induced by phorbol ester treatment would reflect the long half-life of phorbol esters, which are only slowly degraded *in vivo*. It is apparent that antigen receptor-ligation does not induce the association of GFP-PKD with intracellular membranes following its dissociation from the plasma membrane, unlike phorbol esters which target GFP-PKD to the membranes of cytoplasmic granules in RBL 2H3 mast cells. This would indicate that monounsaturated/saturated DAGs, generated by metabolism of PLD-derived phosphatidic acid, during sustained responses to antigen receptor engagement do not regulate PKD localisation *in vivo*.

6.3.3. Spatial and temporal dynamics of the regulation of PKD by the BCR.

Plasma membrane localisation of PKD in BCR-stimulated A20 B cells is transient, with the enzyme returning to the cytosol within 10 minutes following antigen receptor triggering. Strikingly however, this was not accompanied by a downregulation of PKD catalytic activity. Rather, a sustained period of activation of this enzyme within the cytosol was observed, with high levels of PKD activity maintained for over two hours following antigen receptor ligation in B cells. Biochemical fractionation studies indicated that active PKD at these later times points is not tightly associated with intracellular membranes but is present within the cytosol of these cells. Unfortunately, the pS916 anti-serum is not of sufficient purity at present to use in immunofluorescence studies. However, it will be very interesting in the future to use this antibody to visualise the location of active PKD in intact, antigen receptor-activated cells.

Data showing that the total cellular pool of GFP-PKD translocates to the plasma membrane (e.g. Fig. 6.9) in response to antigen receptor engagement support a model whereby active PKD recycles from the plasma membrane back to the cytosol where it is maintained in a catalytically active state. The sustained activation of PKD away from the plasma membrane reflects the observation that PKD activity is dependent on its

phosphorylation status and not on DAG-binding (Chapter 3, (Iglesias *et al.*, 1998b; Zugaza *et al.*, 1996)). Phorbol esters also induce a prolonged activation of PKD in intact cells (Fig. 6.14b; (Rennecke *et al.*, 1996)). However, the localisation data presented here reveals that active PKD is localised to different intracellular sites in cells activated by pharmacological or physiological stimuli. This has an important bearing for future studies where PKD function is probed using phorbol esters or indeed PKD mutants, since constitutively active, delocalised PKD mutants would not reflect the true physiological situation.

To date there have been few reports which attempt to correlate the activity of DAG-regulated kinases with spatial localisation under conditions of physiological stimulation. Frequently, the dissociation of classical/novel PKC enzymes from cellular membranes is associated with their catalytic inactivation (for example, (Feng and Hannun, 1998b; Oancea and Meyer, 1998b)). Recently, Ng *et al* have studied the subcellular localisation of PKCα in phorbol ester-treated COS7 cells and showed that active PKCα is initially localised to the plasma membrane of these cells but is rapidly internalised, while still bound to the membrane, in a manner indicative of PKC downregulation (Ng *et al.*, 1999a). In contrast, the data presented in this chapter indicates that the spatial distribution of active PKD is dynamically regulated during responses to antigen receptor engagement, thus active PKD is initially located at the plasma membrane of these cells but subsequently dissociates back into the cytosol where is remains in an active, non-membrane bound state for prolonged time periods, in agreement with data described earlier in this thesis showing that PKD is not subject to downregulation (Chapter 3). These results reveal a novel mechanism by which DAG signals may be disseminated away from the plasma membrane into the cell interior during sustained responses to antigen receptor ligation.

CHAPTER 7 : General Discussion

The PKC family of serine/threonine kinases, important intracellular signalling molecules, have been implicated in both positive and negative aspects of the regulation of cell survival, proliferation and differentiation. Much of the work concerning the biological functions of classical and novel PKC enzymes has been derived from studies using generic pharmacological agents, including phorbol esters, membrane-permeant DAG-analogues and calcium ionophores. The complexity of biological responses to these agonists, and also in response to receptor-mediated elevation of membrane DAG in part reflects the heterogeneity of the PKC family. However, the growing realisation that other cellular proteins are also regulated by DAG and phorbol esters, through the presence of conserved C1 domains, has added a further level of complexity to this field.

At the start of this thesis, PKD was recognised as a novel serine kinase that could be activated *in vitro* by phorbol esters in combination with phosphatidyl-L-serine. Thus PKD was predicted to be a novel target for DAG signals in intact cells that would potentially operate in parallel to classical and novel PKC enzymes. The experiments described in this thesis were undertaken in order to examine spatial and temporal aspects of the regulation of PKD under conditions of pharmacological and importantly, physiological stimuli. The primary observations made in this thesis, within the context of other studies, are summarised below.

7.1. REGULATION OF PKD ACTIVITY AND LOCALISATION IN INTACT CELLS.

The N-terminal regulatory region of PKD contains a C1 domain and PKD can be activated *in vitro* by phosphatidyl-L-serine in combination with different C1 domain ligands, including phorbol esters, membrane permeant DAG-analogues and bryostatin 1 (Chapter 3 and (Dieterich *et al.*, 1996; Valverde *et al.*, 1994; Van Lint *et al.*, 1995)). Moreover PLC-regulated signalling pathways are both necessary and sufficient for activation of PKD in intact cells, similar to the regulation of classical and novel PKC enzymes. Thus, BCR-induced PKD activity is abolished in Syk or PLCγ_2-deficient DT40 B cells (Sidorenko *et al.*, 1996) and a PDGF receptor in which the phosphotyrosine docking site for PLCγ is mutated cannot activate PKD (Van Lint *et al.*, 1998). Moreover, a PDGF receptor mutant which couples only to PLCγ (and not to Ras or PI3-Kinase) is able to activate PKD as efficiently as the wild-type receptor (Van Lint *et al.*, 1998).

As addressed in Chapter 3 and elsewhere phosphorylation events play a critical role in the regulation of PKD activity. Activators of PKD markedly enhanced the incorporation of ^{32}Pi into PKD in metabolically labelled cells and phosphatase treatment of active PKD inhibits its catalytic activity, an effect that is reversed by inhibitors of protein phosphatases. Identification and mutation of two critical trans-phosphorylation sites within the activation loop of PKD (S744 and S748) has demonstrated the key role played these sites in the regulation of PKD catalytic activity (Iglesias *et al.*, 1998b). Studies using inhibitors of different intracellular protein kinases revealed that inhibitors of classical/novel PKC enzymes (that do not directly inhibit PKD activity, Chapter 3 and (Gschwendt *et al.*, 1996; Johannes *et al.*, 1995)) were able to specifically block the activation of PKD in intact cells induced by pharmacological agonists (Chapter 3) or by antigen receptors (Chapter 5). This is in agreement with similar data described for other receptor systems (Van Lint *et al.*, 1998; Zugaza *et al.*, 1997). A role for PKC enzymes as proximal regulatory kinases for PKD in antigen receptor signalling cascades was confirmed by the ectopic expression of different constitutively active PKC mutants in B cells or mast cells and subsequent analysis of PKD activity (Chapter 5).

Consistent with the hypothesis that PKD is indirectly regulated by DAG in intact cells mutation of the DAG/phorbol ester binding C1 domain of PKD (P287G, such that phorbol ester binding to PKD is severely abrogated, (Iglesias *et al.*, 1998a)) does not alter the kinetics or magnitude of PKD activation compared to wild-type PKD. Indeed, this PKD P287G mutant is activated through the same PKC-dependent signalling pathway as wild-type PKD (Chapter 5). Taken together these data suggest a model in which PKD lies downstream of, and not parallel to, classical/novel PKC enzyme(s) in DAG-regulated signal transduction pathways in a variety of cellular systems.

Several important areas remain to be explored in the future, not least of which is to determine whether there is a direct connection between PKC enzymes and PKD *in vivo,* or whether PKCs regulate an intermediary kinase that phosphorylates and activates PKD. Certainly the basic and hydrophobic amino-acid residues surrounding the PKD activation loop phosphorylation sites (serines 744 and 748) show homology to the described optimal substrate phosphorylation motifs of multiple PKC enzymes, and in particular that of PKC η and PKCε, as shown in Table 7.1.

	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5
S744	R	I	I	G	E	K	S	F	R	R	S	V
S748	E	K	S	F	R	R	S	V	V	G	T	P
PKCα	R	R	R	R	K	G	S	F	R	R	K	A
PKCβ$_I$	K	L	K	R	K	G	S	F	K	K	F	A
PKCγ	R	R	R	R	K	G	S	F	K	R	K	A
PKCε	P	R	K	R	Q	G	S	V	R	R	R	V
PKCη	R	R	R	R	R	R	S	F	R	R	x	R
PKCζ	R	F	K	R	Q	G	S	F	F	Y	F	F

Table 7.1. Comparison of optimal PKC phosphorylation motifs with PKD activation loop sites. The optimal peptide substrate sequences for the PKC enzymes, as determined by peptide library screening, are taken from (Nishikawa *et al.*, 1997). All PKC enzymes prefer substrates with hydrophobic residues at positions +1 and basic residues at positions -6, -4, and -2. Most PKC enzymes also prefer basic residues at positions +2, +3 and +4, although PKCζ shows specificity for hydrophobic residues at these sites. Sequences surrounding the activation loop phosphorylation sites S744 and S748 are shown, with the serine site at position 0. Basic and hydrophobic residues surrounding these activation loop sites that are identical (black) and conserved (grey) with PKC phosphorylation site motifs are highlighted.

Interestingly, complexes have been described between PKD and the novel PKC enzymes η and ε (Waldron *et al.*, 1999b), two isoforms which are strongly implicated in the regulation of PKD activity (this thesis and (Zugaza *et al.*, 1996)). Together, these results suggest that a direct link between PKCs and PKD may exist *in vivo*. Direct phosphorylation and/or activation of PKD by PKC enzymes has not been demonstrated to date, however. Experiments where purified, constitutively active PKCε was added to PKD immunoprecipitates *in vitro* failed to induce PKD activity (J.L. Zugaza and E. Rozengurt, unpublished observations). It is possible that these *in vitro* conditions did not reproduce the intracellular microenvironment required for a productive PKD/PKCε interaction. For example, active PKC enzymes are targeted to specific intracellular sites and to specific substrates by anchoring/interacting proteins, as discussed in Chapter 1. *In vitro* experiments would not take account of a potential role for such complexes in the regulation of PKD activity by PKC. One approach that could be used to detect potential PKC/PKD interactions in intact cells could be FRET based techniques. Here, close molecular interactions between two proteins, labelled with different fluorescent chromophores, can be detected by the transfer of energy from the donor to the acceptor chromophore and consequently, a change in the emission wavelength of these fluorescent molecules.

Another question that remains to be answered is which PKC enzymes can and/or do regulate PKD activity *in vivo*? Activated mutants of the classical PKC enzyme β_{II}, as well as the novel PKCs η and ε, have been shown to induce maximal PKD activity when ectopically expressed in either COS-7 cells (Zugaza *et al.*, 1996) or in hematopoietic cells (Chapter 5). Whether these enzymes can actually regulate PKD under physiological situations remains unclear. Calcium is not required for the regulation of PKD activity by the BCR or by the G-protein-linked bombesin receptor (Sidorenko *et al.*, 1996; Zugaza *et al.*, 1997), consistent with a specific role for a novel PKC enzyme(s) in mediating PKD activation by these receptors. Alternatively multiple, redundant PKCs may be capable of activating PKD within a given cell in response to a stimulus. Future experiments should be directed towards elucidating exactly which PKCs regulate PKD activity *in vivo*. Studies regarding the calcium-requirements for PKD activity could be extended to determine whether different receptors use different PKC-subfamilies to regulate PKD or not. This could be achieved through simple chelation of intracellular calcium by the cell-permeant calcium chelator BAPTA-AM or by pretreating cells with thapsigargin to inhibit the endoplasmic reticulum calcium ATPase, thus depleting intracellular calcium stores. A more elegant approach may be to use IP_3-receptor deficient B cell lines to study the role of calcium in PKD activation by various receptors. A collaboration with the Kurosaki group is in progress to study this. Cells from the available PKC knock-out mice could also be used to study the role of specific PKC enzymes in the control of PKD activity by different receptors. This would be particularly interesting for the PKCβ-deficient mice, which exhibit B cell defects, given that a constitutively active PKCβ_{II} mutant increases the basal activity of PKD *in vivo* and that BCR signals potently regulate PKD activity and subcellular localisation.

The regulation of PKD by DAG and phorbol esters through an indirect mechanism *in vivo* is in marked contrast to data describing the direct activation of purified PKD by these agonists *in vitro* (Chapter 3 and (Dieterich *et al.*, 1996; Valverde *et al.*, 1994; Van Lint *et al.*, 1995)). The discrepancy between the *in vitro* and *in vivo* mechanisms of activation of PKD is unclear. Mutation of residues serine 744 and serine 748 (critical activating phosphorylation sites within the activation loop of PKD) prevents PKC-dependent activation of PKD *in vivo* but significantly does not block direct allosteric activation of immuno-purified PKD *in vitro* (Iglesias *et al.*, 1998b). This indicates that PKD activation *in vitro* occurs through a mechanism which is distinct from the described PLC-DAG-PKC cascade which regulates PKD activity *in vivo*. It is possible that this *in vitro* mechanism

may involve the simple disruption of intramolecular interactions within PKD following non-specific ligand binding. Indeed, such a mechanism might explain the potent activation of PKD *in vitro* by polyanionic compounds such as dextran sulphate or heparin (which would not bind to the PKD C1 domain) and also the inhibition of PKD activity *in vitro* by basic peptides (Gschwendt *et al.*, 1997) but not by calphostin C, a competitive C1 domain antagonist (Johannes *et al.*, 1995).

Catalytic activity is only one mechanism by which antigen receptors regulate PKD, the data presented in Chapter 6 describes spatial aspects of the regulation of PKD localisation by antigen receptors. Here engagement of FcεR1 receptors in the mast cell line RBL 2H3 or of the BCR in A20 B lymphoma cells induces a striking but transient redistribution of PKD from the cytosol to the plasma membrane that is regulated by the PKD C1 domain. Significantly, dissociation of PKD away from the plasma membrane of antigen receptor-activated cells is not accompanied by catalytic inactivation, instead recycled PKD remained active within the cytosol for several hours. This data establishes PKD as a PKC-regulated serine kinase that temporally and spatially amplifies antigen receptor-induced DAG signals *in vivo*.

Potentially, the PKD C1 domain could function to target inactive PKD to the same plasma membrane site as classical/novel PKC enzymes, thus increasing the efficiency of PKD activation by PKC. However, since direct binding of DAG/phorbol ester to the C1 domain and subsequent membrane association of PKD can be dissociated from the induction of PKD catalytic activity, as demonstrated by use of the PKD P287G mutant (Chapters 5 and 6) it is unlikely that this is the major function of the PKD C1 domain. The observation that active PKD is localised to two distinct subcellular sites following antigen receptor ligation in B cells and mast cells would rather suggest that PKD phosphorylates two distinct sets of downstream targets - those localised at the plasma membrane and those localised within the cell interior - and that the C1 domain is required to target PKD to the plasma membrane in order to phosphorylate this first set of downstream targets. Subsequent release of the PKD C1 domain and dissociation of active PKD away from the plasma membrane upon DAG turnover would then allow PKD to target intracellular substrates.

Thus, as summarised in Figure 7.1, PKD is regulated through a dual mechanism *in vivo*: (1) DAG indirectly regulates PKD phosphorylation and activity through a PKC-dependent signalling pathway and (2) DAG directly controls the dynamics of PKD subcellular localisation.

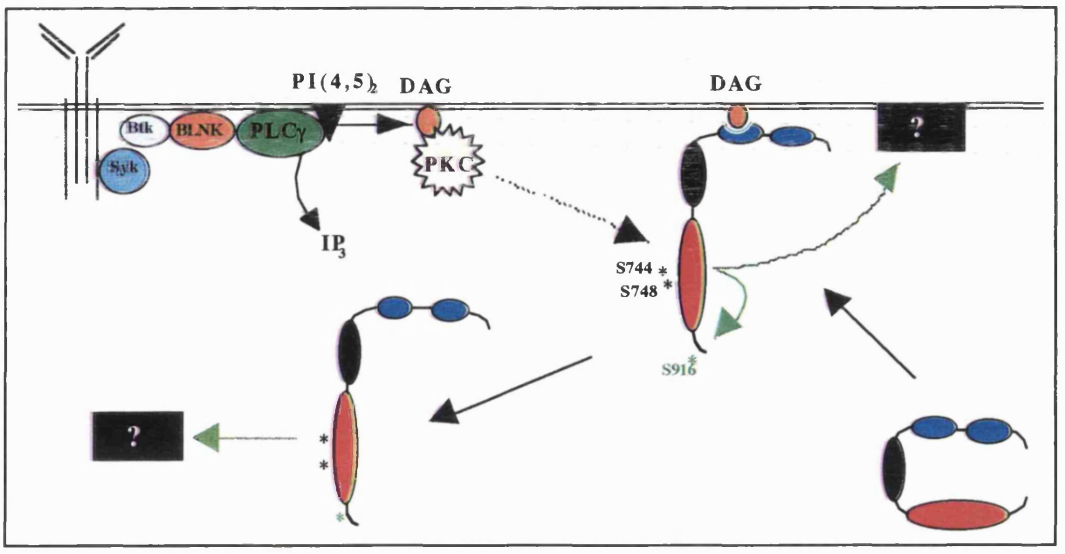


Figure 7.1. BCR regulation of PKD through two distinct mechanisms.

BCR crosslinking increases calcium and DAG levels, leading to plasma membrane recruitment and activation of classical/novel PKCs. Active PKC then induces the phosphorylation and activation of PKD. Once active, PKD autophosphorylates on a number of serine residues, including S916. At the same time, PKD is itself recruited to the plasma membrane, in a DAG-dependent manner, where it potentially phosphorylates membrane-localised targets. Active PKD is subsequently released from cellular membranes into the cytoplasm where it may target a distinct set of substrates. See main text for further details.

7.2. THE PKD PH DOMAIN.

The data presented in this thesis indicates that the PKD PH domain is not essential for antigen receptor-mediated PKD translocation (Chapter 6). Moreover, it is clear that the lipid products of PI3-kinase do not regulate the catalytic activity of PKD, via its PH domain, following stimulation of tyrosine kinase or G protein-coupled receptors (Van Lint *et al.*, 1998; Zugaza *et al.*, 1997), although an indirect role for PI3-Kinase in the regulation of PKD via a Btk-PLCγ-PKC cascade by the BCR has been proposed (Chapter 5). Furthermore, preliminary data suggests that the PKD PH domain does not bind a variety of phosphoinositides [including $PI(3,4)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$] *in vitro*, nor is PKD activity induced by these lipids *in vitro* (Dr. T. Iglesias, ICRF, *Personal communication*). The exact function of the PKD PH domain remains unclear at present although, as discussed below, potential roles for the PKD PH domain as an autoinhibitory domain (Iglesias and Rozengurt, 1999), as a binding site for upstream activators (Jamora *et al.*, 1999; Waldron *et al.*, 1999b) and as a determinant of the basal subcellular localisation of PKD (this thesis) have been proposed.

Many protein kinases are maintained in inactive state by an autoinhibitory domain that interacts with the kinase domain to prevent substrate binding. For example, all PKC enzymes possess an autoinhibitory pseudosubstrate motif that is located just upstream of the C1 domain (Toda *et al.*, 1993). In contrast, PKD does not contain a comparable pseudosubstrate motif. Deletion of or point mutations within the PKD PH domain have been shown to markedly enhance the basal catalytic activity of PKD *in vivo* (Iglesias *et al.*, 1998b). This contrasts with other protein kinases (e.g. PKB/Akt and Btk) where PH domain mutations are linked to the abolition of kinase activity. It is therefore possible that the PH domain functions as an autoinhibitory domain which is released upon PKD activation. A more recent study has demonstrated that mutations within the PKD C1 domain also result in increased basal PKD activity (Iglesias and Rozengurt, 1999). It is unlikely that the C1 and PH domains function as classical autoinhibitory domains *per se*, rather the integrity of the PKD structure may be critical for maintaining PKD in an inactive conformation. Disruption of this structure by mutation of the C1 or PH domains, and potentially also during PKC-dependent phosphorylation *in vivo* or co-factor binding *in vitro*, could result in a conformational change to a "active" state such that the kinase domain is accessible for substrate phosphorylation.

The PKD PH domain may also play a role in controlling the basal cytosolic distribution of PKD *in vivo* since, as discussed in Chapter 6, the PKD ΔPH mutant exhibits a distinct pattern of subcellular localisation compared to that of wild-type PKD or indeed other PKD mutants. A potential role for the PH domain as a "nuclear exclusion" or "cytosolic retention" determinant requires more detailed analysis in the future: would partial PH domain deletions or single amino-acid substitutions also alter the basal subcellular localisation of PKD? Does the PKD PH domain bind a protein/chaperone that localises PKD to the cytoplasm?

A third potential regulatory role for the PKD PH domain could be to mediate interactions with upstream regulatory proteins. For example, the PH domain of PKD has been implicated in the regulation of PKD catalytic activity by Gβγ subunits of heterotrimeric G proteins (Jamora *et al.*, 1999), as discussed below. Moreover, complexes between PKD and the novel PKC enzymes η and ε (but not classical or atypical PKC enzymes) have been described both *in vitro* and *in vivo* (Waldron *et al.*, 1999b). Here the PKD PH domain was both necessary and sufficient for this interaction. *In vitro* and *in vivo* interactions between multiple PKCs and the PH domains of PKB/Akt and Tec family kinases have previously been described (Kawakami *et al.*, 1995; Konishi *et al.*, 1994; Konishi *et al.*,

1995; Yao *et al.*, 1994). Interestingly, there is some evidence to suggest that PKC negatively regulates the catalytic activity of Btk and PKB/Akt kinases *in vivo* (Doornbos *et al.*, 1999; Yao *et al.*, 1994) whereas PKCη and PKCε are positive upstream regulators of PKD (as discussed above). At present PKD/PKC complexes have only been observed in transfected COS-7 cells but in the future it will be interesting to determine whether such interactions occur between the endogenous kinases and whether physiological stimuli would increase this association. Formation of PKD/PKC complexes may be important for enhancing PKD activation by PKC enzymes *in vivo*, although this will be difficult to determine experimentally given that mutations within the PKD PH domain that abolish PKC interactions also result in a constitutively active kinase (Iglesias and Rozengurt, 1998c).

7.3. ROLE OF PKD IN SIGNAL TRANSDUCTION PATHWAYS.

The work described in this thesis has been concerned with the upstream regulation of PKD activity and localisation in response to both pharmacological and physiological agonists. A major question that remains unanswered is what is the biological function(s) of PKD? The ubiquitous pattern of PKD expression, combined with the potent and sustained stimulation of PKD catalytic activity by a wide variety of cell surface receptors highlights the potential importance of this novel kinase *in vivo*. The differential localisation of PKD in different cell types indicates that PKD may have distinct functions depending on the cellular context. Given that PKD is regulated via a PKC-dependent signalling cascade *in vivo* many previously described PKC-responses may be mediated ultimately through PKD. Over the last four years several potential roles for PKD have been proposed, as described below.

7.3.1. PKD as a molecular scaffold.

One emerging theme is that the N-terminal regulatory region of PKD functions as a scaffold to recruit and/or localise other cellular proteins. For example, the PH domain of PKD mediates protein-protein interactions with two distinct upstream regulatory proteins, namely novel PKC enzymes and Gβγ subunits (see above). In addition, as discussed in section 4.3, PKD appears to associate with at least two lipid kinases (Nishikawa *et al.*, 1998) and with 14-3-3 proteins (Hausser *et al.*, 1999). Thus, PKD may play a role in the assembly of specific lipid-protein complexes *in vivo*, although the functional significance of this remains unclear since none of these proteins appear to be substrates for PKD and many of these interactions are constitutive.

7.3.2. PKD as a negative regulator of intracellular signalling cascades.

One interesting potential role for PKD *in vivo* could be as a negative regulator of signal transduction pathways. Bagowski *et al* have observed that PDGF does not activate JNK by itself but will suppress EGF-induced JNK activity and subsequent downstream events such as cell proliferation and transformation in Rat 1 fibroblasts. In these cells PDGF, but not EGF, stimulates PKD catalytic activity. In contrast, both EGF and PDGF are able to activate JNK in SF126 glioblastoma cells, with no inhibitory crosstalk mediated by PDGF. Strikingly, no detectable PKD activity was observed in these cells, indicating that PKD may function as a negative regulator of growth factor-induced JNK signalling pathways *in vivo*. This hypothesis was confirmed when overexpression of PKD in SF126 glioblastoma cells (which induced constitutive PKD activity) resulted in the inhibition of both EGF- and PDGF-induced JNK activity (Bagowski *et al.*, 1999).

EGF receptor downregulation is mediated by the phosphorylation of two threonine residues, T654 and T669, within the juxtamembrane region of the receptor, a process which involves classical/novel PKC enzymes (Hunter *et al.*, 1984; Morrison *et al.*, 1993a). Mutation of the EGF receptor at one or other of these sites prevented the PKD-induced downregulation of JNK activity in SF126 glioblastoma cells (Bagowski *et al.*, 1999), suggesting that PKD negatively regulates an EGF-JNK signalling pathway *in vivo* by either directly (or indirectly) regulating EGF receptor phosphorylation and downmodulation.

The Clark laboratory also suggested a potential role for PKD as a negative regulator of BCR-signalling events when they identified PKD as a target for BCR signals in B lymphocytes (Sidorenko *et al.*, 1996). They observed that co-immunopreciptation of PKD with Syk (from BCR-stimulated cells) partially inhibited the ability of Syk to phosphorylate a GST-PLCγ SH_2/SH_3 fusion protein *in vitro*. Interactions between PKD and the BCR complex was also detected through co-immunoprecipitation. However, these studies were carried out using digitonin as a weak detergent and this technique is not thought to be a reliable diagnosis of protein co-localisation because digitonin is inefficient at solubilising membrane proteins. Thus, *in vitro* associations detected using this detergent may not reflect true *in vivo* interactions. Thus a role for PKD in the negative regulation of BCR pathways cannot be concluded from these experiments.

7.3.3. PKD: a role in Golgi organisation and protein transport.

Prestle and co-workers first identified a potential role for PKD at the Golgi complex from observations that endogenous PKD is partially co-localised with a *cis*-Golgi linked enzyme in human hepatocellular carcinoma cells (Prestle *et al.*, 1996). They went on to demonstrate that cellular sulphate uptake and Golgi-specific glycosaminoglycan sulfation were enhanced by ~30% in PKD-overexpressing HeLa cells (Prestle *et al.*, 1996), suggesting that PKD may be involved in the regulation of basal transport processes within the Golgi compartment. Interestingly PKCε, an isoform implicated in the regulation of PKD activity, has also been reported to be localised to the Golgi compartment, although PKCε inhibits rather than stimulates protein sulfation (Lehel *et al.*, 1995).

The marine sponge toxin ilimaquinone (IQ) induces Golgi vesiculation (breakdown) *in vivo* through activating heterotrimeric G proteins to release active Gβγ subunits. Recently, the Malhotra laboratory have demonstrated that free Gβγ subunits, via interactions with the PKD PH domain, can directly activate PKD *in vitro* (Jamora *et al.*, 1999). Moreover, inhibition of Gβγ/PKD PH domain interactions (thorough microinjection of a monoclonal antibody directed against the PKD PH domain or of excess purified PH domain) prevents IQ-induced Golgi fragmentation and protein transport processes in rat kidney epithelial cells (Jamora *et al.*, 1999). How activation of PKD by Gβγ relates to the dynamics of Golgi membranes during normal protein transport events is still unresolved. It will also be interesting to determine whether PKD regulates Golgi-breakdown in physiological situations, for example during mitosis, and also to identify potential PKD substrates that could mediate these events.

7.3.4. PKD and cell growth/differentiation.

It has been proposed that PKD may be involved in the control of cellular proliferation and differentiation. This was based on observations that (i) PKD expression levels decrease as keratinocytes stop dividing and differentiate within the mouse epidermis and (ii) that a 2-fold overexpression of PKD in NIH 3T3 fibroblasts results in a decreased cell doubling time, from 22 to 16 hours (Renneke *et al.*, 1999). However, a 4-fold overexpression of wild-type PKD in Swiss 3T3 fibroblasts does not alter the growth rate of these cells and constitutively active, kinase-dead or wild-type PKD proteins have no effect in NIH 3T3 transformation assays, either alone or in combination with activated V12-Ras (Dr. T. Iglesias, ICRF, *Personal communication*).

In addition, antisense oligonucleotide experiments in 3T3-F442A pre-adipocytes has demonstrated a negative role for PKD (as well as PKCα and PKCδ) in adipocyte differentiation (Fleming *et al.*, 1998).

7.3.5. PKD and NFκB.

PKC enzymes have been implicated in the regulation of the NFκB transcription factor, as discussed in Chapter 1. Recently, Johannes *et al* have observed that co-expression of wild-type PKD enhances, and a kinase-dead PKD mutant suppresses, TNFα-mediated activation of an NFκB-driven reporter gene (Johannes *et al.*, 1998). In addition, a 4-fold overexpression of wild-type PKD reduces TNFα (but not ceramide) induced apoptosis by ~25%, possibly by enhancing the induction of NFκB-dependent, anti-apoptotic genes by TNFα.

7.4. FUTURE DIRECTIONS.

The work of this thesis has detailed investigations into the upstream regulation of PKD by pharmacological agents and by antigen receptors in hematopoietic cells and mouse fibroblasts. However the function of PKD within antigen receptor signalling cascades remains elusive. A number of different experimental techniques could be used to identify biological roles for PKD in these systems. As discussed in Chapter 1, activated mutants of PKC enzymes have been used to place specific PKC enzymes in antigen receptor signalling pathways. Thus, one approach that could be used in an attempt to define signalling pathways regulated by PKD might be to study the effect of different activated and kinase-defective PKD mutants on transcriptional responses induced by antigen receptors, using reporter gene assays. It should be noted these assays would only give an indication of what PKD can do in these systems however, and may not necessarily reflect the true function of PKD. Moreover, caution would be needed to interpret data obtained using constitutively activated PKD mutants, which would be delocalised compared to endogenous PKD that is activated under physiological conditions.

Identification of proteins that interact with PKD (either constitutively or inducibly) might also provide clues as to the biological role of PKD. A number of different techniques could be employed here, for example yeast 2-hybrid screening and affinity chromatography are both techniques that have been successfully employed to identify interacting proteins (either regulatory proteins or substrates) for multiple signal transduction molecules. Moreover, the PKD consensus phosphorylation motif could be

used to search EST databases to try and identify potential PKD substrates. At present this motif is not specific enough to attempt this, however if the consensus sequence could be defined further such an approach may prove possible in the future.

One approach that may prove successful in the long-term would be to knock-out the PKD gene. At present only two PKC knockout mice have been described in the literature, both of which exhibit different phenotypes: PKC$\gamma^{-/-}$ mice exhibit a mild neurological phenotype (Abeliovich *et al.*, 1993b) whilst the PKC$\beta^{-/-}$ mice are characterised by B cell defects (Leitges *et al.*, 1996). One concern with this approach would be that the relationship between PKD and the related kinase PKCυ has not yet been defined. If PKCυ is expressed in the same tissues as PKD and shares the same regulatory features and substrate specificity the question of functional redundancy between these kinases could arise. Detailed studies on PKCυ will be required to answer these questions.

A more immediate prospect to study the function of PKD in an *in vivo* model system may be to generate transgenic mice expressing different PKD mutants. Here, tissue specific promoters could be used to study PKD function in lymphocytes (e.g. the CD2-promoter for T cells, the Eμ-promoter for B cells).

Finally, the study of PKC homologues in lower organisms, including *Caenorhabditis elegans* and *S. cerevisiae*, has revealed striking parallels with the PKC superfamily in mammalian cells. Indeed, the interaction between Pkc1 and Rho1 was reported in yeast before the discovery of an analogous interaction between PRK1 and RhoA in fibroblasts (Amano *et al.*, 1996; Nonaka *et al.*, 1995; Watanabe *et al.*, 1996). *Caenorhabditis elegans* represents an extremely manipulatable and powerful genetic system and a BLAST search of the *Caenorhabditis elegans* genome database has revealed the presence of two PKD homologues. Study of these *Caenorhabditis elegans* PKD proteins would allow the biology of PKD to be addressed *in vivo* within the context of a multicellular organism and might also provide insight as to the function of PKD in mammalian cells.

These areas and more represent intriguing areas for future research into this novel and exciting kinase.

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