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Selective Regulation of Protein Kinase C Delta in Response to B Cell Receptor Crosslinking

by

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Abstract

One of the most important response-determining cell surface molecules on the mature B cell is the B cell antigen receptor (BCR). Activation of the B cell through BCR crosslinking is known to initiate a protein tyrosine kinase (PTK) cascade, and lipid second messenger production through the activation of phospholipase Cγ (PLCγ) and phosphatidylinositol 3' kinase (PI3'K). This thesis demonstrates how protein kinase C δ (PKCδ) also responds to crosslinking of the BCR by becoming activated and tyrosine phosphorylated within 30 seconds. The contribution of the three major BCR-associated signaling pathways (PTK, PLCγ, PI3'K) was investigated in terms of the activation and tyrosine phosphorylation of PKCδ. PKCδ was determined to be primarily dependent on PI3'K for its activation, also dependent on an upstream PTK, and independent of PLCγ for activation. Preventing PKCδ activation by blocking PI3'K was accompanied by a decrease in the tyrosine phosphorylation of PKCδ.

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Abbreviations Used:

APC, antigen presenting cell
BCR, B cell antigen receptor
C-terminus, carboxy terminus
DG, diacylglycerol
DMSO, dimethylsulfoxide
DT40, chicken B cell line used for its convenience of genetic alteration
ECL, enhanced chemiluminescence
IB, immunoblot (also known as Western blot)
IC_{50}, inhibitory concentration at which 50% of the enzyme is inactive
IP, immunoprecipitate
ITAM, immunoreceptor tyrosine activation motif
kDa, kiloDalton
mAb, monoclonal antibody
mHBS, modified HEPES-buffered saline
N-terminus, amino terminus
PBS, phosphate-buffered saline
PE, phorbol ester
PI3'K, phosphatidylinositol 3' kinase
PIP_2, phosphatidylinositol-4,5-diphosphate
$PI(3,4)P_2$, phosphatidylinositol-3,4-diphosphate
PIP_3, phosphatidylinositol-3,4,5-trisphosphate
PKC, protein kinase C
PLC, phospholipase C
PMA, phorbol-12-myristate-13-acetate
PTK, protein tyrosine kinase
P-tyr, phosphotyrosine
SDS, sodium dodecyl sulfate
SDS-PAGE, SDS polyacrylamide gel electrophoresis
SH, Src-homology; SH1 is tyrosine kinase; SH2 is phosphotyrosine binding; SH3 is proline-rich sequence binding
TPA, 12-*O*-tetradecanoylphorbol-13-acetate
TTBS, tween-20 tris-buffered saline

Chapter One

An overview of signaling from the B cell receptor to protein kinase C.

Introduction

The B cell antigen receptor (BCR) functions as one of the crucial growth- and differentiation-determining signal mediators for the B cell; signals initiated by this molecule through its associated cytoplasmic kinases (see Figure 1-1) can result in activation, tolerance, or apoptosis (Gold and DeFranco, 1994; Gold and Matsuuchi, 1995). Since BCR crosslinking is thought to mimic the process by which a B cell detects an antigen, the membrane proximal signaling events of BCR crosslinking have been extensively characterized. In summary, BCR crosslinking results in protein tyrosine kinase (PTK) activation (Saouaf et al., 1994), which directly leads to the activation of each of the Ras, phosphatidylinositol 3' kinase (PI3'K) and phospholipase $C\gamma_2$ ($PLC\gamma_2$) pathways through separate mechanisms (Carter et al., 1991; Gold et al., 1992; Gold et al., 1994; Hempel et al., 1992) (see Figure 1-2). This cascade provides the necessary machinery to produce the lipid second messengers which then stimulate essential cellular pathways. The purpose of this introduction is to provide an overview of the published literature relevant to the activation and regulation of protein kinase C δ (PKCδ) in B lymphocytes, therefore the emphasis will be placed on the participants whose involvement has been demonstrated. Each of the key steps in generating signals in response to BCR crosslinking will be examined, culminating in the activation of the PKC serine/threonine kinases. Figures 1-1 and 1-2 have been provided to illustrate the key points of the BCR signaling pathways.

The tyrosine phosphorylation cascade that characterizes the initial events of BCR crosslinking has been delineated into several key components, the first of which is the phosphorylation of specific Igα/β tyrosine residues by Src-family kinases, namely Lyn and to perhaps a lesser degree Fyn (see Figures 1-1 and 1-2; for review, see Kurosaki, 1997).

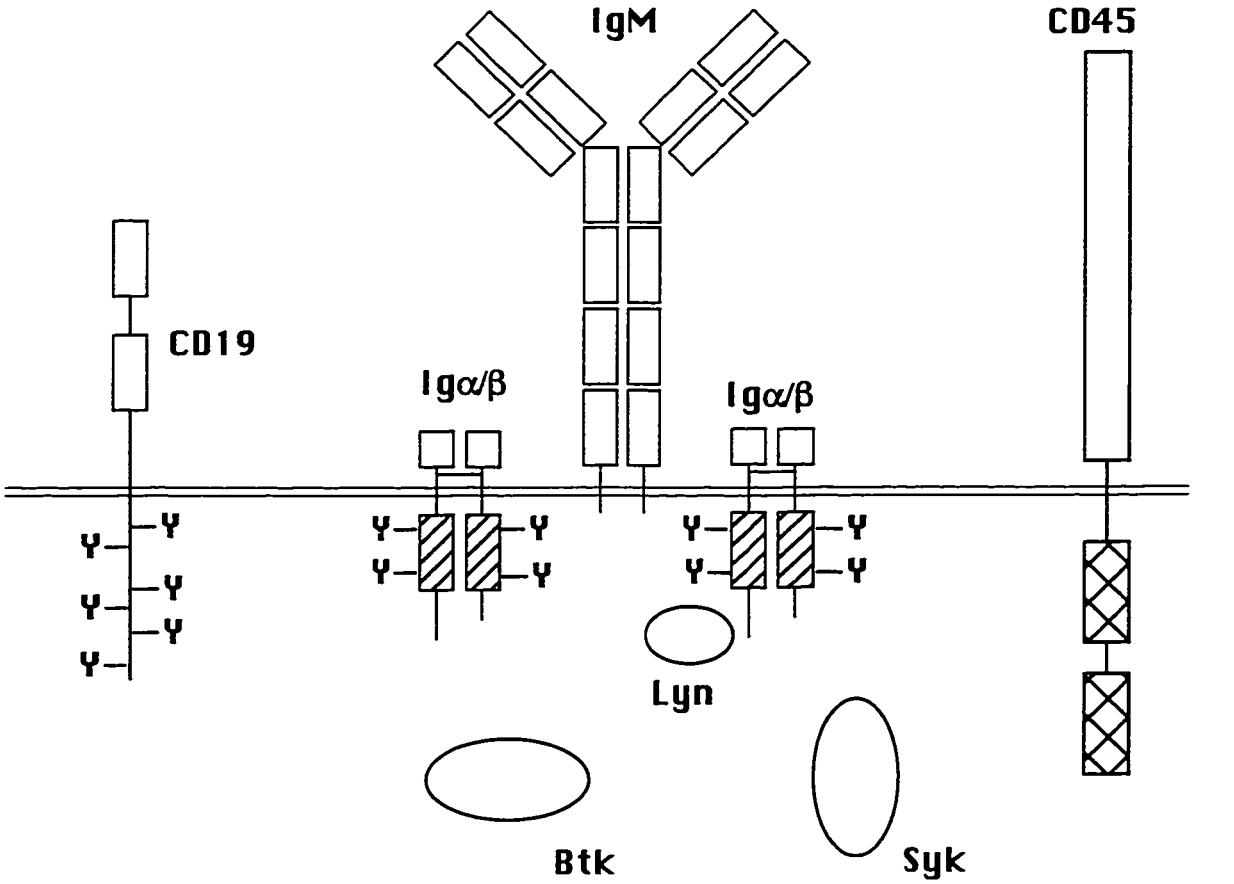


Figure 1-1. The B cell receptor and associated signaling proteins.

Membrane IgM with its associated Igα and Igβ proteins is collectively defined as the BCR.

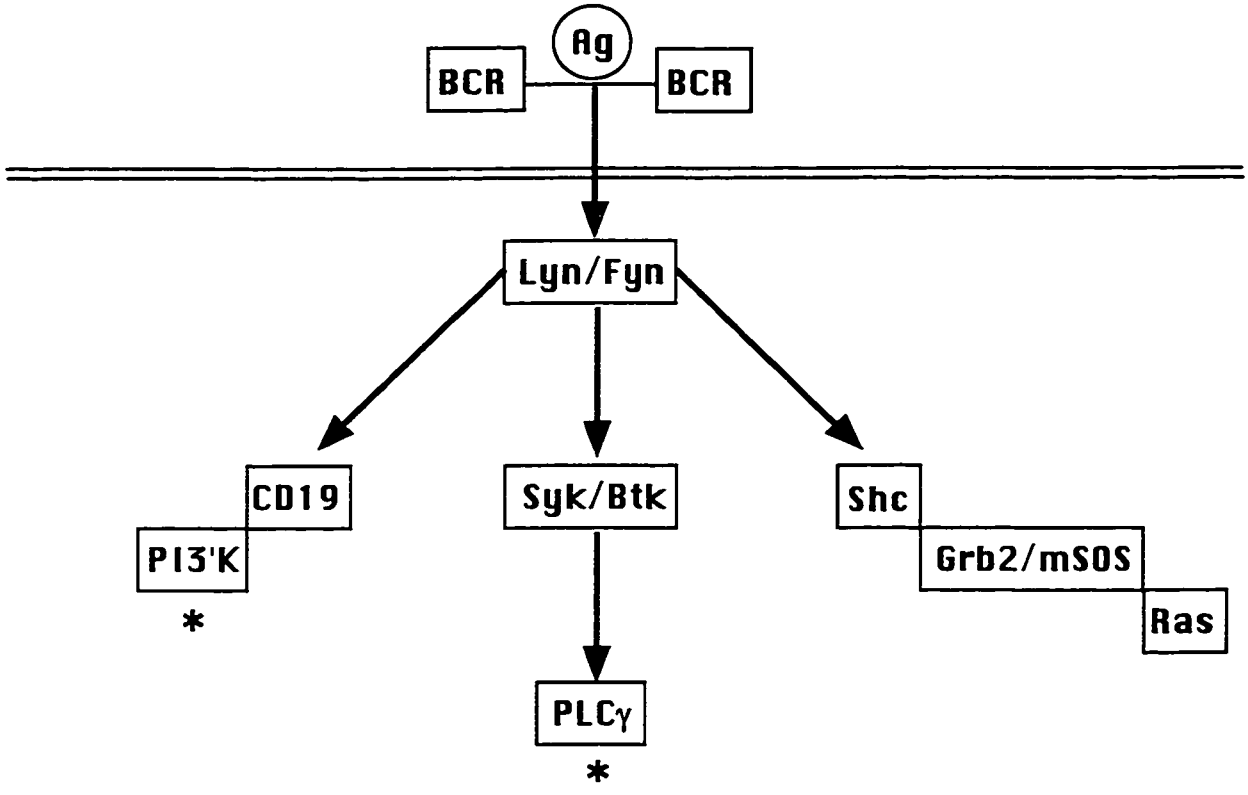


Figure 1-2. Schematic of signal transduction in response to BCR crosslinking by antigen (Ag). Arrows denote activation by tyrosine phosphorylation; connected boxes denote activation by association or recruitment; * denotes second messenger production.

The tyrosines which become phosphorylated upon receptor ligation are present in a region known as an immuno-receptor tyrosine activation motif (ITAM). The ITAM sequence is defined as a sequential twenty six amino acid region (sequence $(D/E)x_7(D/E)xx\mathbf{Y}xx(L/I)x_7\mathbf{Y}xx(L/I)$, single letter amino acid code, x is any amino acid), and is present in both the Igα and Igβ proteins. The activation of Lyn likely involves two cooperating elements: the priming effects of C-terminal dephosphorylation of Y527 by the tyrosine phosphatase CD45 (Pao and Cambier, 1997) (see Figure 1-1), and the localization of Lyn to the BCR complex through its N-terminal binding of Igα (Pleiman et al., 1994). The combination of these two factors allows for the extreme sensitivity of Lyn activation, therefore, when the BCR is crosslinked, proximity and predisposition result in immediate transphosphorylation of Lyn. The primary function of Lyn activation following BCR ligation is the tyrosine phosphorylation of the ITAM tyrosines (a total of four tyrosines per Igα/β heterodimer). The crucial requirement for Lyn in BCR function is demonstrated by the severe effects of its absence ($Lyn^{-/-}$), characterized by systemic autoimmunity and immunodysfunction (Hibbs et al., 1995).

A second PTK known to mediate BCR signals is the ZAP70 homologue, Syk (for review, see (Kurosaki, 1997)). Following ITAM tyrosine phosphorylation by Lyn, Syk is recruited to the two phosphorylated tyrosine residues within the ITAM by its two Src-homology 2 (SH2) domains, resulting in partial activation. Syk is then further activated by Src-PTK tyrosine phosphorylation (Weiss and Littman, 1994), analogous to Lck/ZAP70 cooperation in the response to TCR ligation. Syk is known to play an essential role in mediating BCR signals, as Syk deficiency ($Syk^{-/-}$) in DT40 chicken B cells results in the failure of the BCR to mediate apoptotic signals (Takata et al., 1995). Btk is the third prominent PTK involved in BCR signals, and is likely activated simultaneously with Syk (for review, see (Kurosaki, 1997)). Activation of Btk requires the tyrosine phosphorylation of one site (Y551) by Src-family kinases, then subsequent autophosphorylation of a second site

(Y223) in the Src-homology 3 (SH3) domain (Park et al., 1996; Rawlings et al., 1996). This activation is thought to promote membrane association, possibly through SH2/ITAM interactions (Kurosaki and Kurosaki, 1997) or interactions with PI3'K lipid products (Li et al., 1997). Alternatively, the recruitment of Btk to the membrane in response to PI3'K lipid signals may predispose it for tyrosine phosphorylation by proximity to the BCR complex (Li et al., 1997). The importance of Btk is demonstrated by the clinical condition known as X-linked agammaglobulinemia (mutations in the Btk gene) or the less severe X-linked immunodeficiency in the mouse, caused by a point mutation in the pleckstrin homology domain of Btk. One essential function of both Syk and Btk is the activation of PLCγ_2 through tyrosine phosphorylation, as demonstrated by studies with $Btk^{-/-}$ DT40 cells, in which the activation of PLCγ_2 is compromised (Takata and Kurosaki, 1996). Again, as illustrated in Figure 1-2, PTK activation is central to BCR crosslinking-mediated signaling. From this branch point, the PLCγ, PI3'K, and Ras pathways all become activated.

BCR crosslinking results in the activation of the Ras pathway, which involves tyrosine phosphorylation of the adaptor protein Shc through association with the active PTKs in the BCR complex (for review, see (DeFranco, 1997)). This causes the assembly of Grb2 with mSOS, followed by their subsequent translocation to the plasma membrane, where Grb2/mSOS interacts with both the PTK-associated Shc and membrane associated Ras, forming an activating bridge (Lankester et al., 1994; Saxton et al., 1994; Smit et al., 1994). The regulation of the Ras/MAPK pathway is also believed to involve protein kinase C (PKC), as phorbol ester (PE) stimulation of lymphocytes promotes Ras activation but PKC inhibitors only partially block BCR-induced MAPK activation (Gold et al., 1992).

The activation of PI3'K is known to involve the binding of PI3'K (p85/p110) to tyrosine phosphorylated CD19/Lyn complex *via* the SH2 of its regulatory subunit, p85

(Tuveson et al., 1993). Lyn has been implicated in the activation of the PI3'K pathway (Gold et al., 1993; Yamanashi et al., 1992), as crosslinking of the BCR results in sequential increases in Lyn kinase activity, phosphorylation of CD19, and association of p85 to CD19. PI3'K activation through BCR crosslinking results in the dramatic and immediate increase in the lipid second messengers $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (Gold and Aebersold, 1994) due to the phosphorylation of the 3' hydroxyl of PI(4)P or $PI(4,5)P_2$ by PI3'K. The downstream effects of PI3'K activation are vaguely understood, although the activation of PKB (c-Akt) by $PI(3,4)P_2$, and Btk by $PI(3,4,5)P_3$ have been reported (Franke et al., 1997; Salim et al., 1996).

The PLC pathway in B cells is defined by the hydrolysis of $PI(4,5)P_2$ through the activation of $PLC\gamma_2$, resulting in the production of diacylglycerol (DG) and inositol-1,4,5-trisphosphate (IP_3), ultimately causing the mature B cell to enter the G1 phase of the cell cycle (Gold et al., 1990) (for review, see (DeFranco, 1997)). Textbooks generally attribute the activation of the PKC family of serine/threonine kinases to the production of DG, and the release of Ca^{2+} from intracellular Ca^{2+} stores to the production of IP_3. The activation of $PLC\gamma_2$ is known to involve its tyrosine phosphorylation in response to BCR crosslinking (Hempel et al., 1992). Genetic evidence from DT40 B cells implicates both tyrosine kinases Syk and Btk in the activation of PLCγ (Kurosaki, 1997; Takata and Kurosaki, 1996). In addition, PLCγ has been shown to directly associate with tyrosine phosphorylated Syk; this may represent the mechanism of association of the cytosolic PLCγ with the BCR complex (Law et al., 1996; Sillman and Monroe, 1995).

The adaptor proteins involved in BCR signaling are the least understood. BCR crosslinking promotes tyrosine phosphorylation of Cbl, in addition to assembly of the multi-adaptor Cbl protein complex (Ingham et al., 1996; Panchamoorthy et al., 1996) Although this complex appears to associate preferentially with active PI3'K, Lyn, Fyn, and

Syk following BCR stimulation, the function of this association is not known (Panchamoorthy et al., 1996). This complex has both the potential to amplify the BCR signals by focused signaling, as well as to dampen it by absorbing active kinases into an inactive aggregate.

The regulation of the BCR signal is thought to involve several key players (for review see (DeFranco, 1997)), of which the tyrosine phosphatases are most relevant to this discussion. The involvement of the transmembrane phosphatase, CD45, in the priming of Lyn has already been addressed, however, it is likely involved in the dephosphorylation of many other tyrosine-phosphorylated species as well. Another well characterized phosphatase that is known to participate in the regulation of the BCR-mediated signal is the protein tyrosine phosphatase 1C (PTP-1C). BCR crosslinking causes tyrosine phosphorylation of a cell adhesion molecule, CD22, which then recruits PTP-1C via its SH2. This in turn associates with Syk and PLCγ_2 via their SH2s; tyrosine phosphorylated CD22/PTP-1C may modulate the activity of CD22, Syk, and PLCγ_2, by dephosphorylation of all three members (Law et al., 1996). Another regulatory mechanism relevant to this thesis is the termination of lipid second messenger production by the inositol 5' phosphatase, p145^{SHIP} (SHIP). SHIP is thought to downregulate lipid signals from PLCγ_2 and PI3'K (Ono et al., 1996) by depleting their common substrate, PI(4,5)P_2. Finally, it is believed that PKCμ also plays a regulatory role by down regulating Syk and PLCγ (Sidorenko et al., 1996) by serine/threonine phosphorylation.

The next level of signaling resulting from the receptor proximal events involves many cellular proteins, including the PKC serine/threonine kinases. PKC is known to participate in BCR-mediated signals because calcium ionophores and phorbol esters which induce PKC activation can also induce B cell responses similar to those observed through BCR crosslinking, namely: proliferation, apoptotic signals, growth arrest, and apoptosis in

lymphoma cell lines (Foote et al., 1996; Kim et al., 1992; Muthukkumar et al., 1993). In addition, the activation of the lipid-modulating enzymes which produce PKC-activating lipids is well known and has been extensively characterized in response to BCR signaling (Chen et al., 1986; Gold and Aebersold, 1994; Gold et al., 1992; Nel et al., 1986)

Protein kinase C (PKC) was originally identified by Nishizuka, Inoue, Takai, and Kishimoto because of its potential to become activated by limited proteolysis in frozen brain preparations, and was published in two back-to-back papers in the Journal of Biological Chemistry describing the purification and activation of a cyclic-nucleotide independent inase (Inoue et al., 1977; Takai et al., 1977). Historically speaking, it was really the discovery that Ca^{2+} and lipids could modulate the activity of this enzyme that accelerated interest in this field of research (Takai et al., 1979a; Takai et al., 1979b; Takai et al., 1979c). Currently, it is known that many lipid second messengers can contribute to the activation of PKC including ceramide, $PI(3,4)P_2$, PIP_3, and cholesterol sulfate (for review, see (Liscovitch and Cantley, 1994)).

The structure of conventional PKC is depicted as a linear domain diagram in Figure 1-3a. Briefly, PKC contains several regions, denoted C1 to C4, as well as an N-terminal pseudosubstrate (PSub) region. The PSub, C1, and C2 domains all comprise the regulatory portion of the molecule; the C3 and C4 regions make up the catalytic portion. The function of each is described below, and illustrated as a model of PKC activation in Figure 1-3b.

PKC activation *in vivo* is indeed a complicated phenomenon. Many different compounds have been identified that can increase the activity of PKC both *in vitro* and *in vivo*, however, in most cases the significance of these findings remains to be determined in a natural scenario. The current philosophy on the activation of PKC is as follows (for

model, see Figure 1-3b; for review, see (Newton, 1997)): binding of lipid cofactors to each of the C1 and C2 domains is required to provide the required energy for the removal of the pseudosubstrate from the active site. This involves the binding of DG to one of the two C1 domains, and the binding of phosphatidylserine (PS) to the C2 domain, in complex with Ca^{2+}. The binding of the two cofactors is cooperative; DG binding causes PS to bind with 10x increased affinity compared to other acidic lipids. Binding of either lipid alone is insufficient to cause activation but can cause low affinity membrane localization. Recent crystallographic data have provided evidence that binding of phorbol ester (PE) or DG to the C1 region of PKC occupies a polar site within an otherwise hydrophobic site on the domain, and the hydrophobicity of the DG or PE acyl chains causes the formation of a then contiguous hydrophobic surface (Zhang et al., 1995) which can then insert into membranes. Upon substrate availability, the enzyme binds ATP in the C3 region (GxGxxG), and transfers the γ-phosphoryl group of ATP onto either a serine or threonine hydroxyl of the substrate polypeptide.

PKC is a family of serine/threonine kinases, with a high degree of homology in the catalytic domain (C3/C4), but a variety of preferences for lipid cofactors, and an even more complex tissue and subcellular distribution. The "PKC" that was investigated prior to 1986 was a heterogeneous mix of several proteins (Inoue et al., 1977; Takai et al., 1977), including what we now know to be α, β, and γ (Coussens et al., 1986; Huang et al., 1986; Jaken and Kiley, 1987; Ono et al., 1986). This discovery spawned the cloning efforts of numerous groups who identified a plethora of proteins, each coded by separate genes (Akimoto et al., 1994; Bacher et al., 1992; Johannes et al., 1994; Ohno et al., 1988; Ono et al., 1988; Osada et al., 1990; Osada et al., 1992; Selbie et al., 1993; Valverde et al., 1994). We now know that the identification of PKC isozymes from different species accounts for the extranomenclature of PKCη=L, PKCι=λ and PKCμ=PKD.

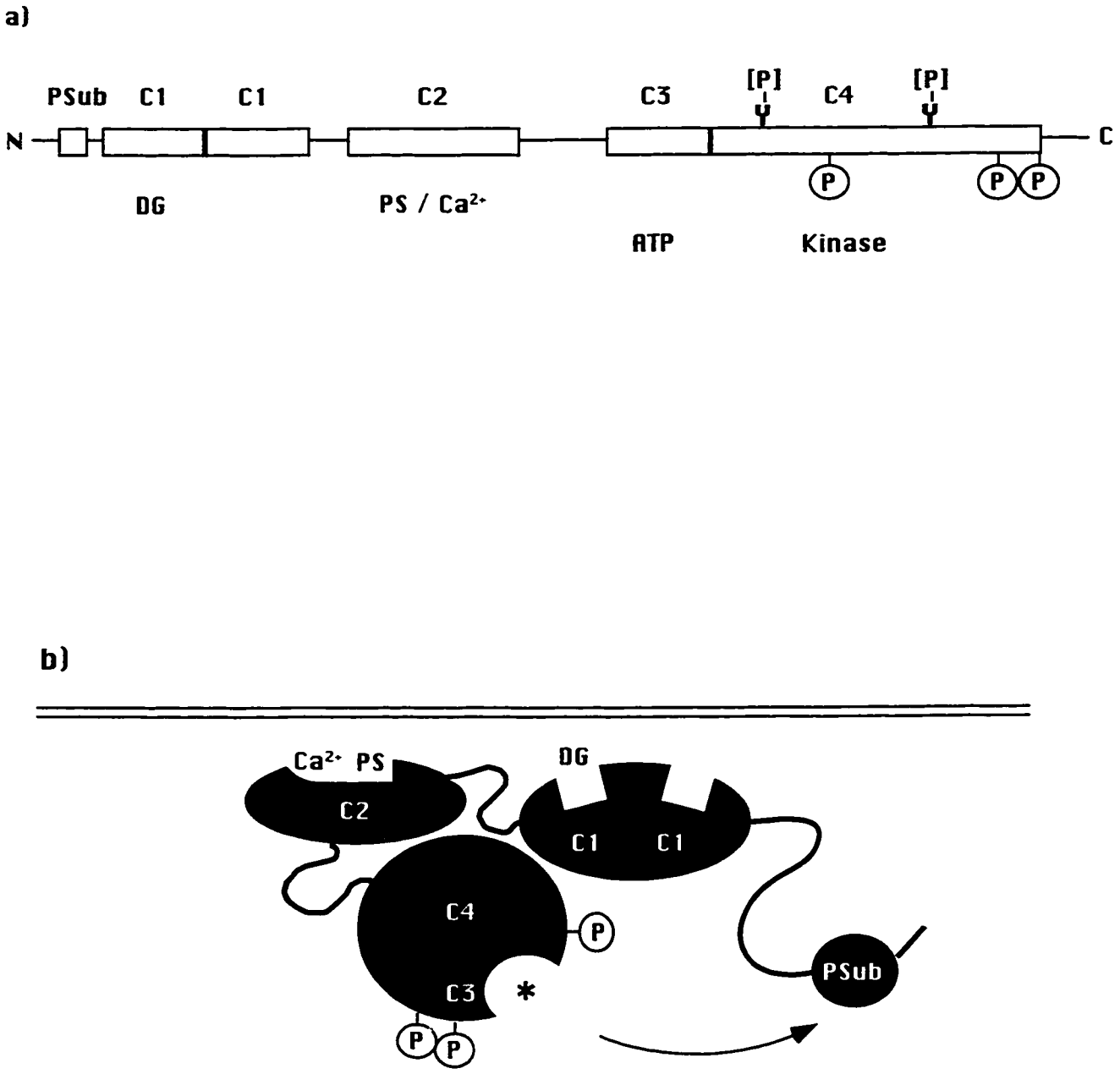
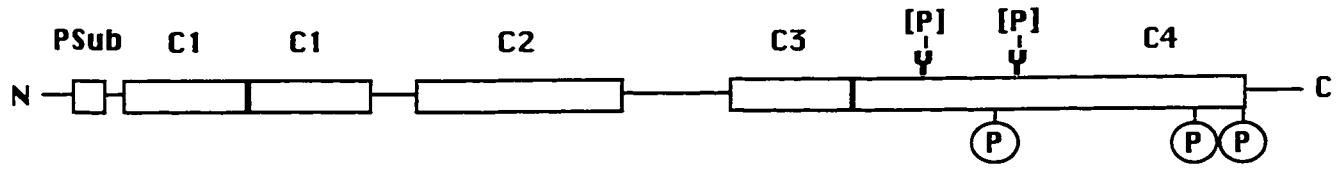


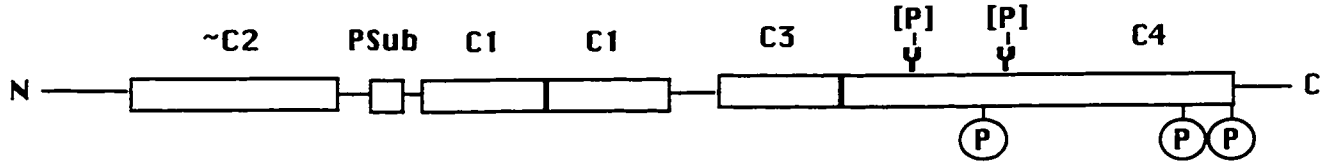
Figure 1-3. Structure and activation of PKC. a) the linear structure of PKC, including conserved phosphorylation sites. b) schematic of PKC activation: binding of DG and PS results in the release of the pseudosubstrate (Psub) from the active site, allowing for enzymatic activity.

PKC has been classified by Groups based on the domains that dictate cofactor preference (see Figure 1-4). Group A (*conventional*) PKCs (α, $\beta_{I/II}$, γ) require both Ca^{2+} in complex with PS, and DG; Group B (*novel*) PKCs (δ, ε, η/L, θ) require only PS and DG; Group C (*atypical*) PKCs (ζ, ι/λ) contain only one of the lipid binding consensus (C1) domains, and are insensitive to both DG and Ca^{2+}; Group D is currently PKCμ alone (PKD in the mouse), which shares similarity with the *novel* isoforms and with respect to their cofactor preference and domain structure, yet behaves differently than other PKCs with respect to catalytic activity (for review, see (Newton, 1997) and (Nishizuka, 1995)). The C1 domain which binds DG has been recently redefined to describe one Cys-rich Zn^{2+}-binding moiety; the *conventional* and *novel* groups therefore have two C1 domains, *atypical* PKCs have only one (Hurley et al., 1997).

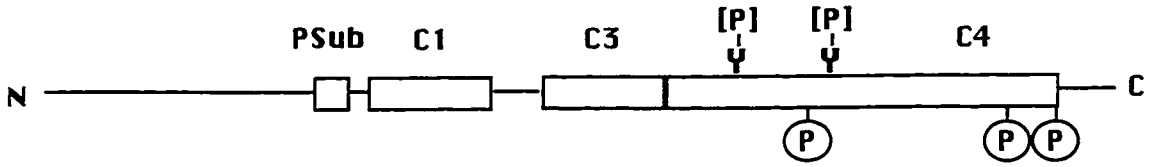
Phosphorylation of PKC is also important in its regulation. Newton's group has shown that PKC is serine/threonine phosphorylated on three distinct sites (Keranen et al., 1995) (see Figure 1-3), and they propose that each site has a different function, all related to producing catalytically competent PKC. These sites (or homologous acidic residues) are highly conserved within all PKC's, the first of which is (in PKCβ_{II}) T500 in the activation loop, which is thought to provide a negative charge for positioning substrates, and is phosphorylated first by an as-yet unidentified PKC-kinase. The phosphorylation of T500 produces catalytically competent PKC, which can then autophosphorylate first at T641, then at S660, at the C-terminus, which are believed to 'lock' PKC in a primed activatable state (Keranen et al., 1995). Keranen, *et al.*, suggest that the PKC that responds to extracellular signals is triply-phosphorylated, competent for activation, although likely not active *per se*. Homologous sequences in this region of human PKCδ give equivalent residues T507, S645, and S664 (Aris et al., 1993; Keranen et al., 1995). It is interesting to note, however, that the hypothesis of phosphorylation of PKC to produce catalytically



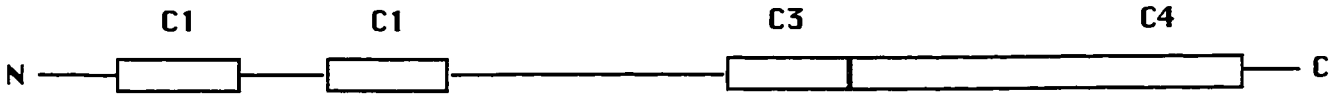
Group A: (conventional) α, βI/βII, γ



Group B: (novel) δ, ε, θ, η



Group C: (atypical) ζ, ι/λ



Group D: μ, PKD

Ⓟ, serine/threonine phosphorylation sites

[P] ψ, tyrosine phosphorylation sites

Figure 1-4. The PKC family of serine/threonine kinases. (see text) ~C2 refers to a C2-homologous region in novel PKCs, which does not bind Ca^{2+}.

competent enzyme is already being challenged; a recent report regarding the activation loop of PKCδ (T505 in the rat) substantiates that preventing phosphorylation of T505 or S504 by site-directed mutagenesis to alanine does not result in any alteration in the activation of recombinant rat PKCδ in their system (Stempka et al., 1997). If nothing else, this study reminds us that subtle differences, even among species, suggests even more complex systems of regulation of individual isoforms.

In addition to serine/threonine phosphorylation, most isoforms of PKC have also been reported to be tyrosine phosphorylated in whole cell systems. PKCα, β_I, γ, δ, ε, μ, and ζ (Haleem-Smith et al., 1995; Konishi et al., 1997; Liu and Roth, 1994; Sidorenko et al., 1996; Soltoff and Toker, 1995; Zang et al., 1997) have all been reported to be capable of being tyrosine phosphorylated. The exact function of tyrosine phosphorylation is not known, however, it is believed that this tyrosine phosphorylation is regulatory to a certain extent, perhaps by controlling subcellular localization. This will be further addressed in Chapters Two, Three, and Five.

PKC binding proteins are quickly becoming an intense area of focus because of their highly selective nature and specific function (for review, see (Mochly-Rosen, 1995)). Included in this supergroup are the receptors for activated C kinase (RACKs) that bind selectively to active PKC (Mochly-Rosen, 1995). Further, this area has progressed to the level of isoform specificity; several studies have suggested preference of one isoform over another for specific PKC-interacting proteins (Mochly-Rosen et al., 1991; Robles-Flores and Garcia-Sainz, 1993; Ron and Mochly-Rosen, 1995) (see Chapter Five).

Thesis Objectives and Rationale

The overall objective of this thesis is to describe to what extent PKCδ responds to signals originating with BCR crosslinking, and which BCR-associated pathways are in fact

responsible for influencing the behavior of this isoform in the B cell. This is in direct response to the hypothesis that **"the state of activation and tyrosine phosphorylation of PKCδ in the mature B cell are regulated by signaling through the BCR."**

The individual objectives of this thesis are described below:

1. **Objective One**: to determine the profile of PKC isoforms in B cells, based on reported observations and recently identified isoforms
2. **Objective Two**: to identify PKCδ as unique compared with other isoforms, in terms of tyrosine phosphorylation in response to BCR signaling
3. **Objective Three**: to characterize the tyrosine phosphorylation of PKCδ in response to the crosslinking of the BCR in terms of kinetics, upstream pathways, and state of activation of PKCδ
4. **Objective Four**: to characterize the activation of PKCδ in response to BCR crosslinking in terms of timeframes and upstream pathways

The rationale to support these studies is that although many investigations have been performed with respect to BCR signaling, limited attempts have been made to elucidate which isoforms of PKC respond to the immediate events of BCR crosslinking, especially with respect to tyrosine phosphorylation. The reported findings that PKCδ has the capability to become tyrosine phosphorylated in response to other physiological (receptor-mediated) stimuli, both in leukocytes (Kent et al., 1996) and other cell types (Denning et al., 1996; Soltoff and Toker, 1995), provides an avenue for investigation in the B cell. Indeed, the change in state of PKCδ from non-tyrosine phosphorylated to tyrosine phosphorylated within seconds of BCR signaling substantiates the involvement of PKCδ in some dimension of BCR-mediated processes. No published reports have yet implicated PKCδ in B cell signaling, allowing for an exciting opportunity to contribute the

knowledge gained from this study to both the fields of B cell immunology as well as PKC isoform specificity and function.

Experimental Model: the Ramos Burkitt's Lymphoma (Human) B cell line

This research utilized a particular B cell line, the Burkitt's lymphoma B cell line Ramos (ATCC CRL 1596), as a model to study the signals generated by the BCR. This has the advantage of large quantities of cells continuously available from *in vitro* culture. The Ramos cell line was selected over other available B cell lines because the BCR is expressed at a relatively high level, and it has been widely used to study signal transduction pathways originating from the BCR as a model of B cell activation.

The Ramos cell line was originally described by Klein, et al., as **Ra No. 1** (Klein et al., 1975), a human American Burkitt's lymphoma that survived *in vitro* culture (Klein et al., 1974). We now know that the immortalization of the Ramos line is due to a 8;14 translocation of all three exons of the c-myc oncogene into the IgM switch site region of chromosome 14 (Wiman et al., 1984), and that the continual expression of this oncogene is due, at least in part, to the co-translocation of two positive regulatory NFκB promoters in the 5' flanking region of the c-myc gene (Ji et al., 1994). This results in the expression of 2- to 5-fold higher levels of c-myc than in other lymphoblastoid cell lines, rendering the cells immortal (Wiman et al., 1984). Ramos cells are CD40L-sensitive, IgM+, of germinal center origin, and therefore represent the mature B cell, and were used for the majority of the studies described in this thesis.

Chapter Two

Isoforms of protein kinase C in B lymphocytes

Introduction

The discovery of PKC isoforms prompted the question of the extent of specificity within the system, both in function, tissue distribution, and subcellular organization. Evolutionarily speaking, the expression of several different isoforms of an homologous kinase is in fact a clever way to allow specific cellular response to a variety of external stimuli through selective receptor coupling to specific second messenger-generating enzymes. In terms of PKC, signals causing the production of cellular second messengers Ca^{2+} and DG will likely induce activation of *conventional* PKCs, whereas signals that cause the production of only DG will likely activate only the *novel* PKCs. Considering the cellular environment in multiple dimensions including time and subcellular location, it is therefore entirely possible that a selective nature of PKC isoform activation exists.

The evidence for isoform-selective function of PKC in a whole cell environment is overwhelming. In order to provide the necessary background, this introduction will focus on several of the important examples of selective isoform function in a detailed manner, rather than simply list all of the published examples of isoform specificity. These examples share the characteristics of singular isoform function in a whole cell environment, in response to physiological stimuli, and closely parallel the data which will be presented with respect to PKCδ later in the thesis.

The selective activation of PKCθ in the T cell upon antigen presenting cell contact was recently described (Monks et al., 1997). In this study, the Kupfer group identified a specific situation in which a single isoform of PKC was being activated and translocated to a specific site at the membrane. The isoform PKCθ in T cells was shown to respond

spatially to the site of contact of the T cell with the antigen presenting cell. Only PKCθ was shown to have increased inherent activity following contact; addition of TPA to unstimulated PKCθ immunoprecipitates increased the activity by 430%, (filling the C1 site, activating the enzyme); however, the PKCθ immunoprecipitated from stimulated cells was not activated further by the addition of TPA, implying that the C1 cofactor site was inherently occupied. The authors compared the activity of PKCθ with PKCδ isolated from either stimulated or unstimulated T cells; PKCδ showed no difference in its state of activation. This further substantiated that PKCθ was being activated prior to isolation. The point of multidimensional signaling involving several receptor-mediated pathways is also made in this investigation, as crosslinking of the TCR either alone or with CD3, CD4, CD28, or LFA-1 did not produce the same result, although PKCθ, like all other isoforms tested associated with the plasma membrane in response to phorbol esters (PE). The kinetics of this specific localization of PKCθ are quite slow (55 minutes) compared with standard activation of PKC, which is usually measured within minutes. These data conclusively demonstrate the specific utilization of PKCθ but not other PKC isoforms by the T cell in response to antigen presenting cell contact.

The response of PKCμ to BCR ligation with CD19 results in the phosphorylation and probable down regulation of Syk and PLCγ (Sidorenko et al., 1996). This report describes a situation in which a protein was identified in complex with the BCR, which exhibits increased activity of PKCμ after co-crosslinking the BCR and CD19. In addition, they provide *in vitro* data that Syk and PLCγ_1 may serve as preferential substrates for this PKC isoform, and that PKCμ causes downregulation of Syk's kinase activity on PLCγ_1. They further substantiate the upstream requirement for Syk in the activation of PKCμ using DT40 Syk$^{-/-}$ cells. Taken together, their results implicate PKCμ in a negative feedback role in response to BCR crosslinking. This study characterizes some of the upstream and

downstream connections of one PKC isoform, but did not compare PKCμ to any other PKC isoform.

Cardiac myocytes selectively use PKCε to protect them from hypoxia-induced cell death (Gray et al., 1997). This investigation from the Mochly-Rosen group elegantly demonstrates how, in response to hypoxia-induced preconditioning, PKCδ and PKCε become activated, but $PKC\beta_I$ does not. Of these three isoforms, only PKCε is capable of mediating long-term protection as a result of this preconditioning-induced activation. The basic premise of their experiment is that pre-starving myocytes of oxygen and glucose for 30 minutes causes an upregulation of a protective mechanism that functions as an effective damage-reducing agent in response to prolonged (9 h) hypoxia. Their protocol included loading myocytes with a selective peptide antagonist of PKCε:RACK binding (not PKCε translocation), which then abolished the protective effect of preconditioning, therefore directly implicating the activated PKCε:RACK interaction as the mechanism of protection.

Since the PKC family of kinases has been implicated in mediating a prominent phase of BCR-mediated signals (for review, see (DeFranco, 1997)), it was necessary to determine which representatives of the Groups of isoforms were present, in addition to update the current literature based on the discovery of additional isoforms, namely PKCμ and PKCθ. The possibility that the BCR could signal preferentially through one isoform over others was of great interest, analogous to the reported selective use of PKCθ in the T cell in response to antigen-presenting cell contact (Monks et al., 1997).

Materials and Methods

The cells used were Ramos lymphoblastoid B cells, collected during log-phase growth (0.6 to $1.6x10^6$/mL). Fresh cultures of Ramos cells were thawed every 6-8 weeks. Live

cells were counted with a Hausman Hemocytometer using the Trypan Blue dye exclusion assay (0.05% Trypan Blue in phosphate-buffered saline (PBS)).

Cell samples were prepared by centrifugation of cells out of culture media, followed by washing in PBS (1 mL/sample). Samples were then lysed in 500 μL of Lysis Buffer (0.5% Surfact-Amps X-100, 10 mM Tris (pH 7.5), 150 mM NaCl, plus freshly added 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM Na_2MoO_4, and 1 mM Na_3VO_4, 1 μg/mL aprotinin and 1 μg/mL leupeptin) and incubated on ice for 20-30 minutes. High purity, low carbonyl and peroxide Surfact-Amps X-100 was obtained from Pierce (active ingredient: Triton X-100). Nuclear fraction was cleared by centrifugation at 13 000 × *g* for 20-30 minutes in a Heraeus refrigerated centrifuge at 4°C. Cleared cell lysate was then decanted, and immunoprecipitation performed, if necessary (below). Alternatively, lysate was added to an equal volume of 2 × SDS sample buffer before boiling (100°C, 5 min) and loading onto 10% Acryl-Bisacrylamide resolving SDS-PAGE for separation followed by transfer to PVDF solid support (Immobilon P, Millipore). It was empirically determined that 1.25×10^6 cell equivalents per lane (ceq/lane) were required for detection of PKC isoforms ε, μ, and θ.

Cell Stimulation Cells were stimulated through crosslinking of the BCR, known to initiate a prominent tyrosine phosphorylation cascade (Gold et al., 1990). Pre-warmed cell samples (5×10^6 cells/sample) were gently shaken to resuspend settled cells, then rested for 2 minutes prior to addition of 20 μg anti-IgM (Jackson Immunochemicals, goat-anti-human IgM, $Fc_{5\mu}$-specific $F(ab')_2$) in an equal volume (125 μL) of modified HEPES-buffered saline (mHBS: 25 mM HEPES (pH 7.5), 125 mM NaCl, 50 μM β-mercaptoethanol, 2 mM GlutaMax (GibcoBRL), 5 mM KCl, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 0.5 mM $MgSO_4$, 1 g/L dextrose, 1 mM NaPyruvate) to ensure efficient sample mixing. Cell stimulation was stopped at exact timepoints (±1 s) by the immediate addition of 250 μL of ice-cold 2 × Lysis Buffer, placed directly on ice, and incubated at 0°C for 20-30 minutes to allow for complete membrane solubilization. Complete cell lysis was

confirmed by microscopy using the Trypan Blue Dye Exclusion assay. Cell lysates were cleared of detergent-insoluble fraction (nuclear and cytoskeleton) by centrifugation at 13 000 × *g* for 20-30 minutes at 4°C. Cleared lysates were transferred to clean, pre-chilled Eppendorf tubes for immunoprecipitation of the relevant isoforms.

Immunoprecipitation PKC isoforms were immunoisolated from cell lysates through the use of polyclonal antibodies against PKCα, β, and ζ (2-5 μL/ 500 μL; GibcoBRL), PKCμ (1 μg/ 500 μL; Santa Cruz Biotechnology, Inc.), and monoclonal anti-PKCδ (0.5 μg /500 μL; Transduction Labs), incubated for 6-12 hours at 4°C. Recovery of immune complexes utilized 25 μL slurry of recombinant Protein A-coupled Sepharose (Repligen) mixed 6-12 hours at 4°C. For monoclonal antibodies, rabbit-anti-mouse IgG (Southern Biotechnology Associates, Inc.) was pre-coupled to Protein A-Sepharose to enhance recovery. Sepharose immune complexes were washed thoroughly with Lysis Buffer (above) and PBS; 2 × SDS sample buffer was added directly to the beads, which were then boiled and proteins separated by SDS-PAGE as above.

Western Blotting Membranes were blocked overnight in ultrapure 5% BSA in tween-20, tris-buffered saline (TTBS) (containing 1 mM Na_3VO_4 for anti-phosphotyrosine blots). Immunoblotting of PKC isoforms utilized anti-PKCα, β, γ, ε, and ζ (GibcoBRL, diluted 1:500) or anti-PKC δ, μ, or θ (Transduction Labs, diluted 1:10 000) as the primary antibody, carried in 1% BSA in TTBS. Anti-phosphotyrosine immunoblots utilized 4G10 mAb (Upstate Biotechnology Incorporated, diluted 1:2000) carried in 1% BSA in TTBS supplemented with 1 mM Na_3VO_4. Secondary reagents (rabbit-anti-mouse IgG, Jackson Immunochemicals; Protein A, Bio-Rad), coupled to horseradish peroxidase were added to detect the primary antibodies using the Pierce Super Signal enhanced chemiluminescence (ECL) method and Kodak X-OMAT film. Standard Western blotting protocols were observed: 3 hour incubation with primary antibody, four changes of TTBS (15 minutes each), followed by 1 hour incubation with secondary reagent, and four changes of TTBS (15 minutes each) prior to ECL.

Results

Expression of PKC isoforms in B cells. The profile of PKC isozymes expressed in Ramos B cells was determined. Since PKC α, β, δ, ε, μ, and ζ have been reported to be expressed in B cells (Baier et al., 1994; Brick-Ghannam et al., 1994; Genaro et al., 1994; Mischak et al., 1993; Sidorenko et al., 1996), these isoforms, as well as the neuronal isoform PKCγ, and PKCθ, were tested for protein expression in Ramos cells by immunoreactivity. It was determined that PKC α, β, δ, ε, μ, θ and ζ are all expressed in this cell line, as shown in Figure 2-1. The specificity of immunoreactivity of each isoform against the commercially available antibodies was confirmed using the provided controls (not shown) or other antibodies, as in the case of PKCδ. Consistent with the reported expression of PKCγ being brain-specific (Brick-Ghannam et al., 1994), this isoform was not detected in B cells.

Tyrosine phosphorylation of PKC isoforms in B cells in response to BCR crosslinking. Selective tyrosine phosphorylation of one protein, but not others, in response to the stimulation of a specific receptor is one way that cells can demonstrate specificity of a cellular signaling response. Taking advantage of the readily available method of anti-phosphotyrosine immunoblotting allows us to determine if certain proteins are involved in the response to a specific receptor. The initial events in BCR signaling are known to involve the activation of a plethora of BCR-associated PTKs within 15 minutes of BCR ligation in addition to the lipid-modifying enzymes which activate PKC (Chen et al., 1986; Gold et al., 1992; Gold et al., 1994; Gold et al., 1993; Gold et al., 1990; Nel et al., 1986; Saouaf et al., 1994). Isoforms of PKC known to be tyrosine phosphorylated in other systems were evaluated in terms of their capability to become tyrosine phosphorylated in response to BCR crosslinking; the isoforms selected included representatives of each Group. The following isoforms were selected: PKCα, which becomes tyrosine

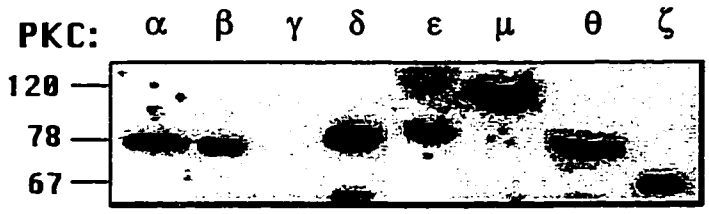


Figure 2-1. Isoforms of Protein Kinase C expressed in Ramos B cells. PKC isoforms (listed by symbol on top of the figure) were individually tested for expression by immunoreactivity to commercially available anti-PKC antibodies. PKC ε, μ, and θ, were detected in whole cell lysates (1.25×10^6 cell equivalents per lane); PKC α, β, δ, and ζ were detected following immunoprecipitation from 5×10^6 cells. PKCγ was not detected in 1.25×10^6 cell equivalents of Ramos B cells.

phosphorylated in response to insulin treatment (Liu and Roth, 1994) or H_2O_2 (Konishi et al., 1997); PKCδ, which becomes tyrosine phosphorylated in response to high-affinity IgE receptor (FcεRI) crosslinking (Haleem-Smith et al., 1995), carbachol, substance P, and PE (Li et al., 1994; Soltoff and Toker, 1995), v-Src overexpression (Zang et al., 1997), oncogenic RasHA expression (Denning et al., 1993), or epidermal growth factor (Denning et al., 1996); PKCμ which is basally tyrosine phosphorylated in B cells, and is reported to associate with the BCR (Sidorenko et al., 1996); and PKCζ, which was chosen to represent the atypical PKCs, and has been reported to become tyrosine phosphorylated in response to H_2O_2 treatment (Konishi et al., 1997). The choice of isoforms was also influenced by which isoforms could be immunoprecipitated by the commercially available isoforms.

Ramos B cells were stimulated by incubation with BCR crosslinking antibody for between 0 and 15 minutes, the cells were lysed, and then each of the selected PKC isoforms (α,δ,μ,ζ) were immunoprecipitated and the status of tyrosine phosphorylation determined by anti-phosphotyrosine immunoblotting. As shown by the anti-phosphotyrosine blot in Figure 2-2a, PKCδ, but not PKCα, μ, or ζ, was tyrosine phosphorylated within 30 seconds of BCR crosslinking, and maintained its tyrosine phosphorylated state for 15 minutes. BCR-associated PKCμ has been reported to be basally tyrosine phosphorylated (Sidorenko et al., 1996), however, this was not detected using the direct immunoprecipitation of whole cell PKCμ. Of some interest is the tyrosine phosphorylated band of ~70 kDa which was phosphorylated in response to BCR crosslinking (Figure 2-2a), and appears to be associated with PKCμ, but does not react with anti-PKCμ immunoblotting antibodies in independent experiments (not shown). Sidorenko, *et al*, (1996) suggest that this ~70 kDa protein may be Syk tyrosine kinase. It was further confirmed that PKCδ also became tyrosine phosphorylated from a basal unphosphorylated

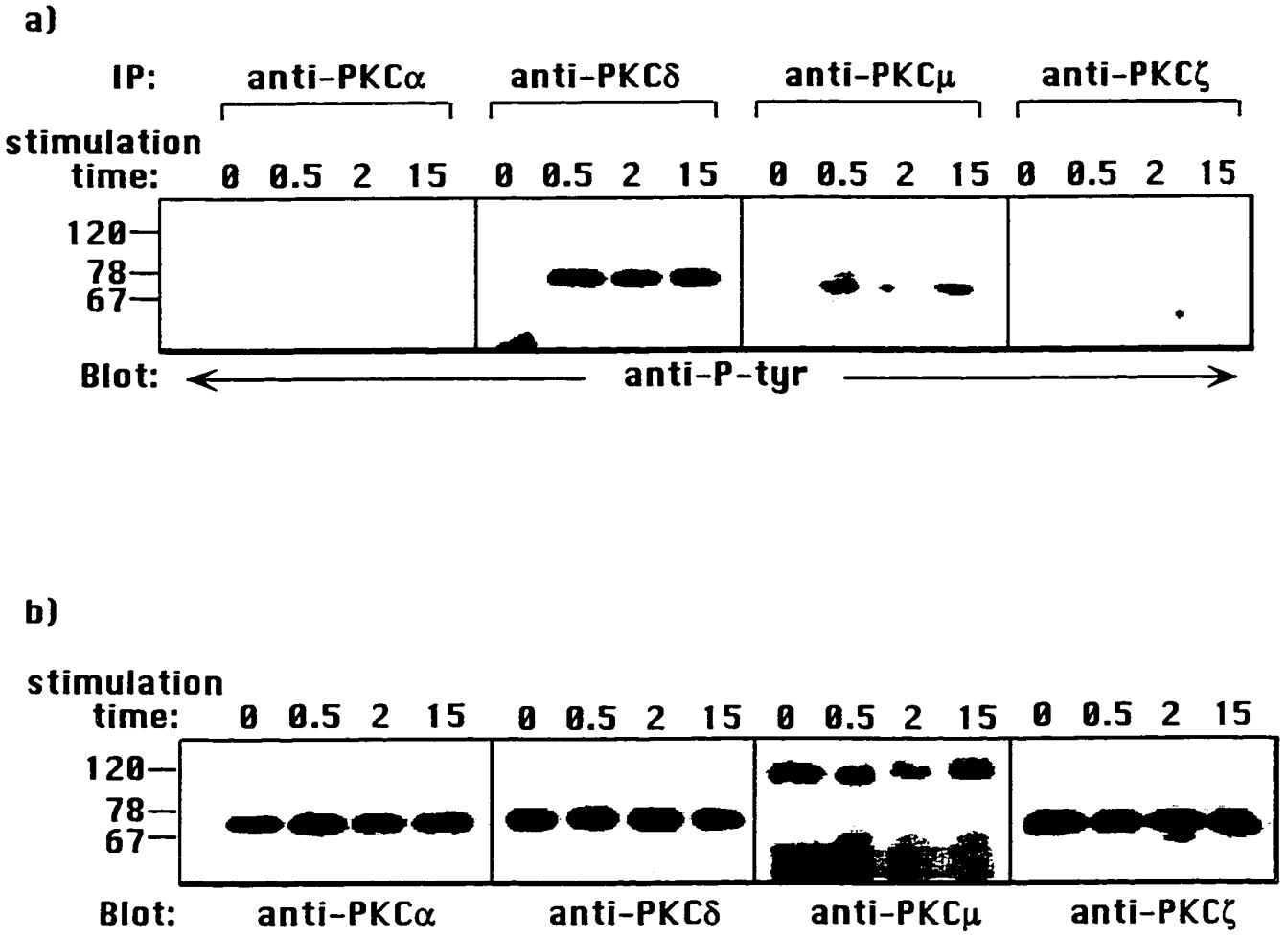


Figure 2-2. PKCδ, but not PKC α, μ, or ζ, becomes tyrosine phosphorylated in response to BCR crosslinking. a) anti-phosphotyrosine immunoblot of PKCα, δ, μ, and ζ immunoprecipitates following BCR crosslinking for the times indicated (in minutes). b) anti-PKC immunoblots of the same membranes, using isoform-specific antibodies listed. The bands appearing at approximately 50 kDa is attributable to heavy chains of the immunoprecipitating polyclonal antibody.

state in response to one minute of BCR crosslinking in human dense tonsillar B cells (data not shown).

Following the anti-phosphotyrosine blot, each membrane was reprobed with the immunoblotting antibody specific for that isoform, confirming the successful immunoprecipitation of each isoform of PKC. As is evident from Figure 2-2b, PKCα (77 kDa), PKCδ (78 kDa), PKCμ (115 kDa), and PKCζ (67 kDa) are all present in all four lanes corresponding to each immunoprecipitate. The bands appearing at approximately 50 kDa in the anti-PKCμ immunoblot are attributable to the heavy chains of the immunoprecipitating polyclonal antibody.

Discussion

PKC isoforms are expressed widely in both neuronal and hematopoietic tissues, with strong representation from novel isoforms in hematopoietic cell types. The expression of several PKC isoforms in B cells has been reported (Brick-Ghannam et al., 1994; Genaro et al., 1994; Sidorenko et al., 1996), therefore, for the purpose of this study, it was sufficient to confirm the presence of relevant isoforms by immunoreactivity with commercially available antibodies, as well as to investigate the expression of PKCθ.

Following the example set by the many groups who also have investigated specificity of PKC isoform function, this research was designed to investigate whether PKCδ was unique in its capacity to respond to the tyrosine phosphorylation cascade in response to BCR crosslinking. This was accomplished through the successful immunoprecipitation of three additional isoforms, namely PKCα, PKCμ, and PKCζ. None of these isoforms were observed to become tyrosine phosphorylated in response to BCR crosslinking, although all have been reported to have the capability to do so in other situations (Denning

et al., 1996; Haleem-Smith et al., 1995; Li et al., 1994; Liu and Roth, 1994; Soltoff and Toker, 1995).

It was indeed curious that PKCθ was detectable in Ramos B cell lysates (see Figure 2-1) especially in light of reports that the protein is predominantly expressed in T cells, and it has yet to be reported that PKCθ is expressed in B cells (Baier et al., 1994; Osada et al., 1992). The protein expression of PKCθ was investigated in Ramos B cells to address a report that low levels of mRNA for PKCθ have also been detected in B lymphocytes and B cell lines (Mischak et al., 1993). It was not possible to test the tyrosine phosphorylation status of PKCθ, however, because the highest quality antibody available against PKCθ did not successfully immunoprecipitate it from Ramos B cell lysate.

Selective tyrosine phosphorylation is a significant indicator of specificity in a whole cell system, as illustrated by the isoform preference for PKCδ in response to B cell stimulation through BCR crosslinking. Considering the wide distribution of isoforms of PKC in B lymphocytes, it is even more significant that the BCR would selectively promote the phosphorylation of PKCδ, and not PKCα, μ, or ζ, considering that all four isoforms are capable of being tyrosine phosphorylated (Denning et al., 1993; Denning et al., 1996; Haleem-Smith et al., 1995; Konishi et al., 1997; Li et al., 1994; Liu and Roth, 1994; Sidorenko et al., 1996; Soltoff and Toker, 1995; Zang et al., 1997). The possibility of tyrosine phosphorylation of other isoforms is further addressed in Chapter Five.

The material presented in this chapter clearly demonstrates that one single isoform of the PKC family can be selectively targetted by BCR-mediated signals. In summary, PKCδ, but not PKCα, μ, or ζ, responds to the BCR by becoming tyrosine phosphorylated.

Chapter Three

Tyrosine phosphorylation of PKCδ in response to BCR crosslinking.

Introduction

The PTK signaling cascade is crucial in mediating the cellular response of the lymphocyte upon BCR stimulation, as demonstrated by the $Lyn^{-/-}$, $Syk^{-/-}$, or $Btk^{-/-}$ systems (Hibbs et al., 1995; Takata and Kurosaki, 1995; Takata and Kurosaki, 1996). The implication that PKCδ could be involved as a target in such an essential signaling pathway generated significant interest in pursuing this phenomenon further. The initial discovery that prompted our interest in this field was one in which the tyrosine phosphorylation of PKCδ was sucessfully induced in response to BCR crosslinking. We observed that BCR crosslinking was exceedingly more effective at inducing tyrosine phosphorylation (within 30 seconds) compared to PMA (5 minutes) (see Figure 3-1) which was used as a control, reproduced from previously published results (Gschwendt et al., 1994; Soltoff and Toker, 1995).

Although PKCδ has been reported to be tyrosine phosphorylated (by PTKs) both *in vitro* and in whole cells, this thesis will focus only on whole cell systems, which may allow for a more realistic evaluation of the function of tyrosine phosphorylation of PKCδ in Ramos B cells. Review of whole cell systems in which PKCδ has been reported to become tyrosine phosphorylated reveals that PKCδ is dynamic in its tyrosine phosphorylation status (see below). To date, the consequence of tyrosine phosphorylation is unclear; however, many have come to the consensus that it downregulates PKCδ activity in many systems or situations.

At this point in the thesis, it is necessary to examine in closer detail several published reports of observed tyrosine phosphorylation of PKCδ. The following descriptions are

prioritized based on relevance to the Ramos B cell system of PKCδ stimulation, and are classified into two groups: endogenous whole cell systems, in which there is phosphorylation of PKCδ in response to receptor mediated stimulation, and transfected expression systems, in which there is phosphorylation in response to constitutively active cytoplasmic signaling components.

PKCδ becomes tyrosine phosphorylated in response to:

1. carbachol (via the muscarinic acetylcholine receptor) and substance P (via the substance P receptor) within 12 seconds of stimulation, and remains phosphorylated for at least 1 minute, in parotid acinar (salivary gland) cells (Soltoff and Toker, 1995),
2. activation of the high-affinity receptor for IgE (FcεRI) within 15 seconds of stimulation, and remains phosphorylated for at least 1 minute, in mast cells sensitized with IgE (Haleem-Smith et al., 1995),
3. epidermal growth factor (EGF) treatment of keratinocytes for 5 minutes, likely through a cytoplasmic PTK associated with the EGF receptor (Denning et al., 1996),
4. stimulation of the (transfected) platelet-derived growth factor β (PDGFβ) receptor with PDGF-ββ for 10 minutes in myeloid progenitor 32D cells (Li et al., 1994).

PKCδ is constitutively tyrosine phosphorylated when:

1. primary keratinocyte cultures are exposed to transforming growth factor α (TGF) for 4 days (Denning et al., 1996),
2. coexpressed with v-RasHA in keratinocytes (Denning et al., 1993),
3. coexpressed in v-Src-transformed fibroblasts (Zang et al., 1997),
4. COS-7 transfectants are treated with 5 minutes of oxidative stress by H_2O_2 (Konishi et al., 1997), implying a stress-activated protein kinase (SAPK) response (Duh et al., 1997; Zu et al., 1997).

The discovery that PKCδ could become rapidly and selectively tyrosine phosphorylated in response to BCR crosslinking provided the opportunity for investigation into a potentially specific mechanism of regulation of one isoform of PKC in response to the BCR.

Materials and Methods

Pharmacological cell treatment Ramos cells were collected and aliquotted to Eppendorf tubes for assay (see Chapter Two). 5×10^6 cells per sample were resuspended in 125 μL of mHBS including the following pharmacological compounds, where appropriate, or their common solvent, DMSO: 20 μM U73122 (Calbiochem; prevents agonist-induced activation of PLC), 50 nM wortmannin (Sigma; inhibits PI3'K activity), 100 μM PP1 unless otherwise stated (Calbiochem; inhibits PTK activity, reported to be Src-family preferential (Hanke et al., 1996)), 50 μM genistein (Sigma or Calbiochem, inhibits PTK activity), 2 μM bisindolylmaleimide I (Calbiochem; PKC selective inhibitor).

Cell Stimulation and Cell Lysis as described in Chapter Two. For PMA stimulation, 0.5 μL of 0.1 mM PMA in DMSO (200 nM) freshly dissolved in 125 μL mHBS was added to samples for 5 minutes at 37°C. Control samples for all experiments received mHBS alone, or appropriately diluted DMSO.

Lyn *in vitro* kinase assay To determine the activity of Lyn under various conditions, cells were pretreated with the appropriate pharmacological compounds, then stimulated as necessary and lysed. Lyn was immunoprecipitated using a polyclonal rabbit antibody, and supplied with 25 μL of a kinase reaction buffer containing 20 mM PIPES, pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 10 μCi [γ-^{32}P]ATP per sample. Samples were incubated 15 minutes at 37 °C before terminating reaction with 25 μL of 2 × SDS sample buffer. The supernatant was resolved by SDS-PAGE, and ^{32}P labeled proteins detected by autoradiography. Activity was correlated to the extent of autophosphorylation of Lyn.

Results

Tyrosine phosphorylation of PKCδ The first observation of BCR-crosslinking-induced tyrosine phosphorylation of PKCδ is depicted in Figure 3-1. For this preliminary experiment, samples of 2×10^7 Raji cells were stimulated by incubation with either 200 nM PMA for 5 minutes or 7.5 μg anti-IgM (Jackson) for 30 seconds. Cells were pelletted for 10 seconds at maximum speed in an Eppendorf centrifuge, then resuspended in Lysis Buffer containing a final concentration of 0.5% Triton X-100. PKCδ was then immunoprecipitated using the polyclonal Gibco anti-PKCδ antibody, followed by recovery with protein A-Sepharose. Immunoprecipitates were washed and resolved using 10% SDS-PAGE, as described in Chapter Two, then blotted with anti-phosphotyrosine. It was found that PKCδ became tyrosine phosphorylated in response to PMA as reported (Figure 3-1, lane 2) (Gschwendt et al., 1994; Soltoff and Toker, 1995), but became much more intensely phosphorylated in response to 30 seconds of BCR crosslinking (see Figure 3-1, lane 3). This result prompted the further investigation of the phosphorylation of PKCδ in response to BCR crosslinking. Continued studies with PKCδ were conducted using the Ramos lymphoblastoid cell line, which unlike many B cell lines, maintains a high expression of the BCR, and has a well characterized BCR response (Chaouchi et al., 1995; Ingham et al., 1996; Kaptein et al., 1996; Padmore et al., 1996; Valentine and Licciardi, 1992). Using the Ramos cell line, the conditions of stimulation were determined and are described in Chapter Two.

Since the rest of the studies described in this thesis focused on PKCδ, it was necessary to obtain a reliable monoclonal antibody against PKCδ which would both immunoprecipitate and immunoblot. The mAb commercially available from Transduction Labs was selected. As depicted in Figure 3-2, the specificity of Transduction Labs anti-PKCδ was confirmed by testing the PKCδ immunoprecipitate against two other antibodies, GibcoBRL anti-PKCδ, and a rabbit polyclonal anti-PKCδ generously provided by Dr. Nigel Groome

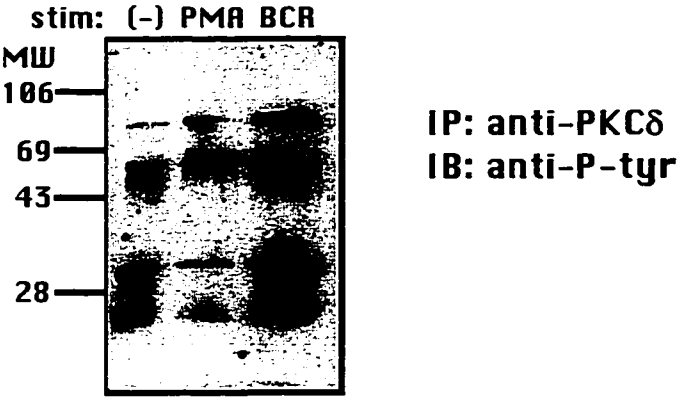


Figure 3-1. Tyrosine phosphorylation of PKCδ. PKCδ (78 kDa) becomes tyrosine phosphorylated in response to PMA treatment or BCR crosslinking in 2×10^7 Raji lymphoblastoid B cells. The bands appearing at approximately 50 kDa can be attributed to the heavy chains of the immunoprecipitating antibodies; the bands at approximately 25 kDa can be attributed to the light chains of the immunoprecipitating antibodies.

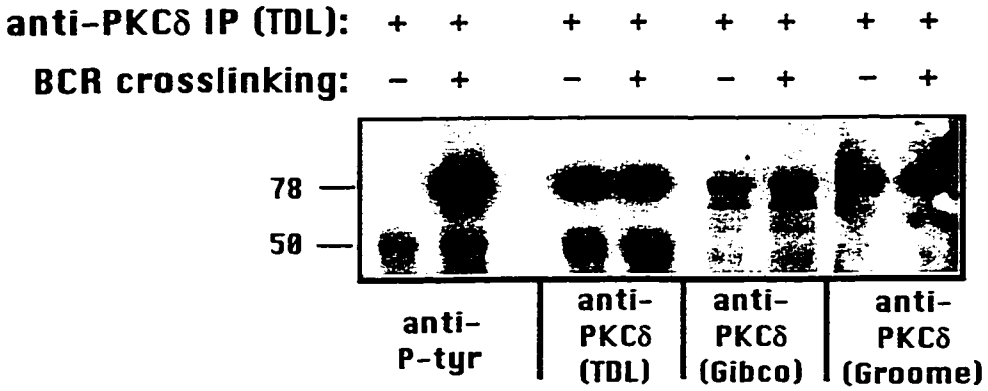


Figure 3-2. Specificity of antibodies against PKCδ. Following stimulation through BCR crosslinking for 30 seconds, PKCδ was immunoprecipitated by anti-PKCδ (Transduction Labs), then blotted with either antiphosphotyrosine, or anti-PKCδ from three different sources.

(Brookes University, Oxford). The tyrosine phosphorylation of PKCδ induced by BCR crosslinking was used as a control. It was determined that anti-PKCδ (TDL) immunoprecipitated a protein of 78 kDa which reacted to all three anti-PKCδ blotting antibodies, thus demonstrating that PKCδ was indeed being immunoprecipitated.

The kinetics of tyrosine phosphorylation of PKCδ were examined. Ramos B cells were stimulated by BCR crosslinking and stimulation was stopped by lysis precisely at each timepoint, prior to PKCδ immunoprecipitation. Following separation by standard SDS-PAGE, Western blotting was performed on resolved PKCδ immunoprecipitates. Anti-phosphotyrosine blotting demonstrated that PKCδ was tyrosine phosphorylated as early as 7 seconds following BCR crosslinking, peaked at approximately 15 to 22 seconds (see Figure 3-3a), then decreased to an intermediate level. The tyrosine phosphorylation of PKCδ was then sustained from 60 seconds to at least 30 minutes (see Figure 3-3b); thereafter, the tyrosine phosphorylation of PKCδ was seen to very gradually decrease over the next four hours (see Figure 3-3c). Anti-phosphotyrosine blotting of 5×10^6 unstimulated Ramos cells (0 seconds) was consistently below the level of detection; note that the unstimulated sample in Figure 3-1 represents PKCδ immunoprecipitated from 2×10^7 Raji cells. Anti-PKCδ immunoblots of an identically prepared parallel set of samples confirmed that an approximately equivalent amount of PKCδ was immunoprecipitated at each time point up to four hours (data not shown). The rapid phosphorylation of PKCδ in response to BCR crosslinking is consistent with the times of activation of BCR-associated Src-family tyrosine kinases Lyn and Blk, which are reported to become active within 5 seconds of stimulation (Saouaf et al., 1994).

Factors influencing the tyrosine phosphorylation of PKCδ To rule out any non-specific tyrosine phosphorylation of PKCδ after cell lysis, as well as to confirm tyrosine specificity of the phosphorylation induced on PKCδ, Ramos B cells were pre-

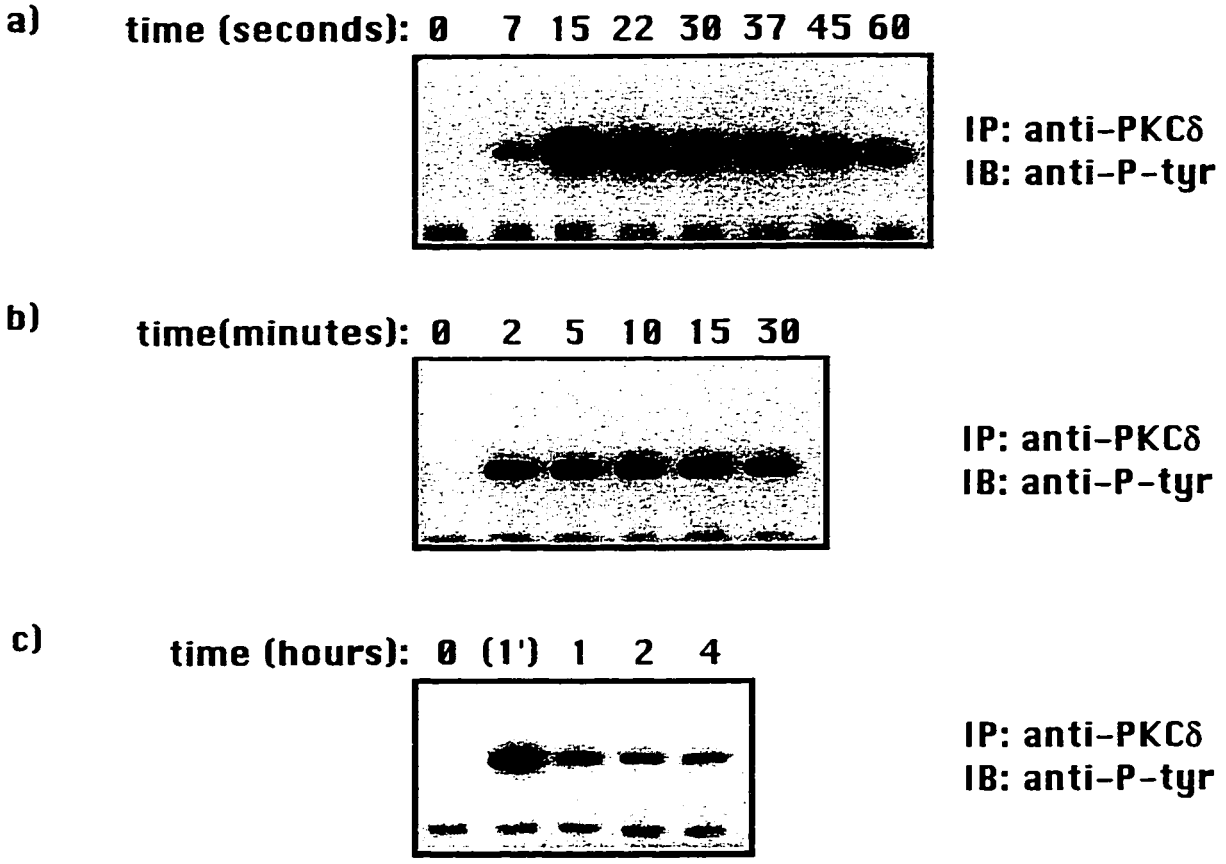


Figure 3-3. Kinetics of tyrosine phosphorylation of PKCδ in response to BCR crosslinking. Ramos B cells were stimulated by BCR crosslinking for the times indicated, then PKCδ immunoprecipitated and subjected to antiphosphotyrosine immunoblotting.

incubated with PTK inhibitors. Comparing the effectiveness of a recently developed PTK inhibitor, PP1 (Hanke et al., 1996), against other inhibitors produced an interesting result, shown in Figure 3-4. As depicted, pretreatment of Ramos cells with 10 μM PP1 prior to either PMA treatment (P) or 30 seconds of BCR-crosslinking (M) significantly reduced the tyrosine phosphorylation of PKCδ more effectively than genistein obtained from two independent sources (Calbiochem, GEN1; Sigma, GEN2) at equivalent concentrations.

A dose titration experiment was performed to determine the concentration at which PP1 would effectively prevent the tyrosine phosphorylation of PKCδ in whole cells. As is shown in Figure 3-5, the tyrosine phosphorylation of PKCδ was significantly reduced by 50 μM PP1, pre-incubated with the Ramos cells for 30 minutes before 30 seconds of BCR crosslinking. This was considered reasonable, since the IC_{50} of PP1 on tyrosine kinase activity *in vitro* has been reported to be in the 1-5 μM range (Hanke et al., 1996).

Since BCR crosslinking was so effective at promoting the tyrosine phosphorylation of PKCδ, it was necessary to find which of the BCR-associated signal transduction pathways were participating in the tyrosine phosphorylation of PKCδ. To establish the factors contributing to the tyrosine phosphorylation of PKCδ, three of the major signaling pathways activated by the BCR were evaluated in terms of their effects on PKCδ tyrosine phosphorylation. This was accomplished by treating B cells with the pharmacological agents described above (U73122, wortmannin, and PP1) before stimulation. Ramos B cells were pretreated for 30 minutes with the pharmacological agent, stimulated by BCR crosslinking for times indicated in Figure 3-6, then lysed. PKCδ was then immunoprecipitated and the state of tyrosine phosphorylation was evaluated by anti-phosphotyrosine immunoblotting. The tyrosine phosphorylation induced on PKCδ as a result of its activation by PMA was included as a constant internal control for anti-phosphotyrosine immunoblotting (Gschwendt et al., 1996; Soltoff and Toker, 1995).

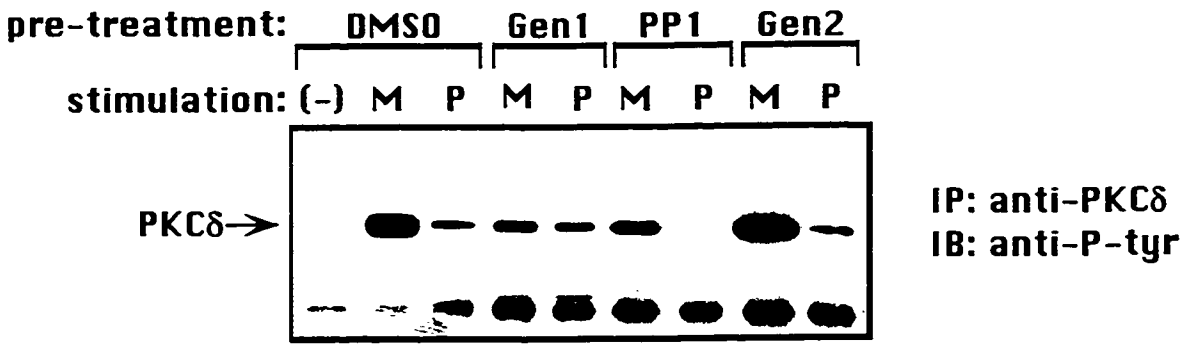


Figure 3-4. Selectivity of PTK inhibitors for PKCδ tyrosine phosphorylation. PP1 (10 μM) is more effective at preventing the tyrosine phosphorylation of PKCδ induced in response to PMA (P) or BCR crosslinking (M) than genistein (also 10 μM obtained from two sources; GEN1, Calbiochem; GEN2, Sigma.

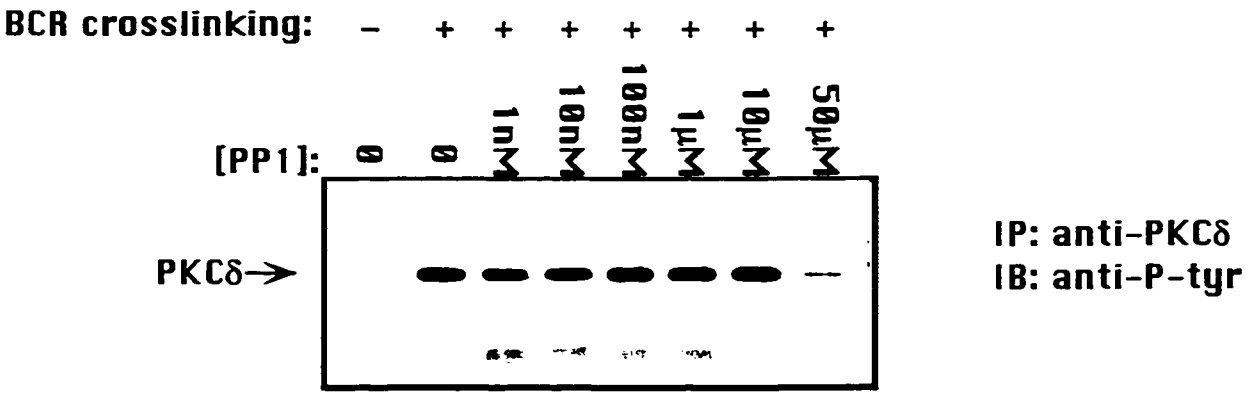


Figure 3-5. Dose/response of PP1 inhibition of BCR crosslinking-induced tyrosine phosphorylation of PKCδ. Ramos B cells were preincubated with the doses of PP1 listed above for 30 minutes prior to 30 seconds of BCR crosslinking.

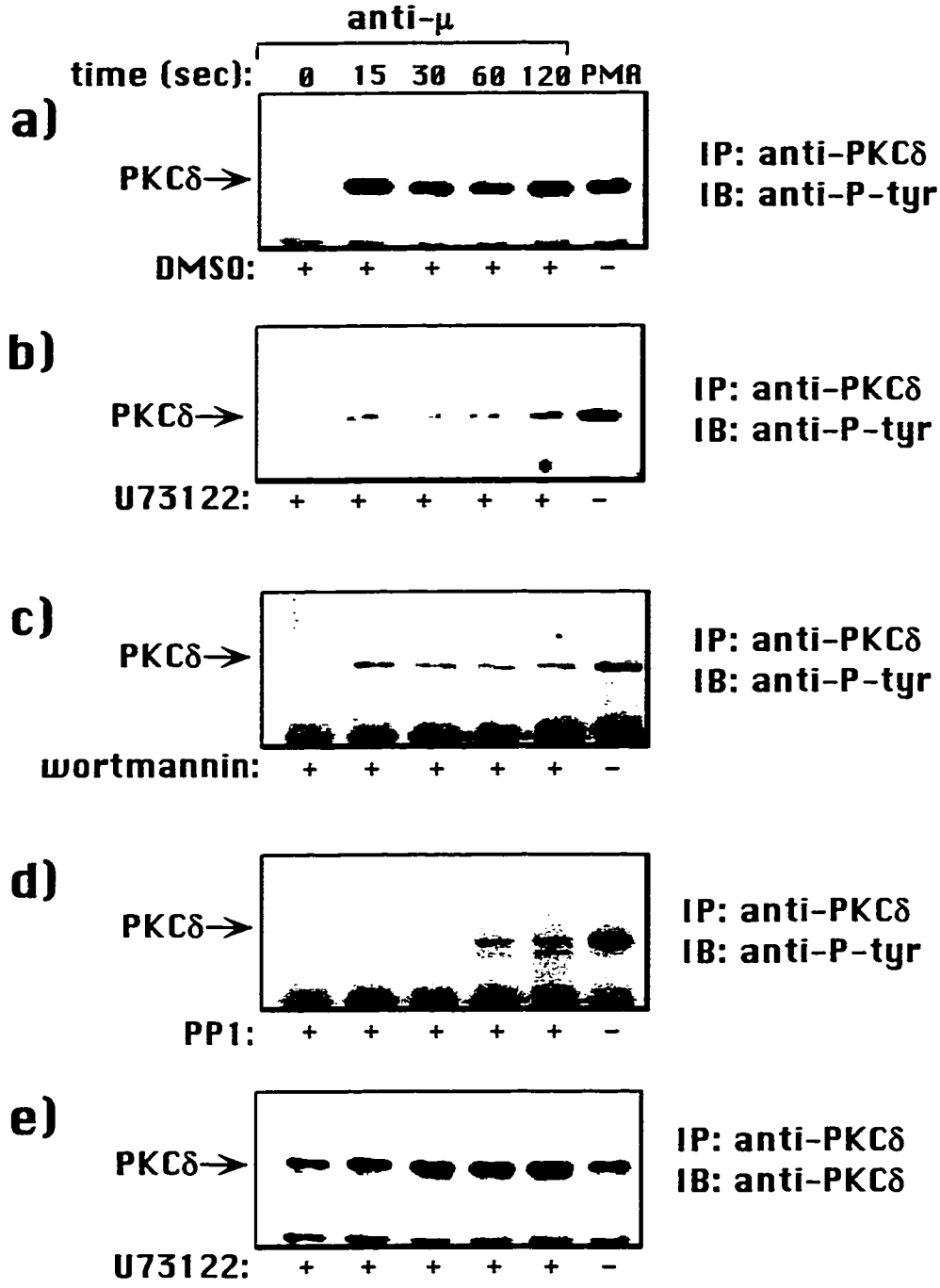


Figure 3-6. Factors contributing to the tyrosine phosphorylation of PKCδ. Ramos B cells were incubated with the compounds listed for 30 minutes prior to BCR crosslinking (see text).

Each experiment was also controlled by an identically prepared set of samples pretreated with DMSO. A typical experiment is shown in Figure 3-6 a and b. Consistent with previous observations, BCR crosslinking induced the rapid tyrosine phosphorylation of PKCδ (a). However, when U73122 was included during the pre-incubation to prevent PLC activation, the early tyrosine phosphorylation of PKCδ was attenuated, as shown in (b). The contribution of the PI3'K pathway to the tyrosine phosphorylation of PKCδ was investigated in a similar manner using wortmannin, as shown in (c). As evident from the immunoblot, the tyrosine phosphorylation of PKCδ was substantially reduced in response to BCR crosslinking, when the samples were pre-incubated in the presence of wortmannin. Control samples (pretreated with DMSO) prepared for the experiment shown in (c) portrayed identical results to those obtained in (a).

As demonstrated in Figure 3-5, PP1 inhibits the tyrosine phosphorylation of PKCδ in response to BCR crosslinking. Here, it was necessary to demonstrate that PP1 selectively inhibited the tyrosine phosphorylation of PKCδ over the entire timecourse investigated. To ensure complete inhibition of the tyrosine phosphorylation, PP1 was used at 100 μM. As is evident from (d), this compound prevented the tyrosine phosphorylation of PKCδ in response to BCR crosslinking. Finally, to confirm the presence of PKCδ in all lanes, the membrane from (b) was reprobed with anti-PKCδ, as depicted in (e).

Inhibition of PKC activity enhances the tyrosine phosphorylation of PKCδ. The induction of tyrosine phosphorylation of PKCδ by phorbol esters (Gschwendt et al., 1994; Soltoff and Toker, 1995) (PMA lane, Figure 3-6 a to d), which activate PKCδ directly, and the effect of U73122 and wortmannin on activation and tyrosine phosphorylation of PKCδ (see Figure 3-6 b and c, and Chapter Four), suggests that activation of PKC may be required for its tyrosine phosphorylation. However, pretreatment of B cells with the selective PKC inhibitor bisindolylmaleimide I prior to

stimulation with either BCR crosslinking or with PMA resulted in enhanced tyrosine phosphorylation (see Figure 3-7a). This suggests that it is not PKCδ activity *per se* which promotes its own tyrosine phosphorylation. It is known that PKC inhibitors such as bisindolylmaleimide I act through binding irreversibly to the ATP-binding site in the catalytic domain of PKC, which is believed to change the conformation to resemble catalytically active PKC (Bradshaw et al., 1993; Kiss et al., 1995; Muid et al., 1991). Thus, we speculate that it is the state of active conformation of PKCδ which increases its substrate potential. The possibility that the increase in tyrosine phosphorylation of PKCδ may be due to the lack of down-regulation of PTKs by serine or threonine phosphorylation was considered (Gold et al., 1994). To address this, the Src-family kinase which was considered to be the most likely candidate for the *in vivo* tyrosine phosphorylation of PKCδ was assayed for its activity in response to bisindolylmaleimide I pretreatment. Pretreatment of Ramos B cells with bisindolylmaleimide I prior to PE stimulation to activate PKC showed no discernible effect on the *in vitro* kinase activity of the Src-family PTK lyn (see Figure 3-7b).

It was necessary to substantiate that the observed effects of the pharmacological compounds (U73122, wortmannin, and bisindolylmaleimide) on the tyrosine phosphorylation of PKCδ were not due to a change in the activity of PTKs in the B cell. To accomplish this, a control experiment was performed in which the autophosphorylation of Lyn was evaluated in samples which had been pretreated with these compounds. Compared to a DMSO-pretreated sample, incubation of Ramos cells with either U73122, wortmannin, or bisindolylmaleimide did not result in decreased activity of Lyn (data not shown). It was further determined that incubation of Lyn immunoprecipitates *in vitro* with either U73122, wortmannin, or bisindolylmaleimide did not substantially inhibit the activity of Lyn *in vitro*, whereas incubation with the PTK inhibitor PP1 substantially attenuated the

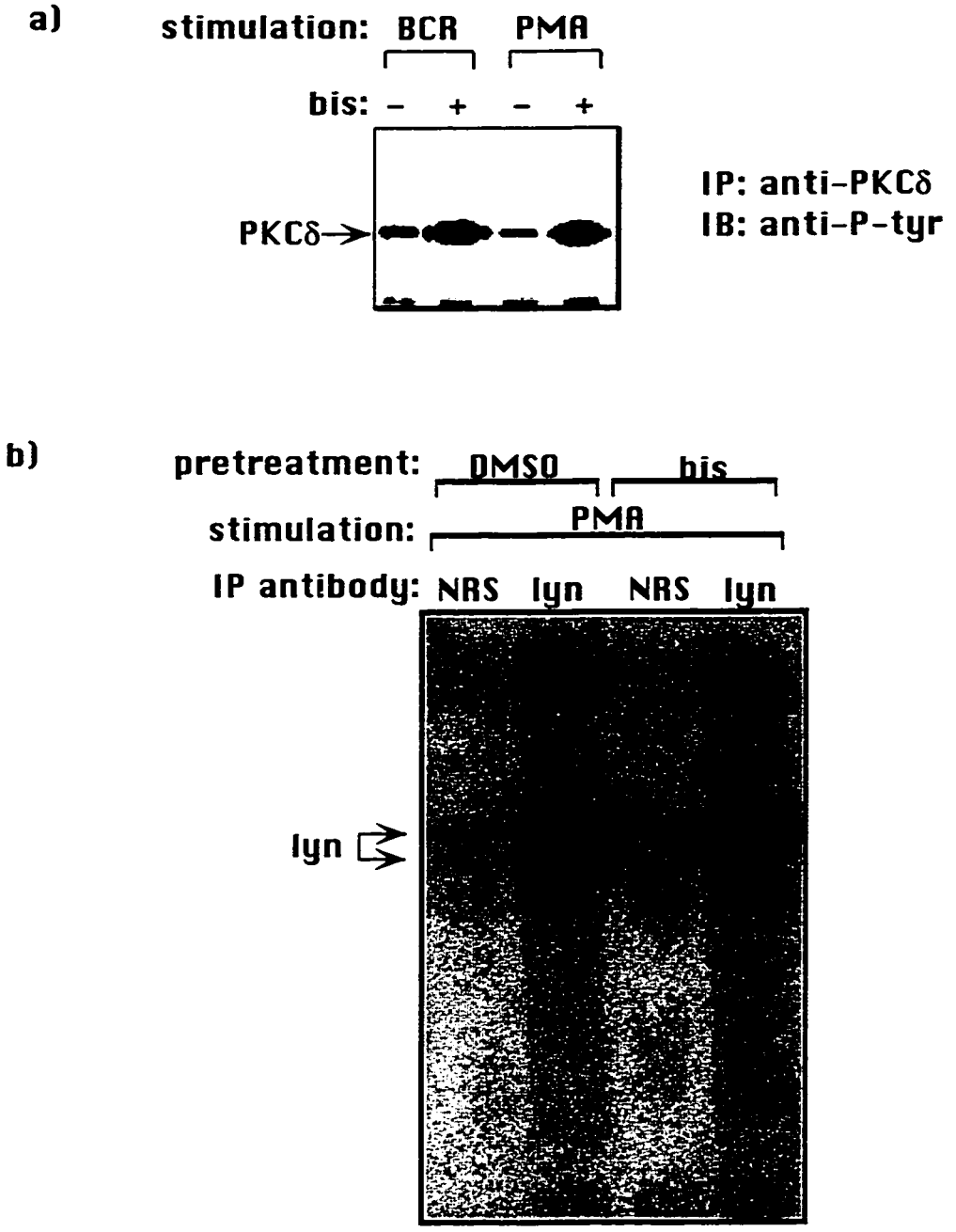


Figure 3-7. Effects of bisindolylmaleimide on PKCδ phosphorylation and Lyn activity. a) Ramos B cells were pretreated with bisindolylmaleimide prior to stimulation with PMA or by BCR crosslinking for 30 seconds. The tyrosine phosphorylation status of PKCδ was determined by antiphosphotyrosine blotting. b) Ramos B cells were pretreated with bisindolylmaleimide prior to PMA stimulation. Lyn activity was determined by *in vitro* kinase assay using [γ◆^{32}P]ATP and autoradiography.

autophosphorylation of Lyn in this *in vitro* kinase system, demonstrating the efficacy of that compound at preventing PTK activity (data not shown).

Discussion

Clearly, PKCδ undergoes tyrosine phosphorylation and dephosphorylation in response to BCR crosslinking. The focus of this chapter has been to describe this phenomenon in light of the known pathways of signaling originating with the BCR. It was found that the PTK, PLCγ, and PI3'K pathways all contributed to the tyrosine phosphorylation of PKCδ. Further, this is dependent on the active conformation of the enzyme, as demonstrated by the data in Figure 3-7a. This discussion will focus on the different aspects of the stimulation and tyrosine phosphorylation of PKCδ in response to BCR crosslinking, then comment on the conditions which regulate it. Discussion relevant to the functional consequences of tyrosine phosphorylation are addressed in Chapter Five.

The stimulation of the B cell through antigen receptor crosslinking was designed to mimic the process by which a B cell would 'see' antigen, and is widely used in the field (Gold et al., 1990; Haggerty and Monroe, 1994; Kaptein et al., 1996; Karras et al., 1996; Sarmay et al., 1996; Takata et al., 1994; Yamanashi et al., 1992; Yang and Desiderio, 1997). One limitation of the technique of cell stimulation as described is the use of the high-affinity anti-IgM $F(ab')_2$ antibody used to crosslink the BCR. This does not necessarily reflect the physiological method of stimulation through the BCR, as the affinity of IgM interactions with antigen are known to be several orders of magnitude lower. It does, however, allow for multiple 'capping' of surface IgM molecules, as would be expected with a polyvalent antigen interacting with the B cell. Indeed, when tonsillar B cells were available, the experiment was performed to determine if PKCδ also became rapidly tyrosine phosphorylated in response to BCR crosslinking of the antigen receptor on heterologous B

cell population, and in fact it became phosphorylated within 30 seconds of stimulation (not shown).

The PTK(s) which performs the duty of tyrosine phosphorylation of PKCδ in the B cell has not yet been identified. However, some evidence presented in this chapter provides an interesting avenue for investigation. PP1 has been reported to be selective for Src-family tyrosine kinases, while genistein is more nonspecific, and inhibits many non-Src-family tyrosine kinases. This also provides evidence to support the theory that it is in fact a Src-family tyrosine kinase such as Lyn which actually functions to tyrosine phosphorylate PKCδ in the B cell. Further, the primary event of BCR ligation involves the activation of Src-family members (see Figure 1-2), thus a Src-selective inhibitor may block all receptor mediated signals. This issue will be further addressed in Chapter Five.

The relative level of tyrosine phosphorylation as depicted in Figure 3-3 raises interesting questions regarding the role of tyrosine phosphorylation of PKCδ in the B cell. As others report (Denning et al., 1996; Haleem-Smith et al., 1995; Li et al., 1994; Soltoff and Toker, 1995), there is a very dynamic response of tyrosine phosphorylation, which rapidly increases above undetectable basal levels to peak by 15 to 22 seconds, then decreases by 60 seconds. The previously unreported finding that PKCδ can be maintained in a constant state of tyrosine phosphorylation for greater than 4 hours may be evidence of protection from dephosphorylation of at least one tyrosine phosphorylation site, possibly by a PKCδ binding protein. This is further addressed in Chapter Five.

In light of the fact that there are twenty tyrosines in the sequence of human PKCδ (Aris et al., 1993), it is very difficult to make interpretations regarding either the stoichiometry or function of tyrosine phosphorylation in a physiological setting. The four tyrosines which have been reported to be phosphorylated in various cellular systems may not even

necessarily be those tyrosines which are being tyrosine phosphorylated in response to BCR crosslinking. In any case, the stoichiometry of tyrosine phosphorylation of PKCδ was attempted, in an effort to determine approximately how many sites per PKCδ molecule were being phosphorylated, both at peak levels, and during the maintained phase of tyrosine phosphorylation. This was performed by stimulating B cells through BCR crosslinking, then immunoprecipitating tyrosine phosphorylated proteins with 4G10 mAb, followed by anti-PKCδ immunoblotting. These experiments were unfortunately not successful, using an immunoprecipitation and immunoblot approach, possibly due to the large proportion of tyrosine phosphorylated proteins in B cell lysates, which could outcompete PKCδ for the phosphotyrosine binding sites of the 4G10 antibody. The purpose of this approach was to determine in a semi-quantitative fashion the proportion of the entire PKCδ population that becomes tyrosine phosphorylated in response to BCR crosslinking. If it was considerably less than the proportionate increase in tyrosine phosphorylation, a conclusion could have been drawn that PKCδ was being phosphorylated on multiple sites, rather than on just one.

In conclusion, the unique profile of tyrosine phosphorylation of PKCδ provides an excellent opportunity for investigation into the selective use of this isoform by the B cell in response to BCR crosslinking.

Chapter Four

Activation of PKCδ in response to BCR crosslinking

Introduction

The observation that PKCδ could become rapidly tyrosine phosphorylated in response to BCR crosslinking, and the well known fact that PKC participates downstream of BCR signaling (Chen et al., 1986; Foote et al., 1996; Kim et al., 1992; Muthukkumar et al., 1993; Nel et al., 1986), raised the immediate question: what are the effects of BCR crosslinking on PKCδ activity? This chapter is dedicated to the activation of PKCδ in response to BCR crosslinking; a discussion on the interaction between tyrosine phosphorylation and activation will follow in Chapter Five.

Two standard methods of evaluating the activation of PKC in response to cellular stimulation predominate in the literature. The first involves measuring the translocation of PKC from a cytosolic environment to membrane compartments, and is either measured by cell fractionation procedures or immunofluorescent confocal microscopy; examples of each are described in (Keenan et al., 1997; Kent et al., 1996; Monks et al., 1997). This system of measurement relies on the principle that PKC must physically redistribute from an aqueous cellular environment to become associated with the necessary lipid cofactors present in biological membranes. Recent studies with green fluorescent protein (GFP)-tagged PKC in fibroblasts have demonstrated that this process is extremely fast, and can occur within a 10 to 30 second timeframe (Dr. Y. Nishizuka, Keystone Symposia, March 1998). The cell fractionation method of activity correlation was considered and attempted, but was not successful in this system of analysis. We believe this to be due to the fact that in our system, stimulation is accomplished by ligation of an high-affinity antibody to the surface BCR molecules on the B cells and must be stopped by cell lysis (see Chapter Two), while in other systems (Kent et al., 1996), using either peptide hormones or neuroagonists,

stimulation can easily be stopped by diluting out the stimulating agent, then proceding with cell fractionation in isotonic buffer (Kent et al., 1996; Soltoff and Toker, 1995). The next commonly used procedure involves the purification of PKC out of the cellular system, then assaying for histone- or myelin basic protein (MBP)-phosphorylation, or phorbol ester-inducible activity *in vitro*, examples of which are described in (Johannes et al., 1995; Li et al., 1994; Sidorenko et al., 1996). It was decided to use this approach as the basis of the assay to evaluate the activation of PKCδ in response to the BCR, because it would accommodate both quantitation of activity and the short time intervals necessary to evaluate immediate activation of PKC.

Methods and Materials

Cell stimulation and Cell Lysis 5×10^6 prewarmed Ramos cells in 125 µL of mHBS were gently shaken to resuspend settled cells, then rested for 2 minutes prior to addition of 20 µg of anti-IgM added in an equal volume (125 µL) of mHBS. Control samples for all experiments received mHBS alone. Cell stimulation was stopped at exact timepoints (±1 s) by the immediate addition of 250 µL of ice-cold 2 × Lysis Buffer. Samples were then placed directly on ice, and incubated at 0°C for 20-30 minutes. Cell lysates were cleared by centrifugation at 13 000 × *g* for 20-30 minutes at 4°C. Cleared lysates were transferred to new, pre-chilled Eppendorf tubes. PKCδ was then immunoprecipitated following the procedure outlined in Chapter Two.

***In vitro* kinase assay** Immunoprecipitated PKCδ was washed 3 times with 500 µL Lysis Buffer then 2 times with 500 µL Buffer K (20 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 10 µg/mL leupeptin, 10 mM EGTA) before addition of the four additional kinase assay ingredients. Each sample received: **(1)** 16 µL 1.5 × [Buffer K], **(2)** 8 µL PSTX (0.5 mg/mL PS sonicated in 0.1% Triton X-100), and **(3)** 1 µL of 1 mM PKCε-derived peptide substrate. Prepared samples were warmed to 37°C for 15 minutes prior to addition of **(4)** 1.0 µL 2.5 mM ATP containing 5-10 µCi [γ-^{32}P]ATP per sample (~500 cpm/pmol)

to initiate the reaction. After allowing the kinase reaction to proceed at 37°C for exactly 2 minutes, phosphoryl transfer was stopped by the addition of 4 μL of 0.5 M EDTA, and the supernatant portion of the reaction was transferred to dry P81 phosphocellulose peptide binding paper (Whatman). Unbound [γ-^{32}P]ATP was washed away with 2 × 500 μL 0.5% H_3PO_4 per sample, followed by 4 × 150 mL bulk washes in 0.5% H_3PO_4. Phosphorylated peptide, immobilized on P81 using a Bio-Dot apparatus, was observed and quantitated using a Storm Phosphoimager (Molecular Dynamics) and a Kodak Storage Phosphor Screen. Given the short reaction time and the vast excess of substrate, the amount of phosphorylated peptide is directly proportional to the amount of activated PKCδ in the assay.

Statistical Analysis of Quantitative Data

With all of the quantitative data collected on the activation of PKCδ in response to BCR crosslinking, it was imperative to perform statistical tests to establish the reproducibility of the data. The book "Mathematics and Statistics for the Bio-Sciences" (Eason et al., 1980) was consulted as a guide for all statistical enquiry. For the purposes of this thesis, a brief description of the statistical analysis will be given, followed by the results of the statistical tests performed on the data (summarized in Table 4-1).

In the field of statistics, a **sample** is defined as the sum of the data collected for one specific set of conditions, for example, anti-IgM stimulated PKCδ activity at the 30 second timepoint. A sample includes all experiments in which the conditions were identical, therefore, is comprised of several data points, each representing a measurement from an experiment conducted on a particular day. A **group** of samples is defined as being the data from related samples, treated under the same conditions except for one variable (usually time), for example, all experiments involving anti-IgM stimulated PKCδ activities from time zero to 60 seconds. **Paired samples** are samples in which each data point was collected in parallel with another related data point, and only differ by one condition, for example, anti-IgM stimulated PKCδ activity at the 30 second timepoint, with and without

the presence of U73122. Paired samples are compared statistically by each set of data points (in other words, experiment by experiment), and generally reflect a higher degree of variability in the data, because in this study only 3 to 9 data points were collected per sample. Higher statistical accuracy is observed when all identical experiments are grouped into one sample. Statistical analysis of quantitative data is accomplished by asking statistical questions in the form of a mathematical test, regarding the distribution of the data in a sample, compared to itself and other samples. Depending on the organization of the data, several different types of statistical questions can be raised. For the purposes of this study, the tests that were appropriate included: the Analysis of Variance (AnOVa) test, which tests groups of samples against each other; the Mann Whitney test, which compares two samples to each other; the Wilcoxon Ranked Signs test, which compares paired samples to each other; and the binomial signs test, which evaluates a single sample in terms of a standard value. The answers to statistical tests are usually given as a probability (p) value which describes the probability with which the data would agree with the null hypothesis purely by chance (see below).

The procedure for conducting a statistical test is outlined below, adapted from (Eason et al., 1980), with practical relevance to this thesis in parentheses:

1. Choose whether or not the data fall into a Gaussian distribution. (A general rule is that more than 30 data points per sample is usually Gaussian, and is treated with 'parametric' statistical tests; less than 30 data points is usually considered non-parametric, consequently, the appropriate statistical tests make no assumption regarding the distribution of the data.)
2. State the null and alternative hypotheses. (The null hypothesis states that two samples are identical in mean and distribution, although they may appear to be different; for example, that anti-IgM stimulated and unstimulated samples have the same PKCδ activity. The purpose of most statistical tests is to prove the null hypothesis wrong, rather than to prove the difference between samples. The alternative hypothesis is

usually that the two samples are not the same, and is accepted when the null hypothesis is proven wrong.)

3. Choose the significance level of the test. (A significance level of 0.05 (5%) means that the results would conform to the null hypothesis purely due to chance 5% of the time.)
4. Choose the statistical test which best tests the variance of the data. (For non-parametric data with one sample, the binomial test; for two samples, the Mann Whitney (non-paired) or Wilcoxon (paired); for three or more, the AnOVa.)
5. Calculate the sample mean, median, rank and variance. (The InStat program automatically performs these calculations.)
6. Calculate the test statistic. (The InStat program automatically performs these calculations.)
7. Convert the test statistic into an acceptance (or rejection) value based on the charts which define the integral area under the curve below. (The InStat program also calculates an exact p value.)
8. State hypothesis to be accepted, based on the probability of the null hypothesis. (This is usually assumed, based on whether the p value falls below the confidence value, for example, if $p<0.05$).

Test statistic values (calculated to describe the distance from mean distribution) are converted to a p value based on the area of the critical region, that is, the area under the Gaussian curve (total area equal to 1.000) which outlies the test statistic. The p value can be interpreted to mean the proportion of outcomes which would NOT agree with the observed outcome (for the null hypothesis, the probability that samples would be identical purely by chance). The statistical questions that were necessary to ask in order to evaluate the data are listed in Table 4-1, with their results in terms of p values. This table describes which sets of data are significantly different from each other, and can be effectively used to comment on the relationships between samples. The following is a brief description of the results of the statistical testing.

In Table 4-1a, the AnOVa results confirm that within the group of stimulated samples (t=0 to 60 seconds), there is an extremely improbable chance that all samples are alike with respect to time of stimulation ($p<0.0001$). Also, it confirms that within the group of unstimulated samples, there is a high probability that these samples are the same ($p=0.9912$), irrespective of time of mock stimulation. The interpretation that we can gain from this test is that there is no time-dependent stimulation of unstimulated samples, however, there is a time-dependent difference between anti-IgM stimulated samples. The p values comparing stimulated with unstimulated samples at each time point demonstrate exactly which stimulated *versus* unstimulated samples are significantly different from each other, namely 20 and 30 seconds ($p=0.0007$ and 0.0209 respectively).

The comparison between stimulation in the presence or absence of U73122 is described in Table 4-1b. As described above, the AnOVa results demonstrate that in this case, stimulated samples treated with either DMSO or U73122 both contain significantly different samples within their respective groups. This is further confirmed by the fact that there is not a high degree of significance at the 20 and 30 second timepoints ($p=0.1905$ and 0.6625). The same comparison is drawn between samples treated with DMSO and wortmannin (Table 4-1c). As is evident from AnOVa results, samples treated with wortmannin do not exhibit significance difference compared to each other ($p=0.4195$). The reason for this result is evident when the two samples are evaluated, especially at the 20 and 30 second timepoints (paired, $p=0.0156$, $p=0.01565$; unpaired, $p=0.0003$, $p<0.0001$, respectively). A very similar result is observed in the comparison between samples treated with DMSO and those treated with PP1 (Table 4-1d). There is no significant difference between PP1-treated samples at different timepoints ($p=0.9981$, AnOVa); in addition, there is a high degree of significant difference when samples treated with DMSO and PP1 are compared at the 20 and 30 second timepoints. Finally, in Table 4-1e the basal activity of samples pretreated with any of the pharmacological agents is evaluated compared to that of

Table 4-1 Statistical Analysis of PKCδ Activity Measurements

a) Is the activity observed upon cell stimulation with anti-IgM the same as that detected in absence of stimulating antibody?

AnOVa (stim): **<0.0001** AnOVa (unstim): 0.9912

Test	p value (Wilcoxon)	p value (Mann Whitney)
t=0 (stim vs unstim)	0.1060	0.1060
t=20 (stim vs unstim)	0.0625	**0.0007**
t=30 (stim vs unstim)	0.1875	**0.0209**
t=45 (stim vs unstim)	0.8125	0.8965
t=60 (stim vs unstim)	1.1250	0.2165

b) Is the increase in activity due to BCR crosslinking significantly different than that observed in presence of U73122?

AnOVa (DMSO): **<0.0001** AnOVa (U73122): **0.0103**

Test	p value (Wilcoxon)	p value (Mann Whitney)
t=0 (DMSO vs U73122)	1.1250	0.7708
t=20 (DMSO vs U73122)	0.2500	0.1905
t=30 (DMSO vs U73122)	0.2500	0.6625
t=45 (DMSO vs U73122)	0.7500	0.2218
t=60 (DMSO vs U73122)	0.7500	0.7270

c) Is the increase in activity due to BCR crosslinking significantly different than that observed in presence of wortmannin (wm)?

AnVOa (DMSO): **<0.0001** AnOVa (wm): 0.4195

Test	p value (Wilcoxon)	p value (Mann Whitney)
t=0 (DMSO vs wm)	0.8438	0.6447
t=20 (DMSO vs wm)	0.0156	**0.0003**
t=30 (DMSO vs wm)	0.0156	**<0.0001**
t=45 (DMSO vs wm)	0.6406	0.4973
t=60 (DMSO vs wm)	0.5469	1.0000

d) Is the increase in activity due to BCR crosslinking significantly different than that observed in presence of PP1?

AnVOa (DMSO): **<0.0001** AnOVa (PP1): 0.9981

Test	p value (Wilcoxon)	p value (Mann Whitney)
t=0 (stim vs unstim)	0.7104	0.7104
t=20 (stim vs unstim)	0.1250	**0.0021**
t=30 (stim vs unstim)	0.1250	**0.0288**
t=45 (stim vs unstim)	1.1250	0.5783
t=60 (stim vs unstim)	1.1250	0.7387

e) Is the activity of samples pretreated with DMSO from the zero second (0 sec) timepoint significantly different than those incubated with either U73122, wortmannin (wm) or PP1?

Sample:	One Sample t test (all data)	binomial signs test (paired)
U73122 (0 sec) vs DMSO	0.7708	1.2500
wm (0 sec) vs DMSO	0.6447	0.8438
PP1 (0 sec) vs DMSO	0.7104	0.8750

DMSO-treated control samples. As evident from the p values, there is no significant difference in basal PKCδ activity when the cells are incubated with any of the compounds.

Results

Optimization of the *in vitro* kinase assay

The final conditions of cell stimulation, cell lysis, PKC immunoprecipitation, and *in vitro* kinase assay (described in Materials and Methods, above) were the culmination of several modifications to the original protocol (Allen et al., 1994). During the early phase of the investigation of the activation of PKCδ in response to BCR crosslinking, cell stimulation was performed by addition of 2 μL of anti-IgM to 500 μL of RPMI containing 1×10^7 Raji cells, stimulation was stopped by pelleting the cells and resuspending them in Lysis Buffer, and the *in vitro* kinase reactions were allowed to proceed for 10 minutes at 37 °C to allow for adequate phosphorylation of the peptide substrate. Quantitation of the assay involved running the entire reaction mixture on resolving SDS-PAGE, followed by autoradiography, excision of the radioactive bands, and measurement of the incorporated ^{32}P by scintillation counting. Using this method and conditions, the absolute PKCδ activity that was detectable was substantially less than that reported by other groups. This was likely due to the small quantity of PKCδ in the assay, estimated to be in the nanogram range. The following modifications were made to optimize the detection of PKCδ activity in the immune complex assay:

1. To measure the activity of PKCδ within seconds of BCR, stimulation needed to be stopped at precise timepoints. This was done by addition of an equal volume of ice-cold 2 × Lysis Buffer (see Chapter Three). A timecourse of cell stimulation is depicted in Figure 4-1. PKCδ activity increased by 30 seconds of BCR crosslinking, then again at 15 minutes (see Discussion, and Chapter Five).

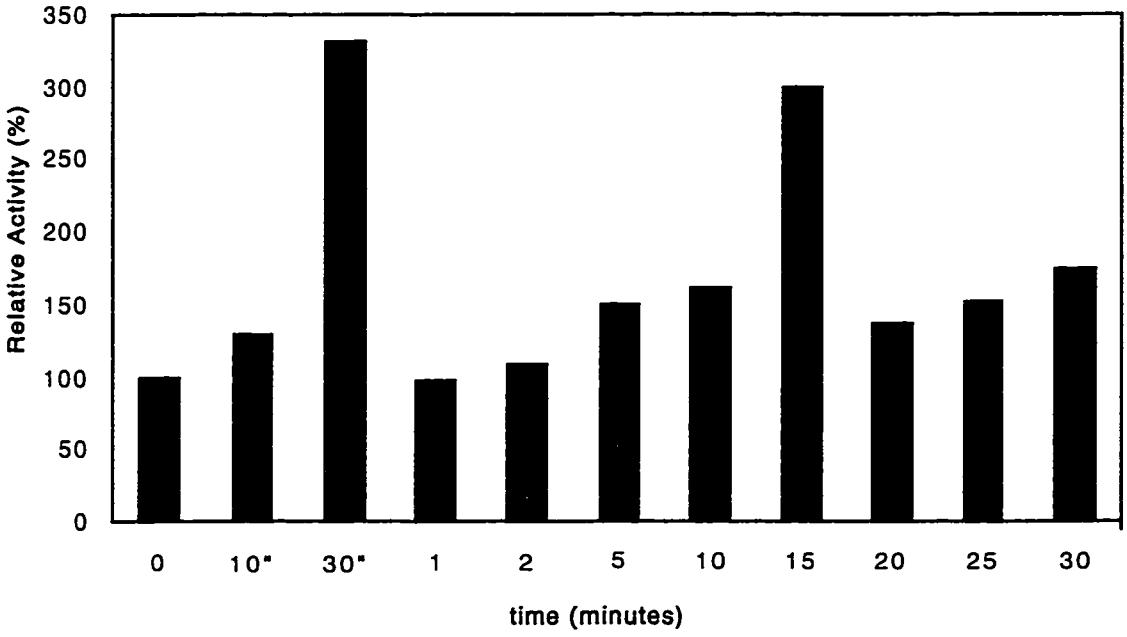


Figure 4-1. Activity of PKCδ in Raji B cells in response to BCR crosslinking. For this pilot experiment, 10^7 Raji cells were stimulated by 3 μg of anti-IgM for the times indicated. Activity was quantitated by scintillation counting of excised bands from PVDF following SDS-PAGE and transfer.

2. The optimal time of the *in vitro* kinase assay was determined (Figure 4-2). PKCδ immunoprecipitated from replicate samples, stimulated by 22 seconds of BCR crosslinking, was incubated for times ranging from 0 seconds to 10 minutes, and the reaction stopped by addition of SDS sample buffer. Following SDS-PAGE, autoradiography, and densitometry, the reaction time of 2 minutes was chosen for future assay, as being within the linear range of reaction.
3. To eliminate sources of error and inconsistency, the samples were not frozen before assay. Further, immunoprecipitates were pre-washed in Buffer K before addition of kinase assay ingredients, and gels were loaded every other lane to improve band excision for quantitation. The cells used were changed from 1×10^7 Raji B cells to 5×10^6 Ramos B cells, largely because Ramos maintain a high expression of surface IgM (see also Chapter One). These improvements were rewarded by the histogram depicted in Figure 4-3. This profile confirmed that a substantial increase in PKCδ activity was detected during the first minute of stimulation through BCR crosslinking, and second, that our preliminary results with Raji cells were reproducible in Ramos cells.
4. To further improve on assay reproducibility, the sample mixing was changed from 2 μL (3 μg) of antibody into 250 μL aliquotted cells to 125 μL of diluted antibody into 125 μL suspended cells, resulting in more homogeneous cell stimulation. In addition, the stimulation medium (RPMI 1640) was compared with medium containing 5% fetal bovine serum, and with modified HEPES-buffered saline (mHBS), in an effort to reduce the background cell stimulation. mHBS was chosen for its diminished contribution to basal PKCδ activity.
5. The performance of exogenous cofactors was evaluated, and it was determined that DG was a much more effective cofactor than PMA for activating PKCδ *in vitro*; however, there was no detectable difference between stimulated and unstimulated samples when incubated with either DG or PMA. Therefore, it was rationalized that either cofactor was contributing to activate PKCδ exogenously, irrespective of the endogenous state of

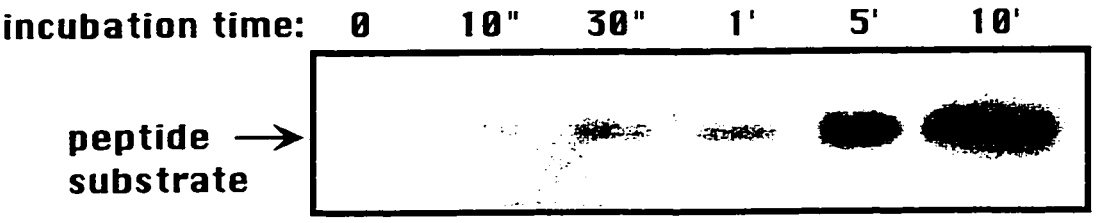


Figure 4-2. Determination of the optimal time of in vitro kinase assay. Stimulated samples were incubated in the *in vitro* kinase assay for times between 0 seconds and 10 minutes, as listed. The time of 2 minutes was chosen, as determined to be within the linear range of reaction by densitometric analysis.

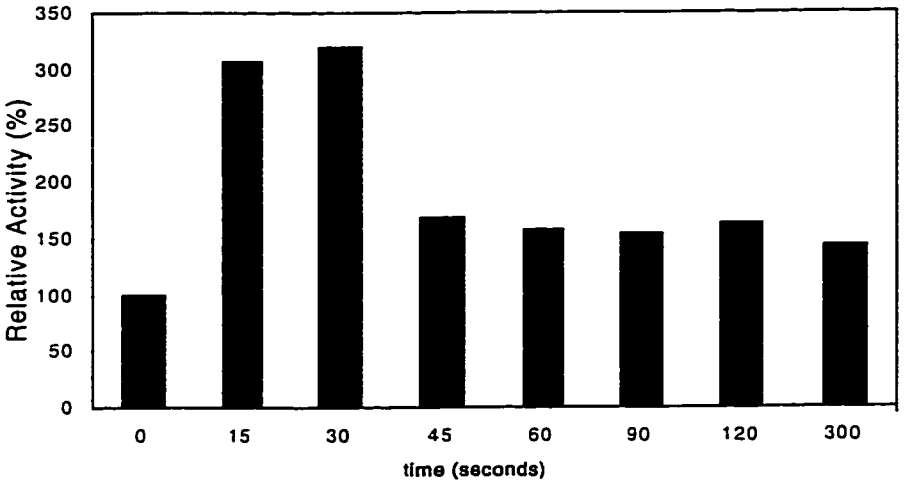


Figure 4-3. Activity of PKCδ in Ramos B cells in response to BCR crosslinking. Following several improvements in the system of activity evaluation for PKCδ, the activation of PKCδ in response to BCR crosslinking was confirmed in Ramos B cells.

activation in response to BCR crosslinking. It was decided to exclude both DG and PMA from the reaction buffer to allow for observation of endogenous activity; in addition, conditions of cell preparation were adjusted to maximize coimmunoprecipitation of PKCδ with endogenous lipid cofactors, produced in response to BCR crosslinking (see Discussion). The concentration of PS in the assay was empirically determined to give maximal PKCδ activity at 0.167 mg/mL.

6. It became necessary to replace the SDS-PAGE method of activity analysis with a more efficient protocol, as the detection method using SDS-PAGE was consuming one third of the overall experiment time. The method that was chosen was the use of large squares of P81 phosphocellulose paper, which is highly negatively charged, binds well to basic peptides, and has been shown to bind well to the peptide substrate derived from PKCε (Allen et al., 1994). Using a Bio-dot apparatus (BioRad), panels of samples were applied to large squares of P81, washed with 0.5% H_3PO_4, and quantitated using the Storm Phosphoimager (Molecular Dynamics). Triplicate aliquots of stimulated samples were also prepared to identify and avoid inconsistency in the *in vitro* kinase assay. Using this detection system, it was possible to complete an entire experiment, including an overnight immunoprecipitation of PKCδ, within two days.
7. A dose response was performed for the quantity of stimulating antibody, as demonstrated in Figure 4-4. Subsequently, 20 μg anti-IgM per 5×10^6 Ramos cells was used to generate maximal activity. This final value is similar to other reports regarding BCR crosslinking (Valentine et al., 1992).

Activation of PKCδ in response to BCR crosslinking. The activation of PKCδ in response to BCR crosslinking was determined. As is evident from the histogram depicted in Figure 4-5, PKCδ was maximally activated by 22 seconds of BCR crosslinking, then essentially de-activated by 45 seconds. Data are normalized to 0 seconds of stimulation as 100%, or basal, activity; statistically significant activation is denoted by *

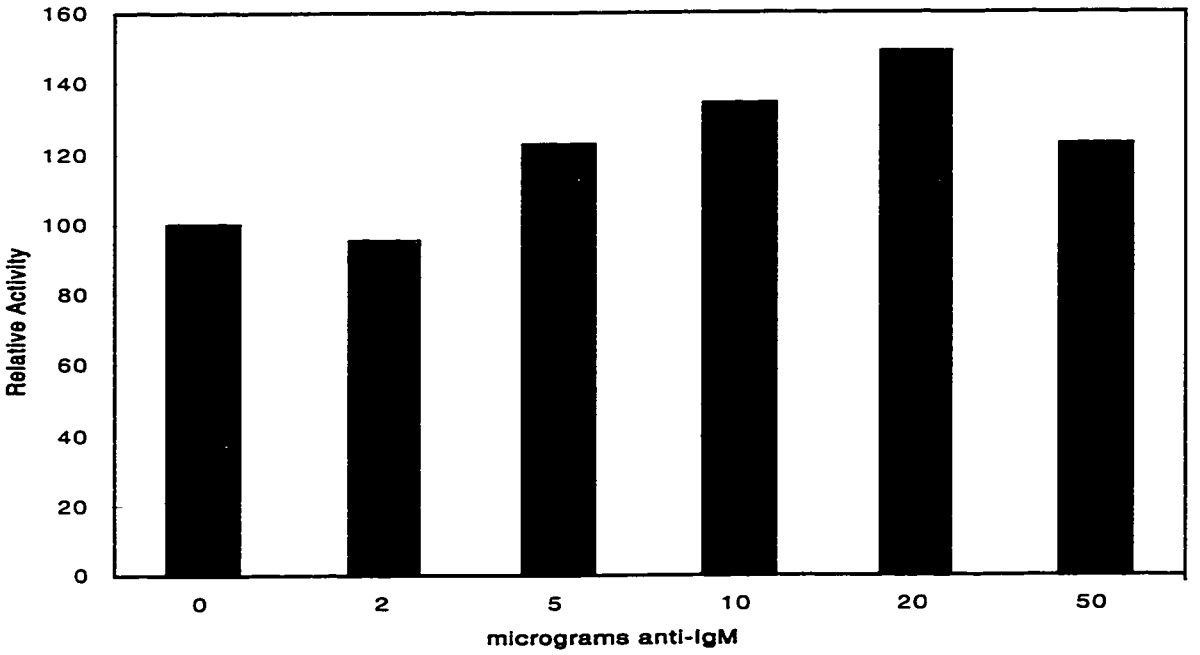


Figure 4-4. Dose/response of anti-IgM for BCR crosslinking in Ramos B cells. 5×10^6 Ramos cells were stimulated by varying quantities of anti-IgM for 22 seconds, followed by PKCδ activity evaluation. The dose of 20 μg per 5×10^6 cells was used for all future experiments.

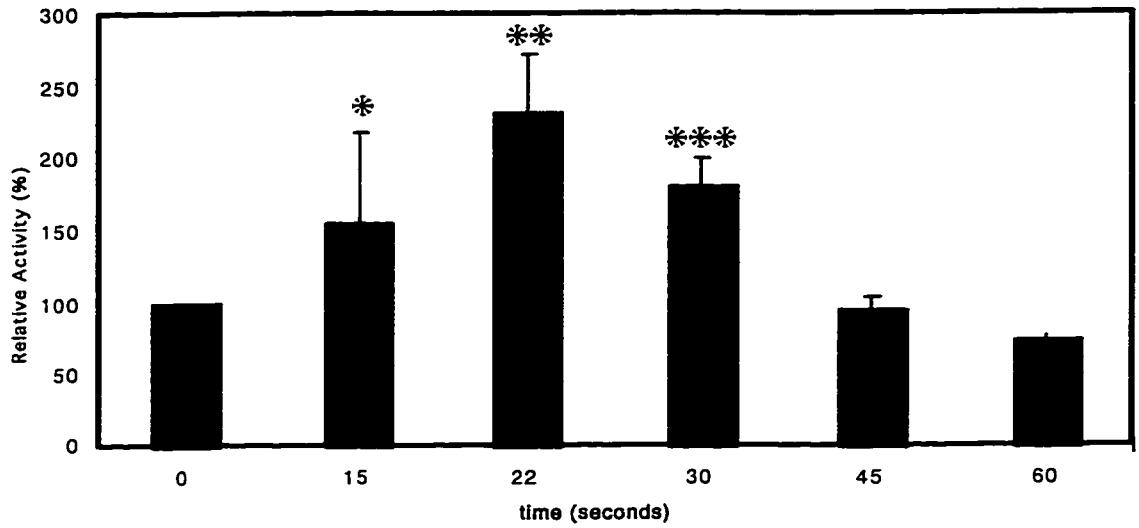


Figure 4-5. Activation of PKCδ in response to BCR crosslinking, relative to unstimulated controls (not shown, n=5). Activity of PKCδ was evaluated using the current assay conditions, compared to samples stimulated with mHBS alone. Activity of unstimulated samples was determined to be insignificant, and independent of time of stimulation (p=0.9912).

(p=0.0571), ** (p=0.0113), and *** (p=0.0209). Control samples were stimulated with buffer alone to control for sample handling effects; the extent of activation of PKCδ in the absence of BCR crosslinking antibody was determined to be insignificant (see Table 4-1a, p=0.9912, n=5). Since there was no detectable ^{32}P incorporation into any species except PKCδ and the peptide substrate in both PKC and PTK *in vitro* kinase assay conditions (Figure 4-2, and data not shown), it is concluded that the activity that is observed under these conditions is due to the immunoprecipitated PKCδ. In addition, all of the detectable activity in the PKC *in vitro* kinase assay was ablated with either a PKC inhibitor peptide or bisindolylmaleimide (data not shown), indicating that the presence of additional kinases is unlikely.

Factors influencing the activation of PKCδ. Diacylglycerol produced by PLC is known to function as a lipid cofactor for PKC; recently, however, products of PI3'K ($PI(3,4)P_2$, PIP_3) have also been shown to function in this role (Mizukami et al., 1997; Nakanishi et al., 1993; Toker et al., 1994). To evaluate the contribution of each second messenger and tyrosine phosphorylation on the activation of PKCδ, the activity of the upstream enzymes PLC, PI3'K, and PTKs was blocked by pretreating cells with U73122, wortmannin, or PP1 (or their common solvent DMSO at an appropriate dilution). Cells were then stimulated and PKCδ was immunopurified and subjected to the *in vitro* kinase assay. The effects of blocking PLC activation with U73122, compared to DMSO (Figure 4-6 b and a, respectively) were consistently observed in 4 out of 4 experiments as a slight increase in the activity at 20 seconds and a slight decrease in the activity at 30 seconds, however this effect was not determined to be statistically significant (20 seconds, † p=0.1905; 30 seconds, †† p=0.6625). These data combined suggest that PLCγ does not contribute to the primary activation of PKCδ. The possibility of incomplete inhibition of PLC activation by U73122 was addressed; using the compound at 50 μM demonstrated an identical profile to that depicted in Figure 4-6b. It was necessary to perform control

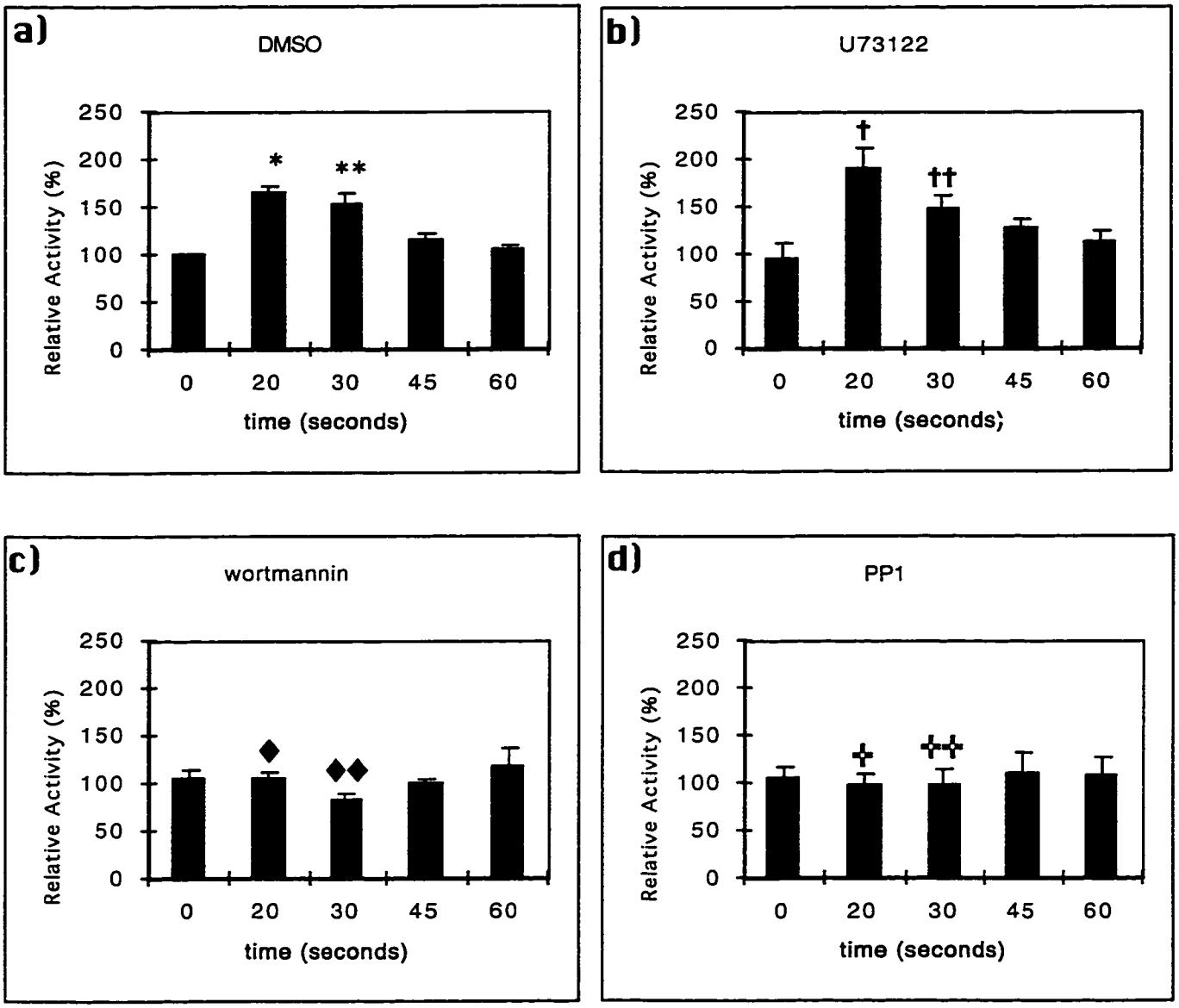


Figure 4-6. Factors contributing to the activation of PKCδ in response to BCR crosslinking. Ramos B cells were pretreated for 30 minutes with the compounds noted at the top of each plot; a parallel set of samples were pretreated with appropriately diluted DMSO. Statistical tests were performed on the activity values, denoted by symbols on top of those values, and referred to in the text.

experiments to address whether the compound U73122 was in fact preventing the activation of PLC in B cells. This was accomplished using the reported mechanism of activation of PLCγ in B cells, namely tyrosine phosphorylation, as a marker of activation (Hempel et al., 1992; Khare et al., 1997; Nishibe et al., 1990). In response to BCR crosslinking, 20 μM U73122 substantially prevented the tyrosine phosphorylation of PLCγ, but not Syk tyrosine kinase, thus confirming the efficacy of U73122 (data not shown).

The participation of the PI3'K pathway in the activation of PKCδ in response to BCR crosslinking was examined by pretreating the cells with wortmannin, as shown in Figure 4-6c. Cells pre-incubated with wortmannin exhibited no increase in activity at early timepoints (20 seconds, ◆ $p=0.0003$; 30 seconds, ◆◆ $p<0.0001$). This result is in agreement with several *in vitro* studies that have demonstrated that PI3'K lipid products can serve as cofactors for the *novel* PKCs including PKCδ (Mizukami et al., 1997; Nakanishi et al., 1993; Toker et al., 1994).

Since PTK activation is known to be the most immediate effect of BCR crosslinking (Gold and DeFranco, 1994; Gold and Matsuuchi, 1995; Saouaf et al., 1994), it was postulated that inhibition of this upstream event would result in the lack of activation of PKCδ. The effects of PTK inhibition on the activation of PKCδ were investigated. As is evident from Figure 4-6d, the inclusion of PP1 during the pre-incubation of B cells completely prevented the activation of PKCδ in response to BCR crosslinking, compared to DMSO pretreated control (20 seconds, ✣ $p=0.0021$; 30 seconds, ✣✣ $p=0.0288$). A control experiment was performed to ensure that the effect of the tyrosine kinase inhibitor PP1 on the activation of PKCδ was not the result of PP1 carried over to inhibit PKCδ in the *in vitro* kinase assay. To test this, both stimulated and unstimulated samples were assayed for PKCδ activity in the presence or absence of 25 μM PP1, added exogenously to the kinase assay. No

significant effect of PP1 on PKCδ activity *in vitro* was observed, confirming that its effect on PKCδ activation in treated intact cells was due to the inhibition of an upstream PTK, activated in response to BCR crosslinking. It is not possible to conclude from these studies that tyrosine phosphorylation of PKCδ itself is required for its activation. Rather, it can be stated that PTK activity is necessary for activation of PKCδ. The target of the required PTK activity is likely to be upstream of PKCδ (see Discussion, and Chapter Five).

Although it was not the focus of this study, the possibility that PKC activation in response to BCR crosslinking was not specific to one isoform was also addressed. In identical experiments substituting Ca^{2+} for EGTA where appropriate in the *in vitro* kinase assay, the activities of PKCβ and PKCδ were compared in response to 30 seconds of BCR crosslinking. It was found that PKCβ did not exhibit any activation under these conditions, even though recombinant PKCα gave strong activity in the same PS- and Ca^{2+}-containing *in vitro* kinase assay, when supplied with DG (data not shown). This does not rule out the possibility that other isoforms of PKC can be activated in response to BCR crosslinking, rather, it strengthens the hypothesis that PKCδ is unique in terms of its ability to become activated and tyrosine phosphorylated in response to BCR crosslinking.

Discussion

This study has provided evidence that two of the major signaling pathways associated with the BCR are implicated in the regulation of PKCδ activation. The involvement of a tyrosine kinase event appears to be crucial in the eventual activation of PKCδ, as demonstrated by Figure 4-6d. Since the primary events of receptor-mediated signaling through the BCR involve Src-family kinase tyrosine phosphorylation of Igα/β proteins, early tyrosine kinase inhibition effectively blocks all BCR-associated signaling, including the activation of lipid second messenger pathways (Gold and Aebersold, 1994; Gold et al., 1992; Saouaf et al., 1994). Again, it cannot be implied that tyrosine phosphorylation is responsible for

activating PKCδ, rather, that upstream tyrosine kinase activity results in the activation of pathways that are themselves directly responsible for activating PKCδ.

The immediate activation of PKCδ in response to B cell crosslinking clearly involves the ability of PKCδ to respond to the activity of PI3'K. This is evident as activation is not significantly attenuated by blocking PLC activation with U73122, but is severely compromised by PI3'K inhibition with wortmannin. While it is believed that this is due to the ability of PKCδ to respond to PI3'K lipid products such as $PI(3,4)P_2$ and $PI(3,4,5)P_3$, as reported for *novel* and *atypical* isoforms in other systems (Mizukami et al., 1997; Nakanishi et al., 1993; Toker et al., 1994), it is also possible that downstream events in response to PI3'K may activate PKCδ (Borgatti et al., 1997; Li et al., 1997). At the concentration used, it is believed that the primary target of wortmannin in the B cell is PI3'K; however, there is the possibility that phosphatidylinositol 4'kinase may also be inhibited (Meyers and Cantley, 1997). The connection between the BCR and PKCδ activation is clearly wortmannin sensitive, as demonstrated by Figure 4-6c, and the most direct interpretation of these data is that PKCδ has the capacity to respond to PI3'K lipid products. Indeed, the potential association of PKCδ with lipid cofactors endogenously produced in response to BCR crosslinking is substantiated by the necessity to titrate the *in vitro* kinase assay conditions (including cell lysis) to exclude high salt or isopropanol, and to have reduced PS relative to other assays (Allen et al., 1994). Finally, it has also been convincingly demonstrated in the recent study on PKCθ in T cells (Monks et al., 1997) that the activity observed was inherent, and that it was due to occupation of the C1 lipid cofactor-binding site.

The effects of preventing PLC activation in response to BCR crosslinking with U73122 on PKCδ activity are curious. Since PLC produces the lipid second messenger DG, which is generally regarded to be responsible for the activation of PKC, it is interesting that blocking

the production of DG did not have a signficant effect on the activation of PKC. Taken together with the results using the PI3'K inhibitor, wortmannin, which support a role for PI3'K products in the activation of PKCδ, it is indeed possible that PLC does not participate in the activation of PKCδ in response to BCR crosslinking. However, as described in Chapter Three, U73122 inhibited the tyrosine phosphorylation of PKCδ, indicating that PLCγ participates in the regulation of PKCδ. It is possible, however, that the assay system was not sensitive enough to detect the involvement of PLCγ in the activation of PKCδ.

The kinetics of activation and deactivation as depicted in Figure 4-5 may only represent part of the activation of PKCδ in response to BCR crosslinking. As demonstrated originally in the extended timecourse (Figure 4-1) and repeated in Ramos cells (not shown), there also appears to be an increase in PKCδ activity in response to 15 minutes of BCR crosslinking. Given the multitude of signals that can occur within 15 minutes of BCR crosslinking, it is very difficult to make any assumptions regarding the nature of the stimulus for this activation; however, one possibility is that PKCδ is responding secondarily to DG produced from sources other than $PI(4,5)P_2$, for example, phosphatidylcholine. In any event, the fact that PKCδ can be activated again at 15 minutes, while tyrosine phosphorylated (see Figure 3-3b), may reveal a facet of interaction between activation and tyrosine phosphorylation (see Chapter Five). Regardless, the secondary signaling required to produce this activation is not believed to be relevant to the initial events of activation, which can be detected as early as 15 seconds. Although this re-activation represents an interesting element of PKCδ's participation in BCR signaling, this observation was not emphasized or further explored.

Chapter Five

Discussion

The B cell antigen receptor is crucial for regulating many of the diverse behaviors of the B cell. There are at least four stages of B cell development in which a signal from the BCR is thought to be critically important (adapted from (Birkeland and Monroe, 1997)): (1) the selection and / or expansion of pre-B cells that have properly rearranged Ig heavy chains, (2) the elimination of self-reactive immature B cells, (3) the activation of the mature B cell, resulting in entry into the cell cycle, (4) elimination of those germinal center B cells in which somatic mutation has resulted in formation of autoreactive antibodies. In light of the variable effects of engagement of the B cell receptor, many attempts have been made to understand precisely the signals that the BCR mediates into the developing lymphocyte. The focus of the research described in this thesis is to address the immediate biochemical events of the activation of the mature B lymphocyte.

The involvement of the PKC family of isozymes in signaling through BCR crosslinking is only beginning to become characterized. It may be that the expression and / or activation of specific PKC isoforms is limited to a specific stage(s) in B cell development. Pre-B cells express an immature version of the BCR composed of μ heavy chains and ψ (surrogate) light chains which is capable of causing a Ca^{2+} influx and activating tyrosine kinases upon ligation. However, it has yet to be determined if PKC participates in pre-BCR signals (Genevier and Callard, 1997). Studies using phorbol ester analogs and ionomycin to induce PKC activation have demonstrated that both *conventional* and *novel* PKC isoforms are involved in different aspects of BCR-mediated signals in mature B cells (Kim et al., 1992). Further, it is known that immature B cells from the spleens of neonatal mice are defective in their ability to hydrolyze phospholipids in response to BCR crosslinking, which would suggest a defect in their ability to activate some of the PKC

family members by DG production (Yellen et al., 1991). The role of PKC isoforms in lymphocyte development has not yet been adequately addressed. PKCμ has been recently identified as performing a regulatory function in the mature B cell, however, although it is ubiquitously expressed in B cells at all stages of maturation, the function of this isoform during development was not explored (Sidorenko et al., 1996). A potential selective role for PKCβ has also been suggested for the aging T lymphocytes in regulating anti-CD3-mediated cell division (Fulop et al., 1995). It is speculated that the profile of expression of isoforms at each different stage of B cell development would change, and that the use of selective PKC isoforms in response to BCR crosslinking would also change. In keeping with the emphasis on mature B cell activation, this thesis is focused on the involvement of PKC in the BCR crosslinking-induced activation of the mature B cell.

The activation of PKC in response to BCR crosslinking has never been clearly understood, and as a result, has been reduced to a simple model. This is that ligation of the BCR induces the activation of PLCγ, which then produces the two second messengers IP_3 and DG, which synergize to activate PKC through the release of Ca^{2+}, and interaction with DG at the membrane (Chen et al., 1986; Nel et al., 1986). Several recent devolopments in the field brought this model into question. The discovery of isoforms of PKC as described in Chapter Two raised the immediate question of which PKC(s) was in fact responding to the BCR-mediated signals. The discovery that other lipid second messengers besides DG could function to activate some of the *novel* and *atypical* members of the PKC family further complicated the issue (Liscovitch and Cantley, 1994; Mizukami et al., 1997; Nakanishi et al., 1993; Toker et al., 1994), considering that PI3'K was also known to become activated in response to BCR crosslinking (Gold et al., 1992). The question of regulation of the PKC family by tyrosine phosphorylation added yet another dimension to the complex situation, since BCR crosslinking generates a well characterized PTK cascade

(Gold et al., 1990). The research described in this thesis has been focused at addressing some of these unanswered questions experimentally.

Recently, exciting developments in this area have implicated multiple members of the PKC family in mediating lymphocyte function. Chronic PKCα stimulation has been implicated in mediating immunosenescence through the selective depletion of naive T cells in a transgenic mouse aging model (Ohkusu et al., 1997). PKCβ has also been shown to mediate B cell activation, as mice with homozygous disruptions in the PKCβ gene display impaired humoral responses, and reduced cellular responsiveness of B cells, in a phenotype that resembles X-linked immunodeficiency (Btk mutation) (Leitges et al., 1996). These studies suggest that PKCβ has a positive role in the regulation of lymphocyte function. Conversely, studies with the Ramos and Daudi B cell lines have demonstrated that PKCμ has a negative regulatory role in BCR-mediated signaling, likely through the downregulation of Syk and PLCγ (Sidorenko et al., 1996). The relationship between function and isoform Group is not easily defined, however, because the novel PKCθ is thought to function in an activating role in T cells upon antigen-presenting cell contact (Monks et al., 1997).

The research that contributed to this thesis describes the participation of another isoform of PKC in B cells, namely PKCδ. In summary, PKCδ has been found to respond to crosslinking of the BCR by becoming both activated and tyrosine phosphorylated. The consequences of either of these events remain to be determined. This discussion will first present a summary of the conclusions drawn from the previous chapters, then focus on the interaction of activation and tyrosine phosphorylation in light of the other data.

Activation of PKCδ in response to BCR crosslinking To summarize the discussion on the activation of PKCδ in response to BCR crosslinking as presented in

Chapter Four, the data indicate that two pathways associated with BCR signaling are involved in the activation of PKCδ. The PTK pathway is likely involved as the first signal instigator, translating the extracellular ligation of the BCR into a cytoplasmic tyrosine phosphorylation cascade, resulting in the activation of both PLCγ and the PI3'K pathways. The PI3'K pathway is clearly involved in PKCδ activation, as blocking PI3'K activity with wortmannin completely abrogates activation. It is unresolved whether this is mediated through lipid second messengers themselves, or rather through a downstream effect such as PKB activation. PLCγ does not appear to play a role in the activation of PKCδ, since preventing PLCγ activation with U73122 had no effect on PKCδ activation.

Tyrosine phosphorylation of PKCδ in response to BCR crosslinking To summarize the data on tyrosine phosphorylation of PKCδ, it is clear that PKCδ becomes rapidly tyrosine phosphorylated, possibly on multiple sites, in response to BCR crosslinking. The dynamic tyrosine phosphorylation that parallels the profile of activation of PKCδ may reflect the state of activation, however, any functional relationship has yet to be demonstrated. The extended tyrosine phosphorylation of PKCδ in the B cell, not observed in other systems (Denning et al., 1996; Haleem-Smith et al., 1995; Soltoff and Toker, 1995), suggests that this aspect of tyrosine phosphorylation of PKCδ may be specific to B cells, and likely has an unique function.

Relationship between activation and tyrosine phosphorylation of PKCδ At least four possible connections between tyrosine phosphorylation and activity exist for PKCδ in the B cell. Each will be critically evaluated here in light of published reports and the data presented in this thesis.

First, tyrosine phosphorylation may serve to activate PKCδ, as demonstrated in some other systems (Gschwendt et al., 1994; Konishi et al., 1997; Li et al., 1994). The parallel

kinetic profiles of tyrosine phosphorylation and activation of PKCδ as presented in this thesis would support this hypothesis (see Figure 3-3 and 4-5). However, considering the fact that activation of PKCδ is observed in the presence of U73122 (Figure 4-6b) while tyrosine phosphorylation is not (Figure 3-6b), it does not seem possible that the activation of PKCδ is dependent on its tyrosine phosphorylation, in response to BCR crosslinking. The fact that PP1 prevented activation of PKCδ in response to BCR crosslinking (Figure 4-6d) does not demonstrate that tyrosine phosphorylation is required for activity, because PP1 is thought to affect the BCR signal at the level of the initial activation of the BCR-associated cytoplasmic tyrosine kinases; by inhibiting these primary signaling enzymes, the complete signal from the BCR is terminated, including the PTK which phosphorylates PI3'K, PLCγ, as well as PKCδ. A recent report (Konishi et al., 1997) identified a distinct tyrosine phosphorylation phenomenon, which may be an indication of a particular type of stress response. Briefly, they report that all isoforms of PKC may become tyrosine phosphorylated in response to H_2O_2 treatment resulting in increased activity, however, the mechanism of this phenomenon is not known. The tyrosine phosphorylation induced by such agents as H_2O_2 may likely cause a "stressed" response, potentially involving the stress-activated protein kinase (SAPK) pathway. It is not thought that this participates in the activation and tyrosine phosphorylation of PKCδ as observed in the Ramos lymphoblastoid B cell line in response to crosslinking of an endogenous cell surface molecule, the BCR. Considering the data together, it is unlikely that tyrosine phosphorylation activates PKCδ in response to BCR crosslinking.

Second, tyrosine phosphorylation may serve to downregulate the activity of PKCδ, as suggested by other reports (Denning et al., 1993; Denning et al., 1996; Zang et al., 1997). It seems possible that tyrosine phosphorylation may function in this role, as the incidence of tyrosine phosphorylation of PKCδ is directly followed by a decrease in the activity from 22 to 60 seconds. However, considering the fact that another increase in PKCδ activity

was observed following 15 minutes of stimulation (Figure 4-1, and data not shown), while the enzyme is still tyrosine phosphorylated (Figure 3-3), this suggests that the tyrosine phosphorylated PKCδ is activatable. This does not exclude the possibility that more than one tyrosine phosphorylation site is involved, one which functions in a downregulatory role, and another of yet unknown function. It is demonstrated in this thesis that the tyrosine phosphorylation in response to BCR crosslinking is likely to be dependent on the active conformation of the enzyme, rather than simply on the active state, as previously described (Gschwendt et al., 1994; Li et al., 1994; Zang et al., 1997) (see Figure 3-5a). The fact that PMA treatment results in tyrosine phosphorylation of PKCδ suggests that activation indeed precedes tyrosine phosphorylation. In light of the requirement for activation, and the influence of blocking the activating cofactors on the tyrosine phosphorylation of PKCδ, it can be reasonably speculated that the most probable effect of tyrosine phosphorylation on activity, if any, is downregulatory.

Third, tyrosine phosphorylation may prevent the activation of PKCδ in the B cell. This is clearly not the case, as described above, since rather than observing the increase of activity of PKCδ above basal levels following BCR crosslinking (Figure 4-5), we would have expected to see reduced activity of PKCδ when it became tyrosine phosphorylated, below unstimulated (resting) levels. Again, an increase in the activity of PKCδ following 15 minutes of stimulation was observed suggesting that the tyrosine phosphorylated protein is activatable.

Finally, tyrosine phosphorylation may not be regulating activity, rather, it may function in some other realm, such as subcellular localization. Since the relationship between activation and tyrosine phosphorylation is not clear, this option is certainly attractive. As described in Chapter One, BCR crosslinking promotes tyrosine phosphorylation and assembly of the multi-adaptor Cbl protein complex (Ingham et al., 1996; Panchamoorthy et

al., 1996); this complex is known to recruit other tyrosine phosphorylated species, so it is entirely possible that PKCδ may be one of the associated proteins. Alternatively, PKCδ may interact with many other proteins, unrelated to the BCR complex, as a result of its tyrosine phosphorylation.

The phosphotyrosine(s) of PKCδ may serve to promote a certain cellular sequestration of PKCδ by a variety of mechanisms, and for several possible reasons. First, PKCδ may interact with one or more of the numerous Src-homology 2 (SH2)-containing proteins, such as PI3'K (p85) (Ettinger et al., 1996), or even Src itself (Zang et al., 1997). To this extent, an attempt was made to determine whether PKCδ selectively associated with Lyn, which is a key Src-family kinase in the B cell; no association between PKCδ and Lyn was ever detected in Ramos B cells, by several separate approaches, including co-precipitation and immunoblotting, re-immunoprecipitation, or *in vitro* kinase labelling with [γ-^{32}P]ATP (not shown). In fact, the sequence of PKCδ would not predict that any of the phosphotyrosines would bind to SH2 domains, which recognize the phosphotyrosine in the consensus sequence of pYEEI (Aris et al., 1993; Brown and Cooper, 1996), making interactions via SH2 domains unlikely. Another phosphotyrosine-binding (PTB) domain has also been described (reviewed in (Pawson and Scott, 1997)). This domain is distinct from the SH2 domain in that it is usually found in scaffolding or docking proteins, near activated receptors. The PTB domain prefers to recognize the sequence of amino acids ϕXNPXpY, where ϕ is any hydrophobic amino acid (Pawson and Scott, 1997), however, this is also not found in PKCδ (Aris et al., 1993), making it unlikely that PKCδ would interact with a PTB domain.

The fact that consensus sequence homologies do not predict interaction specifically with the phosphotyrosine(s) does not render the tyrosine phosphorylation phenomenon irrelevant; it is equally possible that tyrosine phosphorylation induces a change in PKCδ, which

promotes binding to an as-yet unidentified protein through an exposed site. A preliminary metabolic cell labeling experiment demonstrated that PKCδ has the potential to associate with other proteins upon activation and tyrosine phosphorylation, perhaps members of the Cbl adaptor complex, or even the RACK family (see below). Associations with other proteins, regardless of the mechanism, further indicate the selectivity of function of PKCδ.

Subcellular localization of PKCδ may serve one of several functions. First, PKCδ may be recruited into the proximity of specific cellular substrates, the nature of which is discussed further in Chapter Six. This would allow PKCδ to perform a specific, localized function during its brief period of activation in response to crosslinking of the BCR. Another possibility is that PKCδ is erroneously activated in response to BCR crosslinking, and to compensate for this (hence, to prevent it from phosphorylating the 'wrong' substrate), it may be selected to be effectively removed from the site of concern by labelling by tyrosine phosphorylation. This would give reason to why PKCδ is unique among other isoforms in its capacity to become phosphorylated in response to BCR crosslinking. Finally, as discussed with respect to scaffolding or adaptor proteins (Chapter One), tyrosine phosphorylation may serve to group many PKCδ proteins into an inactive aggregate, which only has activity when removed from the cellular environment. In any case, the selective nature of the tyrosine phosphorylation is an indication of the specific function of PKCδ, but not other isoforms, and the sustained nature of the tyrosine phosphorylation is an indication that this function is important over a long time period.

Possible involvement of PKC binding proteins Many PKC binding proteins have been described, including PKC substrate binding proteins (SBPs) (Chapline et al., 1996; Chapline et al., 1993; Dong et al., 1995) such as the recently identified GAP-43, which specifically interacts with the N-terminus of PKCδ (Dekker and Parker, 1997), proteins that interact with C kinase (PICKs) (Staudinger et al., 1995), and the receptor for activated

C kinase (RACK) family (Mochly-Rosen, 1995). It is possible that PKCδ may selectively interact with any of these proteins. Regulation of the activation of PKCδ may be dependent on its interaction with a specific member of the RACK family of PKC-binding proteins, since the potential for isoform specificity of the RACKs has been suggested (Mochly-Rosen et al., 1991; Robles-Flores and Garcia-Sainz, 1993; Ron and Mochly-Rosen, 1995). Since PLCγ also contains a RACK-binding site, and is capable of interacting with RACKs in its activated form (Disatnik et al., 1994), its role in the activation of PKCδ may function in the form of protein-protein interaction. Again, the result from the metabolic labeling experiment would suggest specific interaction of active, tyrosine phosphorylated PKCδ with other proteins, however, the procedure used for the isolation of PKCδ does not parallel the isolation of RACKs, which are reported to be Triton X-100 insoluble (Mochly-Rosen et al., 1991).

Identification of the PTK responsible for phosphorylation of PKCδ in response to BCR crosslinking The identity of the PTK responsible for phosphorylating PKCδ in the B cell has not been determined, but preliminary circumstantial evidence has suggested some likely candidates. First, since tyrosine phosphorylation of PKCδ occurs in response to BCR crosslinking, we may reasonably assume that it is one of the PTKs which are activated upon BCR crosslinking that is responsible for tyrosine phosphorylating PKCδ, the most well known being Lyn, Fyn, Blk, Syk, and Btk. This would be consistent with the kinetics of activation of BCR-associated pathways (Carter et al., 1991; Gold and Aebersold, 1994; Gold et al., 1992) and the activation of Lyn and Blk (Saouaf et al., 1994). Since PP1 is a preferential Src-family protein tyrosine kinase inhibitor and prevented both the PMA-induced and the BCR crosslinking-induced tyrosine phosphorylation of PKCδ more effectively than genistein, it seems likely that a BCR-associated Src-family kinase such as Lyn, Fyn, or Blk is the kinase responsible for phosphorylating PKCδ (see Figure 3-3b). Further, it has been

recently demonstrated that membrane localization of Src results in the increased tyrosine phosphorylation of PKCδ (Zang et al., 1997), and it has also been shown that Src and Lyn can phosphorylate PKCδ *in vitro* (Gschwendt et al., 1994). For the purposes of this study, it may be reasonably concluded that the most likely candidate for *in vivo* phosphorylation of PKCδ in response to BCR crosslinking is Lyn.

The potential relationship of PKCδ to anti-IgM-induced apoptosis was considered. Within 4 hours of ligation of the BCR, Ramos cells begin to show signs of apoptosis, and as a result, several investigations in the area of apoptosis have used the Ramos cell line as a model to characterize the induction of apoptotic pathways (Chaouchi et al., 1995; Kaptein et al., 1996; Padmore et al., 1996; Valentine and Licciardi, 1992). Since it is also known that the ICE-family proteases are hallmarks for apoptosis, and that BCR ligation in Ramos B cells induces apoptosis within four hours through the utilization of caspase family members (Andjelic and Liou, 1998; Valentine and Licciardi, 1992), it was postulated that activation and tyrosine phosphorylation of PKCδ might be one of the immediate events in promoting these phenomena. Further, the proteolysis of PKCδ was also considered as a mechanism of its activation, being the basis of the discovery of PKC (Inoue et al., 1977; Takai et al., 1977), and considering the recently reported activation of PKCδ by proteolysis by ICE-family proteases (Emoto et al., 1995). However, as demonstrated in Figure 3-2, PKCδ migrates at 78 kDa throughout the four hours of stimulation; further, no significant decrease in the 78 kDa band was ever observed in anti-PKCδ immunoblotting experiments carried out to four hours (data not shown), and no 40 kDa PKCδ-immunoreactive proteins were detected, although the blotting antibody was capable of recognizing such cleavage products (not shown). The early activation of PKCδ in response to the BCR described here likely represents a distinct role for PKCδ in the B cell, compared to that induced later by proteolytic activation in other cell types.

While it was clearly demonstrated that PKCδ was unique among the other isoforms tested in its capacity for tyrosine phosphorylation in response to BCR crosslinking, the question is raised as to whether PKCδ is unique among the *novel* isoforms of PKC. The possibility of tyrosine phosphorylation of other isoforms of the *novel* subclass is indeed relevant. PKCδ has been reported to be tyrosine phosphorylated on only 4 of its 20 tyrosines (sequence of human PKCδ from (Aris et al., 1993)), namely Y52 (Szallasi et al., 1995), Y187 (Li et al., 1996),Y514, and Y525 (Konishi et al., 1997), however, the exact role of even one of those tyrosines has yet to be securely demonstrated. PKCη and PKCθ share homology to all four of these phosphorylation sites, and PKCε to three of the four, making it very possible that these novel isoforms may also become tyrosine phosphorylated in a whole cell system, perhaps even in response to BCR crosslinking. However, the literature supports the notion that PKCδ behaves quite differently from other *novel* isoforms (see below). This would imply that PKCδ would also be unique in its activation and tyrosine phosphorylation compared to other *novel* isoforms of the PKC family. Indeed, in the T cell, PKCθ selectively localizes and becomes activated, but neither PKCη nor PKCδ do, in response to antigen presenting cell contact (Monks et al., 1997). In a separate study, Keenan, *et al*, (1997) showed that PKCδ and PKCθ exhibit individual phenotypes of response in human lymphocytes, when stimulated by anti-CD3. In the mast cell line RBL-2H3, a distinction between PKCδ and PKCη was observed in terms of their effects on cell proliferation (Chang et al., 1997). Finally, the study on the effects of overexpression of isoforms on the growth, anchorage dependence, and tumorigenicity of NIH-3T3 fibroblasts demonstrated opposite effects of PKCδ and PKCε (Mischak et al., 1993). Taken together, these studies clearly demonstrate that each isoform functions as an individual, and the specificity within the system and even within Groups is quite significant. Applying this directly to PKCδ in the B cell, it can be hypothesized that PKCδ would be unique among the *novel* isoforms, as well as across Groups, with respect to its activation and tyrosine phosphorylation.

Conclusions The activation and tyrosine phosphorylation of PKCδ in response to BCR crosslinking clearly demonstrate selective control of this isoform. The intriguing observations that three pathways cooperate to regulate the tyrosine phosphorylation and activation state of PKCδ are indicators of multifaceted regulation, which provides countless possibilities of cellular regulation.

Chapter Six

Conclusions and Future Work

Conclusions from this thesis

This study has demonstrated how a single isoform of PKC can be activated and selectively tyrosine phosphorylated in response to BCR crosslinking, a signal that is crucial in the growth and development of the B lymphocyte. PKCδ was found to be capable of responding to the signals generated by BCR crosslinking by becoming concurrently activated and tyrosine phosphorylated within 30 seconds of stimulation. Subsequently, both the activity and tyrosine phosphorylation state were downregulated, and an intermediate level of tyrosine phosphorylation was sustained for at least 4 hours. Pharmacological evaluation of the well characterized BCR-associated signaling pathways demonstrated that three of the primary signaling pathways participated in either the activation or tyrosine phosphorylation of PKCδ. The BCR-associated PTKs were determined to be crucial signal mediators, affecting both activation and tyrosine phosphorylation. The PI3'K pathway was found to directly influence activation, which we speculate predisposes tyrosine phosphorylation. The PLCγ pathway was found to only have an effect on the tyrosine phosphorylation of PKCδ. Thus, the cooperation of three essential signaling pathways demonstrates the multifaceted regulation of PKCδ in response to BCR crosslinking.

Future work in this field

Several key openings for continued research have arisen as a consequence of the investigation of the activation and tyrosine phosphorylation of PKCδ in B cells, in response to BCR crosslinking. These are briefly outlined below, with an experimental approach which could be used to address each issue.

Since the response of PKCδ to BCR crosslinking has been demonstrated, one of the foremost questions concerns the importance of this event in terms of B cell function. To address this, several experimental approaches can be undertaken using various methods of PKCδ depletion, with the overall intention of defining the physiological consequence of the activation and tyrosine phosphorylation of PKCδ. Since signals from the BCR can serve to propagate many different cellular responses (Gold and DeFranco, 1994; Gold and Matsuuchi, 1995), many different readouts of B cell function would need to be considered to evaluate the role of PKCδ in the B cell. First, selectively depleting B cells of PKCδ through the use of targetted antisense oligonucleotides would elucidate the effects on the BCR crosslinking-induced apoptosis of Ramos B cells (Chaouchi et al., 1995). Since PKCδ was not proteolytically cleaved within 4 hours in Ramos B cells, any observed effects of PKCδ depletion on BCR crosslinking-induced apoptosis would imply a function distinct from proteolytic activation. Second, the effects of PKCδ depletion on the BCR crosslinking-induced entry of mature B cells into the G1 phase of the cell cycle (Gold et al., 1990) would provide useful information as to the regulation of B cell cycle progression, in parallel to studies evaluating the effects of other isoforms of PKC on cell cycle regulation (Livneh and Fishman, 1997). Alternatively, the increased expression of class II major histocompatibility (MHC) molecules or cytokines could be evaluated in the situation of PKCδ depletion, as an indication of the antigen-presenting function of B cells.

The immediate nature of the activation and tyrosine phosphorylation of PKCδ in response to BCR crosslinking suggests that PKCδ mediates one of the early signals generated from the BCR. Thus, the potential for B cell activation through the BCR would also be considered, perhaps utilizing an *in vivo* approach of PKC depletion with a $PKC\delta^{-/-}$ (knockout) mouse. This model would allow for many readouts of B cell function in terms of the humoral immune system, including antibody production, isotype switching,

proliferation in response to antigenic challenge, and maturation and development of the humoral response.

The kinase which phosphorylates PKCδ on tyrosine residues has not yet been identified in the B cell. This could be accomplished by using the DT40 chicken B cell lines which lack Lyn, Syk, or Btk, or B cells from the specific PTK knockout mice could also be used to characterize the effects of the BCR crosslinking signal on PKCδ in the absence of selected PTKs. Further, the cellular consequence of such signaling effects on PKCδ could be evaluated, in terms of the role of tyrosine phosphorylation in the activation and regulation of PKCδ. Alternatively antisense oligonucleotides to the prominent PTKs which have been reported to become activated in response to BCR crosslinking could be used to evaluate the function of tyrosine phosphorylation in human B cells. The far downstream effects would be evaluated in terms of activation of normal tonsillar B lymphocytes, isolated from human patients.

The participation of the Ras pathway in the activation and tyrosine phosphorylation of PKCδ needs to be addressed. The Ras pathway has been shown to become activated in response to BCR crosslinking, including the selective activation of the p42 isoform of mitogen activated protein kinase (MAPK) (Gold et al., 1993; Gold et al., 1992). While the literature alludes to Raf-1 being downstream of PKCα and associated with PKCε (Kolch et al., 1993; Ueffing et al., 1997), it is still not clear whether PKC can activate Ras, or whether activation of Ras through its adaptor proteins (Shc, mSOS, Grb2) in response to BCR crosslinking would promote the activation of PKCδ. In any event, PKC's participation in the Ras pathway is upstream of MAPK, and preventing activation of this pathway with compounds such as PD 98059 (selective MAPK kinase (MEK) inhibitor) or SB 202190 (p38 MAPK inhibitor) would not likely affect the activation or tyrosine phosphorylation of PKCδ.

The result from the study with H_2O_2 (Konishi et al., 1997) suggests very strongly that the stress-activated protein kinase (SAPK) pathway is a separate pathway influencing the activation and tyrosine phosphorylation of all PKC isoforms, and with the extensive crosstalk between the SAPK and MAPK pathways (Duh et al., 1997; Zu et al., 1997), it is possible that this pathway contributes to the regulation of PKCδ, however, it is not thought that this process occurs in response to BCR crosslinking. Rather, the SAPK response may function as a "panic" response, which is likely to have very different cellular consequences than receptor-mediated stimulation.

As alluded to in Chapter Five, the phosphorylation of the other *novel* isoforms of PKC in response to BCR crosslinking needs to be examined. Although this thesis clearly demonstrated that PKCδ is unique among the cross section of isoforms across Groups including PKCα, δ, μ, and ζ, and that PKCδ is unique among the *novel* isoforms with respect to its behavior in other cellular systems, the fact still remains that the remainder of the *novel* isoforms have the potential, based on sequence analysis, to become tyrosine phosphorylated in response to BCR crosslinking in B cells (Aris et al., 1993; Bacher et al., 1991; Baier et al., 1993; Basta et al., 1992). PKCδ has been reported to be tyrosine phosphorylated at four sites; PKCη and PKCθ share all four of these phosphorylation sites, and PKCε three of the four, making it very possible that these isoforms may also become tyrosine phosphorylated in a whole cell system, perhaps even in response to BCR crosslinking. This could be addressed provided immunoprecipitating antibodies against each isoform could be generated or identified, in a manner similar to that conducted with PKCδ. If other isoforms become tyrosine phosphorylated, the investigation into the activation of these in response to BCR crosslinking would naturally follow.

Perhaps most relevant to this study on PKCδ is the identification of PKCδ–interacting proteins which may preferentially associate upon BCR crosslinking. This would likely include a member of the RACK family (for review, see (Mochly-Rosen, 1995) and (Mochly-Rosen and Gordon, 1998)), and may be dependent on tyrosine phosphorylation. This could be explored using many strategies based on co-association of the proteins, including metabolic cell labeling, and Western blot detection. An interesting follow up to this study would be to screen a peptide library for sequences that would prevent the association of PKCδ to its binding proteins, and potentially prevent activation and tyrosine phosphorylation. This may result in clinical relevance with respect to leukemia therapy.

Identification of the downstream effects of PKCδ activation would prove beneficial to the field of BCR signaling. Considering that PKCδ is selectively activated during the first minute of BCR crosslinking suggests that PKCδ has a very specific function to perform during this time, which is most likely the serine/threonine phosphorylation of a limited number of substrates. Proteins that are known to become serine/threonine phosphorylated in response to BCR stimulation include Lck (Gold et al., 1994; Watts et al., 1993), PKCμ (Sidorenko et al., 1996) and CD20 (Valentine et al., 1989). Of these, both PKCμ and CD20 are phosphorylated within the appropriate timeframe; the reported activation of PKCμ in response to the BCR may therefore be regulated by the activation of PKCδ. In addition, the serine/threonine phosphorylation of CD20 that is induced by either PMA or BCR crosslinking (Valentine et al., 1989) has recently been further characterized in our laboratory. Preliminary evidence has demonstrated that stimulation of the cell either with PMA or BCR crosslinking prevents CD20 from actively redistributing into a detergent-insoluble membrane compartment (Deans et al., 1998); furthermore, this effect occurs during the timeframe of maximal PKCδ activity. This suggests that PKCδ activation through BCR signaling may be an essential step in regulating the activity of CD20 in the B cell. Regardless of the identity of the substrate, this study would also prove essential in

demonstrating how a specific isoform of PKC is utilized by a single receptor to accomplish a very selective task, with respect to time and subcellular localization.

In conclusion, this study on the selective regulation of PKCδ in response to BCR crosslinking has contributed to the growing field of knowledge of BCR-mediated signals, in addition to providing an additional example of the selective use of one isoform of the PKC family. Future work in this area is sure to further elucidate the intricate mechanisms of cellular regulation.

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IMAGE EVALUATION
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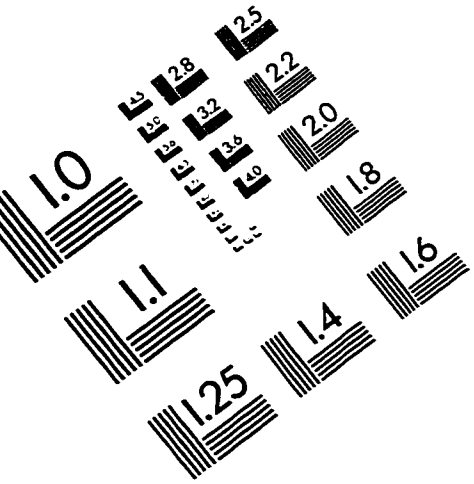

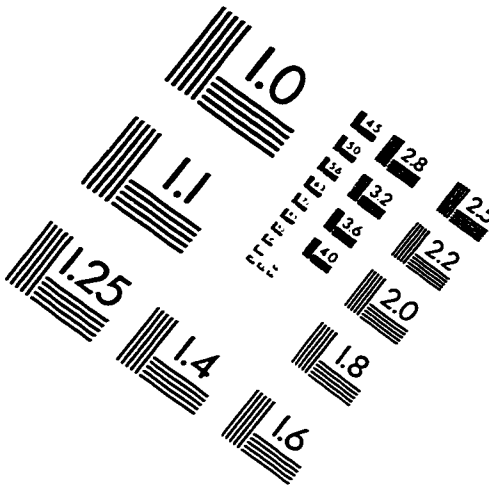

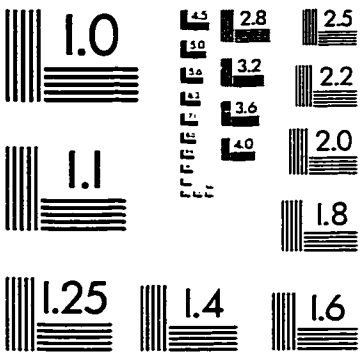

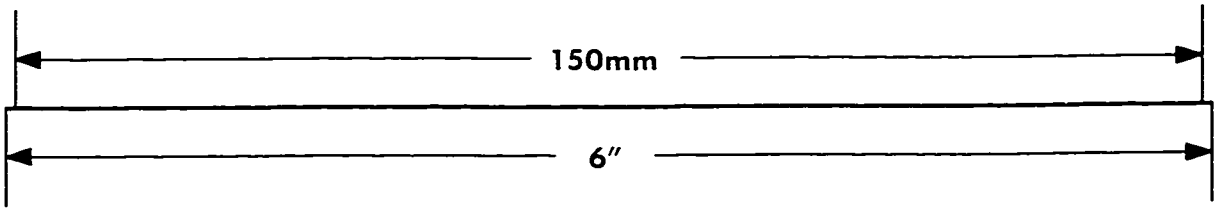

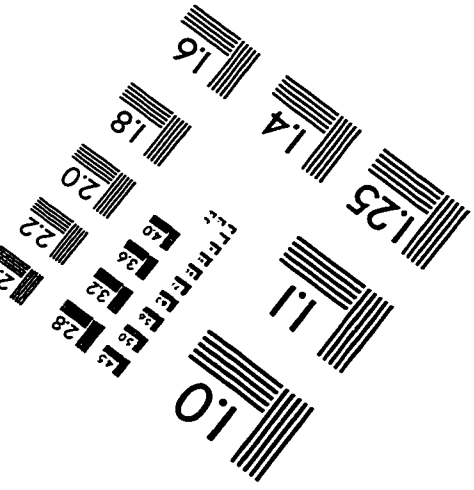

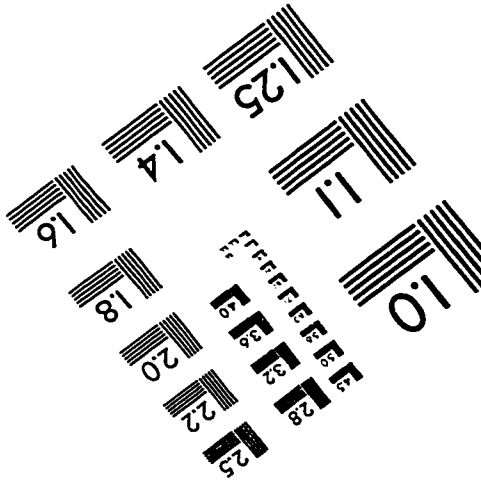

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