THE ROLE OF LIPID SIGNALLING IN LYMPHOCYTE ACTIVATION, MATURATION AND CELL DEATH.

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ABBREVIATIONS

adenosine 5'trisphosphate	ATP
antibody	Ab
antigen	Ag
APC	antigen presenting cell
B cell antigen receptor	BCR
ceramide-activated protein kinase	CAPK
concanavalin A	Con A
cyclic AMP	cAMP
diacylglycerol	DAG
eicosatriynoic acid	ETI
epidermal growth factor	EGF
fMetLeuPhe	fMLP
follicular dendritic cell	FDC
free fatty acid	FFA
G protein	GTP-binding protein
germinal centre	GC
guanine diphosphate	GDP
guanine trisphosphate	GTP
immunoglobulin	Ig
immunoreceptor tyrosine-based activation motif	ITAM
inositol phosphate	InsP
inositol trisphosphate	$InsP_3$
interleukin	IL
lipid second messenger	LSM
lipopolysaccharide	LPS
lysophosphatidic acid	LPA
lysophosphatidylcholine	LPC
major histocompatibility complex	MHC
pervanadate	pV
phorbol-12-myristate acetate	PMA
phosphate buffered saline	PBS
phosphatidic acid	PtdOH
phosphatidylbutanol	PtdBut
phosphatidylcholine	PtdCho
phosphatidylethanolamine	PtdEtn

phosphatidylinositol bisphosphate	$PtdInsP_2$
phosphatidylinositol	PtdIns
phosphatidylinositol trisphosphate	$PtdInsP_3$
phospholipase A_2	PLA_2
phospholipase C	PLC
phospholipase D	PLD
phytohaemaglutinnin	PHA
platelet-derived growth factor	PDGF
protein phosphatase 2A	PP2A
protein tyrosine kinase	PTK
sphingosine-1-phosphate	SPP
surface immunoglobulin	sIg
T cell antigen receptor	TCR
T_H cell	T helper cell
thymus-dependent	TD
thymus-independent	TI
tumour necrosis factor	TNF

SUMMARY

Lymphocytes respond to antigen receptor stimulation in a maturation stage-dependent manner. Thus, whereas mature lymphocytes become activated and proliferate in response to antigen receptor crosslinking, immature lymphocytes become unresponsive or undergo apoptosis. Initial studies, however, failed to identify any differences in the signals generated immediately following the crosslinking of antigen receptors on mature and immature lymphocytes: protein tyrosine kinase activation, inositol phospholipid hydrolysis and the mobilisation of calcium. Recent reports have indicated a key role for the stimulated hydrolysis of phosphatidylcholine in the control of a variety of cellular responses including membrane trafficking, the respiratory burst, proliferation and apoptosis. This investigation has focused on the role of phosphatidylcholine hydrolysis in the regulation of lymphocyte activation, maturation and apoptosis. Two key enzymes which mediate the hydrolysis of phosphatidylcholine are phospholipase D and phospholipase A_2.

Phosphatidylcholine-specific phospholipase D has been found to play a role in the transduction of intracellular signals initiated by mitogenic stimulation of a variety of cell types. This investigation has identified a number of differentially-regulated phospholipase D activities which may play key roles in mediating the transduction of mitogenic and antiproliferative signals in B cells. Initial pharmacological studies identified multiple phosphatidylcholine-specific phospholipase D activities, which were regulated by protein kinase C-, tyrosine phosphorylation- and G protein-dependent mechanisms, in B cells. However, it was found that stimulation of B cells with anti-Ig antibodies did not induce activation of phosphatidylcholine-specific phospholipase D, indicating that the B cell antigen receptor is not coupled to phosphatidylcholine-specific phospholipase D in these cells. Moreover, phosphatidylcholine-specific phospholipase D was not stimulated following crosstalk between surface immunoglobulin, interleukin-4 receptors, Class II molecules, and other T cell interacting coreceptors such as CD40, conditions designed to mimic T cell-dependent B cell activation. However, further investigation identified an as

yet undefined phospholipase D activity which could be stimulated via sIg on B cells, and phospholipid labelling studies indicated that this could be a phosphatidylinositol-specific phospholipase D activity. Further investigation of the role of phosphatidylcholine-specific phospholipase D found that stimulation of B cells with adenosine trisphosphate induced phosphatidylcholine-specific phospholipase D activation. As adenosine trisphosphate was found to suppress B cell proliferation, this suggests that a phosphatidylcholine-specific phospholipase D activity(ies) could be involved in the transduction of negative, anti-proliferative signals in B lymphocytes. Further investigation of a role for phosphatidylcholine-specific phospholipase D in negative signalling in B cells found that the lipid second messengers sphingosine and ceramide, which are implicated in the regulation of apoptosis in many cell types and exert potent anti-proliferative effects on B cells, induced the activation of phosphatidylcholine-specific phospholipase D in B cells. These results therefore indicate that phosphatidylcholine-specific phospholipase D is not involved in the transduction of signals regulating the initiation or maintenance of antigen-driven B cell proliferative responses, and could indicate a role for phosphatidylcholine-specific phospholipase D in the negative modulation of B cell activation.

Phospholipase A_2 has been implicated in the control of proliferation and apoptosis in a number of cells types, and this investigation has generated a number of findings which indicate a key role for cytosolic phospholipase A_2 in lymphocyte maturation. Firstly, cytosolic phospholipase A_2 is expressed by B and T cells in a maturation state-specific manner. Western blotting studies demonstrated that mature lymphocytes, and a number of cell lines representing this maturation state, do not express cytosolic phospholipase A_2. In contrast, cytosolic phospholipase A_2 was found to be expressed in immature murine splenocytes, murine thymocytes, T3 murine thymoma cells and WEHI 231 murine immature B cells, although not in the pre-B cell lines 697, 207 and REH. Secondly, the antigen receptors on immature B and T cells were found to be coupled to cytosolic phospholipase A_2 activation, indicating that arachidonate release may be involved in the antigen receptor-mediated induction of growth arrest and/or apoptosis. Pharmacological studies supported this, demonstrating that certain agents which induce

apoptosis (eg. ionomycin in T3 cells) activate cytosolic phospholipase A_2, whereas those which do not induce apoptosis (eg. phorbol ester in all the immature lymphocytes investigated) failed to induce arachidonate release.Thirdly, stimulation of WEHI 231 immature B cells with specific stimuli known to rescue these cells from sIgM-mediated apoptosis, including interleukin-4, CD40 crosslinking and lipopolysaccharide, did not stimulate cytosolic phospholipase A_2 activity. Moreover, interleukin-4 was found to block anti-Ig-mediated release of arachidonate, suggesting that these stimuli may effect B cell rescue from apoptosis through the inhibition of intracellular signals such as cytosolic phospholipase A_2 activation. The findings of this investigation therefore strongly implicate the antigen receptor-mediated release of arachidonate in the induction of growth arrest and/or apoptosis. Furthermore, sphingomyelinase, a signal transducer which has been shown to be activated by released arachidonate in other cell types, was found to be temporally downstream of cytosolic phospholipase A_2 activation following crosslinking of the antigen receptors on WEHI 231 immature B cells.

1. INTRODUCTION

The experiments described in this thesis have investigated the role(s) of lipid signalling in the maturation and proliferation of B lymphocytes. However, where appropriate, parallel studies have also been performed on T cells in order to determine whether such lipid signals play a universal role in lymphocyte development. Therefore, since many of the signals resulting from ligation of the antigen receptors on B and T cells are essentially similar, this introduction focuses almost exclusively on the intracellular signals involved in the regulation of maturation, activation, and apoptosis in B cells.

1.1 The function of B lymphocytes in the humoral immune response.

B lymphocytes are the principal cellular mediators of the specific humoral immune response to infection. In this context, they perform both recognition and effector functions within the immune system. Thus, B cells recognise and respond to specific antigen via clonally distributed antigen receptors, and recognition of foreign antigen by mature B cells leads to their activation, proliferation and differentiation, ultimately culminating in the development of antibody-secreting plasma cells. The physiological function of secreted antibodies is to neutralise and initiate the elimination of foreign antigen by several phagocytic effector mechanisms.

Activation of B cells by foreign antigen can also lead to the development of memory B cells, which serve as a repository of immunological memory and are responsible for the secondary humoral immune response to antigen. Memory B cells respond to their specific antigen more rapidly than naive resting B cells, and their activation results in differentiation to plasma cells, secreting antibody with increased affinity for antigen. Thus, repeated challenge of the immune system with an antigen (eg. from an endemic pathogen) will induce an humoral immune response which increases in rapidity and efficacy with each subsequent infection. It is this phenomenon which forms the basis for vaccination against specific pathogens.

1.2 B cell development, maturation and activation.

B lymphocytes arise from pluripotent hematopoietic stem cells in the bone marrow, via a number of stages comprising well defined precursor B cell phenotypes. This is a complex process integrating signals generated by a number of soluble factors (including cytokines) and specific immunoregulatory receptors on several cell types, often in specialised environments (reviewed by Cushley and Harnett, 1993) (1). This phase of B cell development is termed the antigen-independent phase, and culminates in the development of immature B cells expressing functional antigen receptors (sIg) of the IgM isotype, with each clone expressing receptors specific for only one antigen epitope. The cellular and molecular events of antigen-independent B cell maturation are summarised in Figure 1.

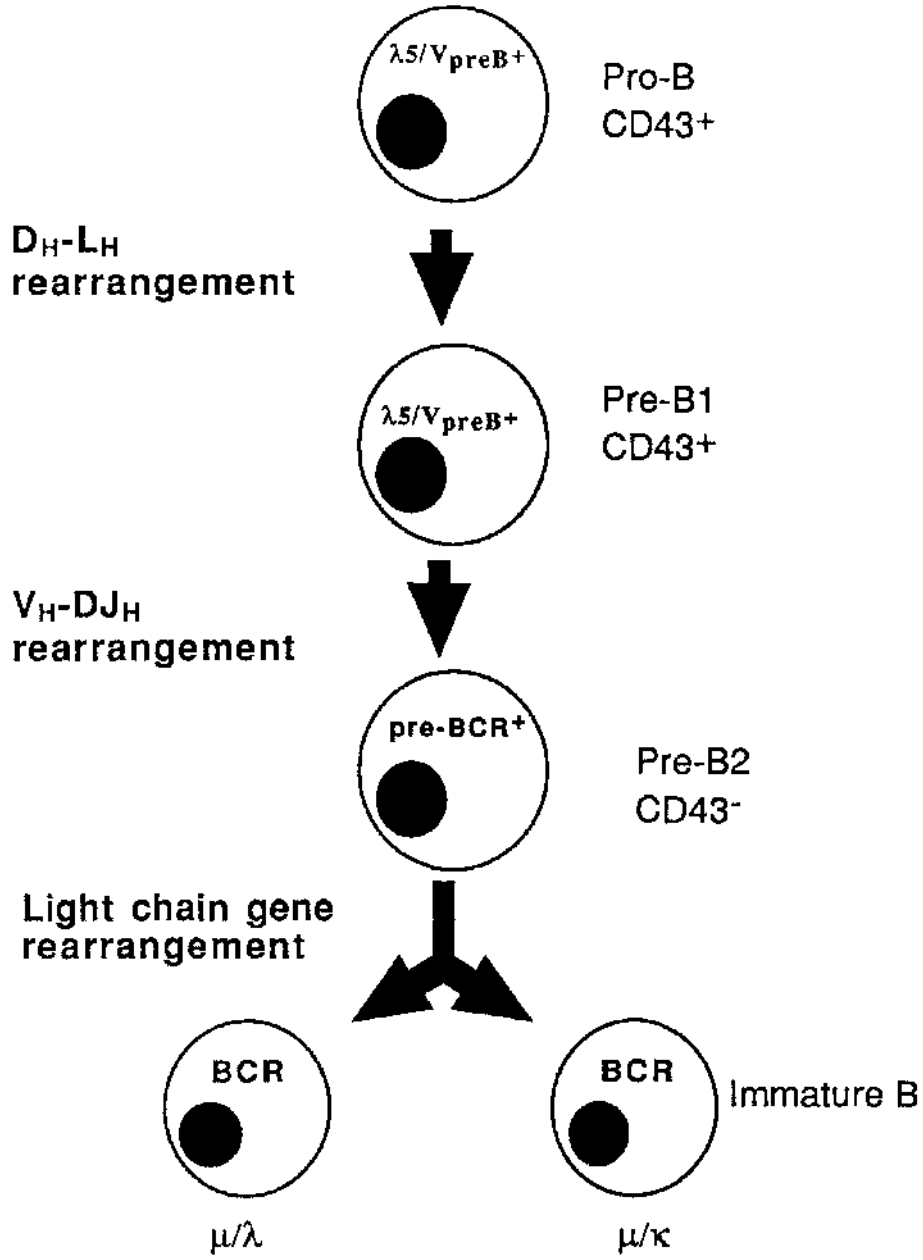
The antigen-independent phase is followed by the antigen-dependent phase of B cell maturation which occurs in the periphery, predominantly in secondary lymphoid organs such as the spleen and tonsils. This phase involves the selection of appropriate B cell clones by antigen, leading to (i) cellular anergy and/or apoptosis of immature B cells (2-4), (ii) activation and proliferation of mature B cells or (iii) rescue of germinal centre cells from cell suicide followed by differentiation into antibody-secreting plasma cells or memory B cells (reviewed by Cushley & Harnett, 1993; and MacLennan, 1994) (1, 5). Thus, the first stage of antigen-dependent B cell maturation involves the negative selection of IgM^+ immature lymphocytes, resulting in the anergy or deletion of autoreactive B cell clones. This mechanism gives rise to a self-tolerant naive mature B cell population expressing both sIgM and sIgD, and is followed by a process of positive antigenic selection, in which the appropriate B cell clones become activated, proliferate and differentiate to generate antibody-secreting plasma cells or memory B cells. These selected cells may express immunoglobulins of the A, E or G isotype, according to the requirements of the particular response to antigen (5). In contrast, unless mature B cells are selected by antigen, they have been found to die by apoptosis with a half-life of 3-4 days These mechanisms of B cell generation and selection account for the dynamic nature and plasticity of the immune system: the generation of a population of B cells capable of

responding to a vast array of different antigens, the deletion of autoreactive B cells, and the constant renewal of the pool of B lymphocytes capable of recognising and responding to foreign antigen.

1.2.1 The role of germinal centres in the development of memory B cells

Memory B cells develop in secondary lymphoid organs such as lymph nodes, the spleen, tonsils and Peyer's patches. The generation of memory cells from naive resting B cells occurs via a number of discrete stages and is mediated by stimuli derived from a number of other cell types, including T cells (5). Resting B cells circulate in the blood, and migrate across high endothelial venules to sites of trapped antigen in the secondary lymphoid organs (6). Those B cells bearing antigen receptors specific for trapped antigen enter the T cell-rich paracortical regions of the secondary lymphoid tissues, where the captured Ag are processed by proteolysis and presented as peptide fragments in the context of class II MHC molecules. Recognition of MHC-bound Ag fragments induces the activation of specific T helper (T_H) cells, resulting in the expression of T cell coreceptors and the release of cytokines. These stimuli induce the activated B cells to migrate into B cell follicles (5).

Follicles consist of a network of follicular dendritic cells (FDCs) (1), which specialise in binding unprocessed antigen in immune (Ag-Ab) complexes and in inducing B cell proliferation and differentiation. Following entry into the FDC network, the sIg^+ B cells proliferate exponentially, filling the network. This proliferation gives rise to a dark zone on the edge of the developing germinal centre (GC). The B cells then differentiate to centrocytes, which lose expression of sIg and introduce mutations into their Ig V-region genes as they continue to proliferate (1). This process is known as somatic mutation, and gives rise to sIg with altered antigen binding properties. As the centrocytes proliferate, they give rise to centroblasts, non-proliferating cells which express the mutated immunoglobulin genes as sIg, before migrating into the light zone of the germinal centre, which possesses a dense network of FDCs, and some T cells (5). The centrocytes then undergo a process of positive selection, in which they compete for the


λ5/VpreB+
Pro-B
CD43+
DH-LH
rearrangement
λ5/VpreB+
Pre-B1
CD43+
VH-DJH
rearrangement
pre-BCR+
Pre-B2
CD43-
Light chain gene
rearrangement
BCR
BCR
Immature B
μ/λ
μ/κ

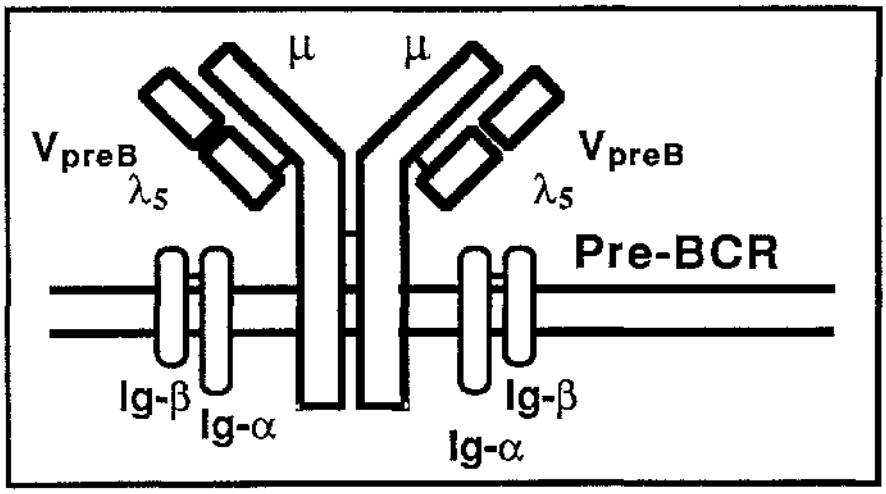

μ
μ
VpreB
λ5
VpreB
λ5
Pre-BCR
Ig-β
Ig-α
Ig-β
Ig-α

Figure 1. Antigen-Independent B cell maturation and the role of the pre-BCR. Developing B cell precursors go through a series of discrete stages, comprising distinct cellular phenotypes. During this process developing B cells rearrange their immunoglobulin genes to allow the production of functional antibody molecules. The gene for immunoglobulin μ heavy chain is the first to be rearranged (in two maturation stages encompassing pro-B to Pre-B2). The rearranged gene is then expressed and forms a complex with two surrogate light chains, called λ5 and V_{preB1}. λ5 has a domain that is homologous to immunoglobulin constant regions, and V_{preB1} is homologous to Ig variable domains. "Knock-out" experiments have shown that these molecules are required for expression of μ-chain at the B cell surface, and it is likely that λ5 and V_{preB1} are involved in receptor assembly. Another possibility is that λ5 and V_{preB1} mediate the cell-cell contact needed by B cells in the bone marrow for full development. The μ-λ5-V_{preB1} complex also associates with Ig-α and Ig-β, and this complex is termed the pre-BCR (B cell antigen receptor). The expression of the pre-BCR has been found to be required for progression from the pre-B1 to the pre-B2 stage. Following expression of the pre-BCR, the immunoglobulin light chains (κ or λ) are rearranged. The expression of functional light chains as components of the B cell antigen receptor is then required for the development of immature B cells which can exit the bone marrow.

antigen bound on the surface of the FDCs. Those centrocytes expressing antigen receptors with the highest affinity for antigen survive, as the result of sIg crosslinking by antigen, and the remainder die by apoptosis (5). The surviving centrocytes also require T cell-derived signals for longterm survival. Antigen captured by specific binding to sIg is internalised by the centrocytes, then processed and presented in the context of class II MHC. If the antigen is recognised by T helper cells, then the B cells receive a signal via CD40 following its ligation by CD40L (7), which is expressed on the surface of the activated T cells. If they do not receive this signal the B cells die by apoptosis. The selected centrocytes may then differentiate to memory cells or plasmablasts (which further differentiate to nondividing plasma cells following migration to sites of infection). The generation of memory cells appears to require IL-4, IL-10 and other cytokines, in addition to CD40 crosslinking In contrast, the generation of plasmablasts appears to take place in the apical light zone, and is mediated by FDCs providing stimulation of B cells via IL-1, CD23 or other surface receptors (8). Such a tightly regulated mechanism has presumably evolved to prevent the development of autoreactive memory B cells during somatic mutation. (See Figure 2).

Typically, B cells undergoing differentiation in germinal centres undergo immunoglobulin class switching from IgM to IgA, IgE, or IgG (5). The isotype expressed usually depends on the secondary lymphoid organ in which the memory cell or plasmablast develops. Thus, B cells leaving the follicles of Peyer's patches or mesenteric lymph nodes are almost all committed to IgA expression (5, 9, 10). In contrast, memory B cells or plasmablasts leaving tonsil, spleen, or peripheral lymph nodes are mainly committed to IgG production with some expressing IgA, and very few expressing IgM (8). The time at which class switching occurs is not clear, however, in human tonsils most centroblasts have already deleted their heavy chain genes, indicating that switch signals are often delivered prior to the selection of centrocytes (5). Some specific signals have been identified which induce the production of specific Ig isotypes by B cells: i) the combination of soluble CD23 and IL-1 induces differentiation of centrocytes to IgG-secreting plasmablasts (8); ii) IL-2 induces a specific subpopulation of $CD5^+$ IgM^+

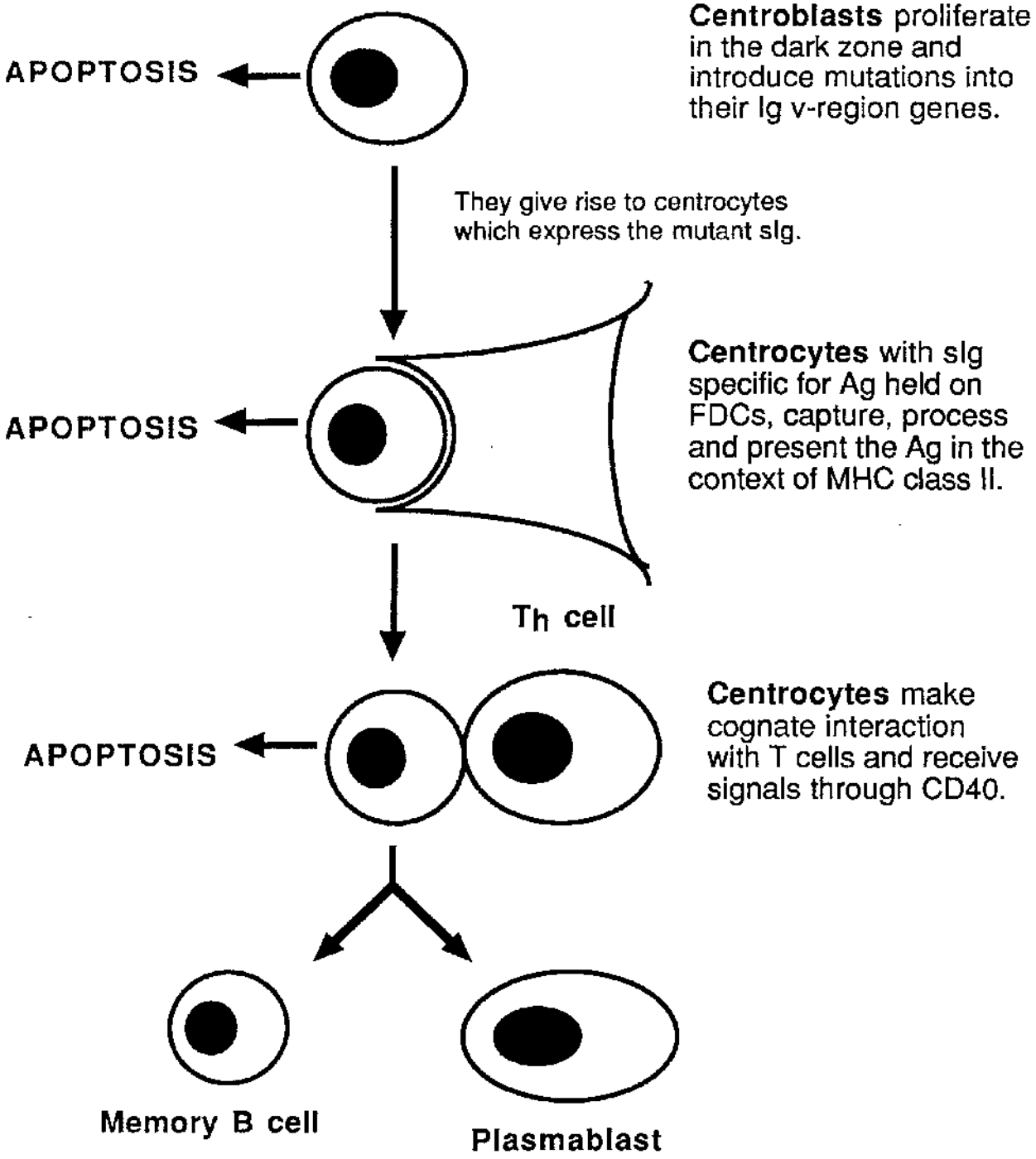

APOPTOSIS
Centroblasts proliferate in the dark zone and introduce mutations into their Ig v-region genes.
They give rise to centrocytes which express the mutant sIg.
APOPTOSIS
Centrocytes with sIg specific for Ag held on FDCs, capture, process and present the Ag in the context of MHC class II.
Th cell
APOPTOSIS
Centrocytes make cognate interaction with T cells and receive signals through CD40.
Memory B cell
Plasmablast

Figure 2. The role of germinal centres in the development of memory B cells and plasmablasts.

germinal centre B cells to become IgM-expressing plasmablasts (11) and iii) co-culture of B cells with FDCs and T cells (secreting IL-4), plus stimulation with IL-1, induces their proliferation and the production of IgG1 and IgE (12).

The function of germinal centre formation is therefore to generate memory B cells, expressing antigen receptors with increased affinity for antigen and Ig of an isotype suited to the specific requirements of the specific humoral response to infection.

1.2.2 Models of B cell activation.

Antigens were originally classified according to their ability or inability to stimulate specific antibody production in athymic individuals, (ie. individuals incapable of T cell production), and are thus termed thymus-independent (TI) or thymus-dependent (TD) antigens (13, 14). TI antigens are typically large molecules consisting of repeating immunogenic epitopes, which efficiently crosslink sIg and induce B cell proliferation and antibody production without a requirement for T cell help. The vast majority of proteins, however, are thymus-dependent antigens which crosslink sIg relatively inefficiently, and require the presence of T cells for the induction of B cell proliferation and antibody production. The study of receptor crosslinking by specific antigens is very problematic, due to the variation in antigen specificity of B cell receptors. As a result of this, the crosslinking of sIg with anti-Ig antibodies has been used extensively as an experimental model for antigen receptor crosslinking by specific antigens. Crosslinking of the B cell antigen receptor (BCR) with relatively high concentrations of anti-Ig has been found to induce the proliferation of B cells, and is used as a model for the activation of B cells by TI-2 antigens.

1.2.2.1 Thymus-independent antigens

There are many antigens which will stimulate antibody production in the absence of T cells, and these are termed thymus-independent (TI) antigens. TI-antigens have been subdivided into two types:

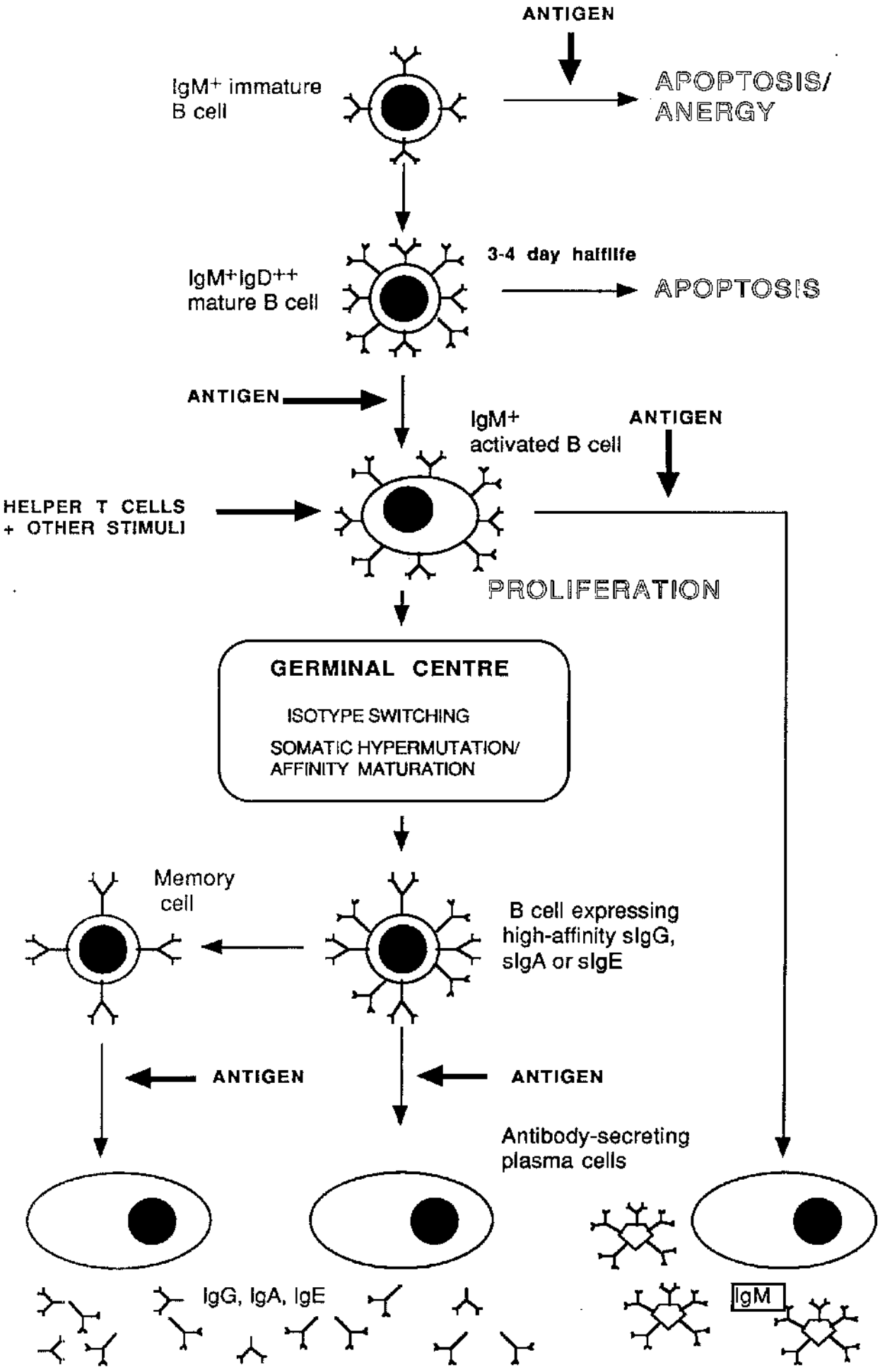

ANTIGEN
IgM+ immature B cell
APOPTOSIS/ ANERGY
3-4 day halflife
IgM+IgD++ mature B cell
APOPTOSIS
ANTIGEN
IgM+ activated B cell
ANTIGEN
HELPER T CELLS + OTHER STIMULI
PROLIFERATION
GERMINAL CENTRE
ISOTYPE SWITCHING
SOMATIC HYPERMUTATION/ AFFINITY MATURATION
Memory cell
B cell expressing high-affinity sIgG, sIgA or sIgE
ANTIGEN
ANTIGEN
Antibody-secreting plasma cells
IgG, IgA, IgE
IgM

Figure 3. Antigen-dependent B cell activation. Engagement of the antigen receptors on immature B cells results in cellular anergy or apoptosis. Crosslinking by antigen of the B cell receptors (BCR) on mature B cells results in their activation and proliferation, and if a mature B cell clone is not selected by antigen it dies with a half-life of 3-4 days. Further antigenic stimulation of activated B cells can result in their direct differentiation to IgM-secreting plasma cells. T_H cell-dependent stimulation of activated B cells leads to the development of germinal centres in secondary lymphoid organs which mediate the development of B cells bearing sIg of higher affinity for antigen, and typically of a different isotype (IgG, IgE or IgA). These cells differentiate to produce memory cells or antibody-secreting plasma cells.

i) TI-1 antigens, which are completely T cell-independent, are usually polyclonal mitogens for B cells. At high concentrations, they induce the proliferation of multiple B cell clones, irrespective of their antigenic specificity, and without necessarily binding to antigen receptors(15). This suggests that B cell activation by TI-antigens occurs independently of the antigen receptor . However, at lower concentrations (10 to 100-fold lower) TI-1 antigens bind to and stimulate specific B cells only. The classic example of a TI-1 antigen is lipopolysaccharide (LPS), which is a component of the cell walls of several gram-negative bacteria. Thus, at concentrations of 10 μg/ml or higher, LPS binds to and activates the majority of B cell clones *in vitro*. However, at significantly lower concentrations LPS only stimulates B cells expressing appropriate antigen receptors. It is proposed that a portion of LPS may directly stimulate B cells, possibly by mimicking the action of a lipid second messenger. B cell responses to TI-1 antigens are typical of those that would be expected in the absence of T cell help and cytokines. For example, LPS induces the proliferation of B cells and the secretion of high levels of IgM (and IgG3), but does not stimulate class switching to other isotypes, affinity maturation or development of memory B cells(1).

ii) TI-2 antigens induce the production of specific antibodies *in vivo* in athymic mice, but will not stimulate antibody production *in vitro* in the absence of T cells. Most TI-2 antigens are polysaccharides composed of repeating antigenic epitopes, eg. dextrans and pneumococcal polysaccharides(15). These antigens only stimulate specific B cells via antigen receptors and do not function as polyclonal mitogens. Although TI-2 antigens cannot be processed by B cells and presented with class II MHC, they do appear to require small numbers of T cells for full B cell activation and the production of antibodies, possibly because certain cytokines are required. Like the responses to TI-1 antigens, most TI-2 antigens elicit the production of only IgM antibodies, although some do induce class

switching to other isotypes, possibly by stimulating the production of specific cytokines.

The activation of B cells by TI-2 antigens is mimicked experimentally by the crosslinking of B cell antigen receptors with mitogenic concentrations of anti-Ig antibodies. B cell activation by polyclonal mitogens is typically modelled experimentally by stimulation of B cells with LPS (50 μg/ml).

1.2.2.2 T cell-dependent B cell activation.

Crosslinking of sIg using submitogenic concentrations of anti-Ig in the presence of T cell-derived cytokines and ligands has been employed as an experimental model for B cell stimulation by TD antigens. This model has been used in many studies characterising the T cell-derived signals which are required, in addition to antigen receptor crosslinking, for the activation of B cells and the production of antibodies. Limited sIg crosslinking, mediated by TD antigens or submitogenic concentrations of anti-Ig, induces abortive activation of B cells. This stimulates the internalisation of captured Ag, which are processed by proteolysis and presented as peptide fragments in association with MHC class II molecules, promoting the "cognate" recognition of the antigen by MHC class II-restricted T cells (ie. $CD4^+$ T helper (T_H) cells) (1). B cells therefore also function as antigen-presenting cells (APC). Such recognition of presented antigen results in activation of the cognate T cells, leading to the production of cytokines and upregulation of expression of specific receptors on the T cell surface (6). Interaction with appropriate counterstructures on the antigen-presenting B cell, which is the preferential recipient of such signals due to its proximity, leads to T cell-dependent B cell activation and differentiation into plasma cells (1, 6). Experiments reconstituting T cell-dependent B cell activation *in vitro* have identified many of the principal T cell-derived signals required for the activation of B cells (1). For example, submitogenic concentrations of anti-Ig were found to be co-mitogenic for B cells with the T cell-derived cytokine, IL-4. This led to the hypothesis that T cell-derived signals may interact with

signals generated via the B cell antigen receptor, possibly to "prime" B cells for additional signals generated during B-T cell co-operation. Indeed, Cambier and coworkers demonstrated that crosslinking of MHC class II molecules on IL-4/anti-Ig-primed murine B cells, is sufficient to induce the proliferation of resting B cells (16). Moreover, further addition of cytokines (IL-4 and IL-5) was observed to induce differentiation of such B cells into antibody-secreting plasma cells (16). This model of T cell-dependent B cell activation was supported by reconstitution experiments which showed that incubation of B cells with fixed, pre-activated T cells or T cell membranes, in the presence of IL-4 and IL-5, was sufficient to induce B cell proliferation and differentiation to antibody-secreting cells(17-19).

The finding that MHC class II crosslinking is involved in the T cell-dependent activation of B cells, serves to underline the fact that the specific response of B cells to TD antigens is inextricably linked to their function as antigen-presenting cells (APCs). Further investigation has identified a number of additional B-T cell contact-dependent signals that mediate the bidirectional signals occurring during T cell-dependent B cell activation and maturation (see Figure 4) (6). One of the most important of these appears to be the crosslinking of CD40, a glycoprotein (45-50 kDa) related to the receptor for tumour necrosis factor (TNF)-α, which is expressed on the surface of late pre-B cells, immature B cells, mature B cells and some accessory cells. The T cell counterstructure for CD40 is the CD40 ligand (CD40L) (gp39), a TNF family member which is expressed on the surface of activated T cells. Crosslinking of CD40 has been shown to promote B cell proliferation, prevent apoptosis of germinal centre B cells and promote Ig isotype switching (1, 5). Other receptors involved in B cell-T cell bidirectional signalling include CD80 (B7/BB1) which is expressed on the surface of B cells activated by BCR or CD40 crosslinking. The interaction of CD80 with CD28, its coreceptor on T cells, stimulates T cells to express CD40L and to make the cytokines needed to induce Ig class switching in B cells. Thus, there is bidirectional B-T cell contact-dependent signalling mediating the activation of both cells, and this can be initiated by the prior activation of either cell by antigen receptor crosslinking. This complex mechanism of bi-directional signalling is

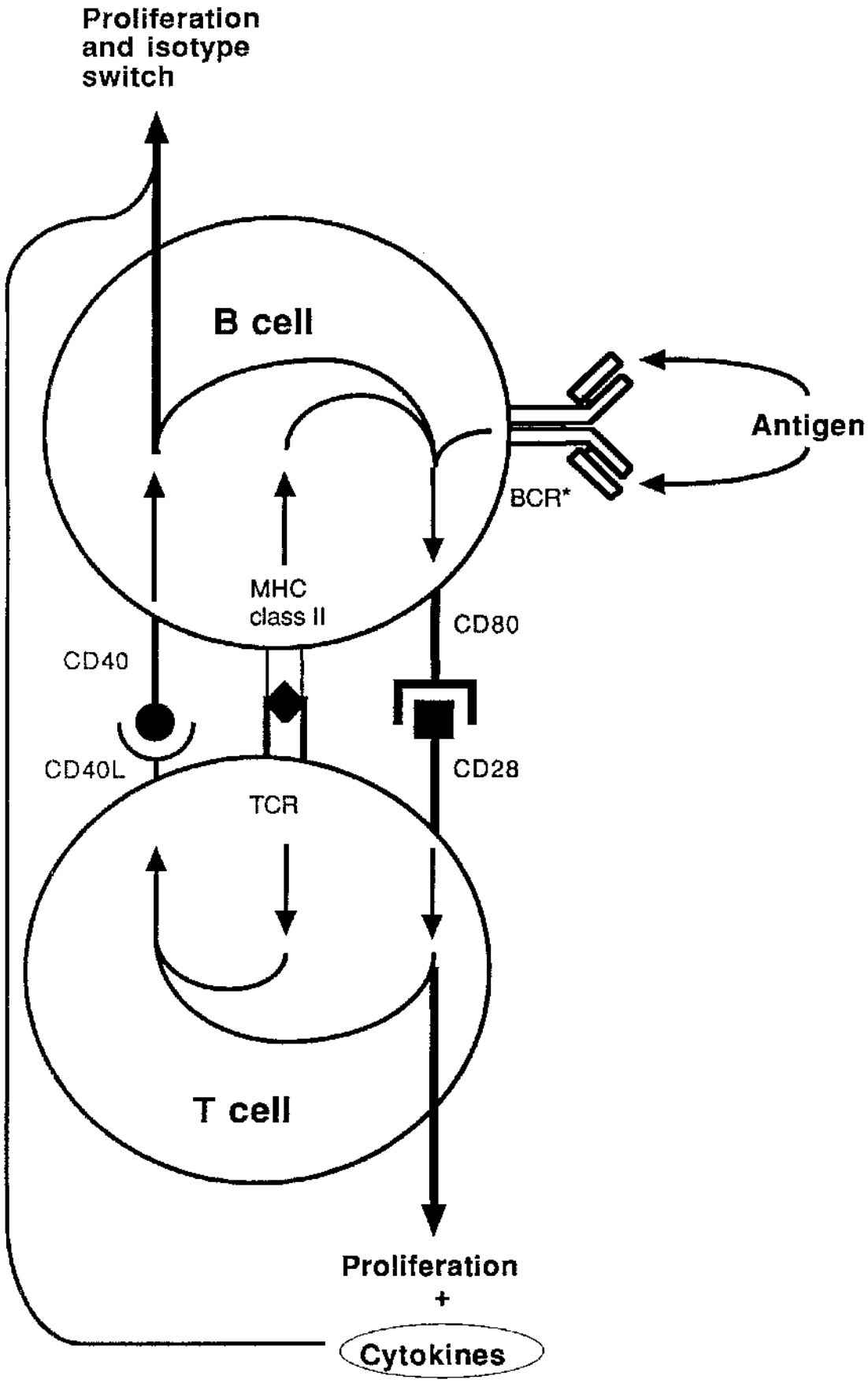

Proliferation
and isotype
switch
B cell
Antigen
BCR*
MHC
class II
CD80
CD40
CD40L
CD28
TCR
T cell
Proliferation
+
Cytokines

Figure 4. B-T cell contact-dependent bi-directional signalling. Recognition of antigen by B cells via sIg results in antigen processing and the presentation of peptide fragments in the context of MHC Class II molecules. Recognition of presented peptide by T cells leads to their activation and expression of CD40L. Crosslinking of class II molecules activates B cells and induces expression of CD80 (B7/BB1). Engagement by CD40L and CD80 of their respective counterstructures, CD40 on B cells and CD28 on T cells, amplifies the initial reaction and stimulates B and/or T cell proliferation. This bi-directional signalling also induces T cells to make the cytokines required by B cells for Ig class switching.

summarised in Figure 4. In addition there are a number of coreceptors of the B cell receptor which appear to act to reduce the threshold of antigen required for B cell activation. These include CD19/CD21 (20, 21), CD22 (22) and CD38 (23), as well as CD54 which interacts with counterstructures on T cells (CD11a/18) (6) possibly stabilising and modulating lineage-specific signalling.

1.3 The B cell antigen receptor as a transmembrane signal transducer.

1.3.1 Structure of the B cell antigen receptor.

Recent studies have defined the BCR as a multiprotein complex comprising a clonatypic antigen binding component, sIg, and its accessory signal transducing molecules, Ig-α and Ig-β (reviewed by Cambier *et al.*, 1994 (24); Pleiman *et al.*,1994 (25); DeFranco, 1995 (26)). Surface immunoglobulin consists of homodimeric heavy chains that are each covalently associated with a light chain, forming a symmetrical four chain structure with two antigen binding sites. There are five classes of immunoglobulin, IgM, IgD, IgG, IgA and IgE, and although all of these can be expressed as B cell antigen receptors, only IgM and IgD are expressed on mature naive B cells, which constitute the vast majority (>90%) of all peripheral B cells. Analysis of μm and δm heavy chain structure predicts that each heavy chain forms a single transmembrane-spanning domain with a short cytoplasmic tail. There are two algorithms which have been used to predict BCR structure: Kyte & Doolittle (1982) (27) predicted a charged cytoplasmic tail of only three amino acids - KVK- for μ, whereas Klein, Kanehisa and Delisi (1985) (28) predicted a cytoplasmic tail of 11 residues. The other Ig isotypes were predicted to have longer cytoplasmic tails when expressed as sIg: whereas Kyte and Doolittle predicted that sIgG and sIgE would each have tails of 28 residues, and that sIgA would have a tail of 14 amino acids, Klein, Kanehisa and Delisi predicted tails of 36 residues for sIgG and sIgE, and 22 for sIgA. These cytoplasmic domains are clearly too small to possess any

intrinsic catalytic activity, suggesting that BCR transmembrane signalling is probably mediated via the interaction of sIg with accessory signal transducing molecules.

Recent studies have found that sIg associates with a protein complex comprising two glycoproteins expressed as a disulphide-bonded heterodimer (29, 30). This heterodimer is composed of the Ig-β (CD79b), which is encoded by the B29 gene, and the Ig-α chain (CD79a), which is encoded by the *mb*-1 gene (31). Both molecules have only been found to be expressed in B cells (24-26, 32). Ig-α and Ig-β are transmembrane glycoproteins which are structurally very similar to the T cell CD3 γ, δ, and ε chains and can associate with all classes of sIg. The stoichiometry of the complex formed between the clonotypic sIg chains and the Ig-α/ Ig-β heterodimer remains to be defined, but a ratio of 1:2 has been suggested by the bilateral symmetry of sIg (see Figure 5).

Ig-α and Ig-β have been implicated in antigen receptor assembly and intracellular trafficking, internalisation and signal transduction (24-26, 32). Studies involving mutational analysis have indicated that Ig-α and Ig-β associate with the CH3 and/or CH4 domains and the transmembrane spanning region of μ (29, 31). A polar patch within the transmembrane-spanning region,-TTAST- has been identified which signals retention in the ER unless Ig-α and Ig-β are also expressed (33). It has been proposed that the engagement of this site by Ig-α/Ig-β facilitates transport of the assembled receptor through the ER. Thus, interactions between sIg and the Ig-α/Ig-β heterodimer are required for receptor assembly and transport to the plasma membrane. Interestingly, however, IgD and certain IgG sub-classes can be expressed at the cell surface in the absence of the Ig-α protein, although the functional significance of this is not yet known (24, 34).

Both Ig-α and Ig-β have sizeable intracellular domains (61 and 48 amino acids respectively), and, although these domains do not possess any intrinsic catalytic activity, there is considerable evidence that they are involved in signal transduction via B cell antigen receptors. Firstly, chimeric receptors of CD8, IgM or FcγRII expressing the cytoplasmic tails of Ig-α and Ig-β are effective signal transducers (24, 35). Moreover, Ig-α and Ig-β chimeras were found to differ in their ability to activate specific BCR-

coupled signalling events, suggesting some compartmentalisation of signalling functions between the two proteins. Secondly, Ig-α and Ig-β possess motifs which are also found in the cytoplasmic domains of a variety of multimeric receptors including the TCR ζ chain and CD3 γ, δ, and ε complex components (24, 36). This motif consists of the conserved sequence, D/E-X_7-D/E-X_2-Y-X_2-L/I-X_7-Y-X_2-L/I, and has been termed the "immunoreceptor tyrosine-based activation motif" (ITAM) (37). ITAM motifs have been demonstrated to recruit cytoplasmic signal transducers and are capable of transducing signals leading to the activation of protein tyrosine phosphorylation and calcium mobilisation (24). Phosphorylation of the tyrosine residues within the ITAM motif appears to be the critical event regulating signal transduction via these accessory molecules: even conservative mutations (eg Y > P) abrogate receptor-mediated signalling (25).

However, in addition to the proposed role for the Ig-α/Ig-β heterodimer, the C-terminus of μm has been implicated in BCR signal transduction. Recently, analysis of μ mutants has found that point mutations in a sequence of polar amino acids in the C-terminus of μ can abrogate anti-Ig antibody-induced signalling without affecting Ig-α/Ig-β binding (24). This region was predicted, using the algorithm of Klein et al. (28), to lie in the 11 residue cytoplasmic domain of m and therefore to be capable of interaction with cytoplasmic effector proteins. Thus, sIg may couple to a signal transducer(s) through direct interaction, as well as indirectly through the Ig-α/Ig-β heterodimer (24, 34).

1.3.2 Signalling via the B cell antigen receptor.

One of the most immediate events following the ligation of B cell antigen receptors is the activation of mulitple protein tyrosine kinases (PTK). These include members of the Src family: Fyn, Lyn, Blk and Lck, and the more distantly related kinase, Syk (24, 25, 38-42). Although there is some debate as to the precise sequence of activation of these PTKs, recent kinetic studies have indicated that, following ligation of the BCR, increased activity of Src family members Blk, Lyn and Fyn precedes that of Syk, suggesting that the activation of Src kinases may initiate BCR signal propagation

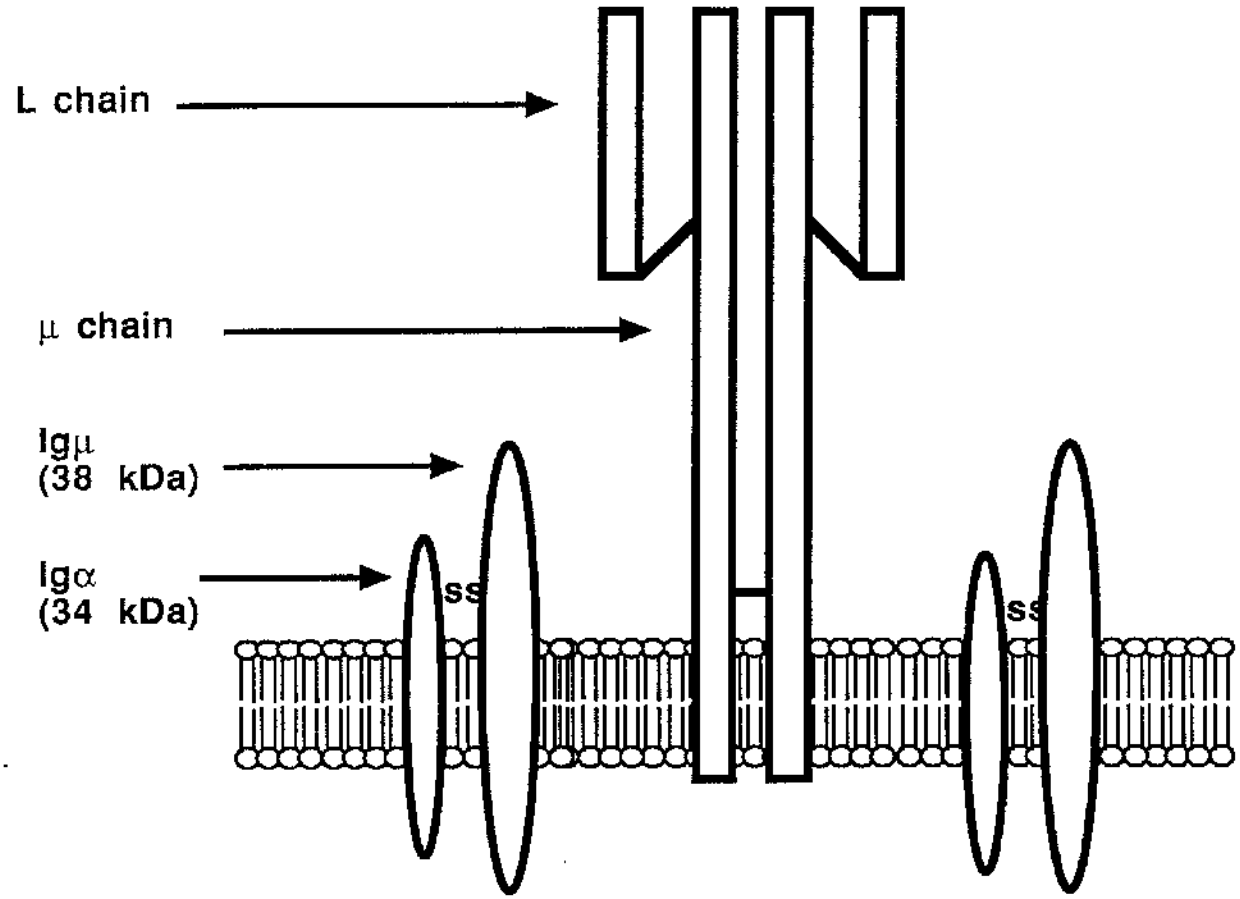

L chain
μ chain
Igμ
(38 kDa)
Igα
(34 kDa)
ss
ss

Figure 5. The B cell antigen receptor. Surface immunoglobulin is composed of a homodimer, each component consisting of a μ heavy chain and a light chain (λ or κ). sIg associates with a disulphide-linked hertodimer composed of Ig-α and Ig-β.

(43). These Src-related kinases associate with the BCR in unstimulated cells via interaction of their N-terminal domains with nonphosphorylated Ig-α. One of the first events to occur following BCR ligation is the tyrosine phosphorylation of ITAM motifs which leads to a dramatic increase in the binding and activity of Src-related PTKs (24, 25, 34, 36). This is associated with a reorientation of the kinases and is mediated via the interactions of their Src homology domain 2 (SH2) regions with the phosphorylated tyrosine residues of the ITAM motif. Further evidence for the involvement of Src tyrosine kinases, rather than Syk, in the initiation of BCR signal transduction includes:

i) crosslinking of the antigen receptors on T cells, which may prove analogous to stimulation via the BCR, induced the activation of Src family PTKs more rapidly than syk or ZAP-70 (a related PTK) (44, 45).

ii) the targeted disruption of Lyn in a chicken B cell line, which express only this member of the Src family, results in a severe decrease in BCR signalling and a disruption of the tyrosine phosphorylation and activation of Syk, suggesting that Lyn acts upstream of Syk (46);

iii) stimulation of the BCR when it is expressed in AtT20 - a non-lymphoid B cell line which expresses Lyn but not Syk, results in the normal tyrosine phosphorylation of Ig-α and Ig-β, but very few other signalling events, suggesting that Lyn will phosphorylate ITAM motifs, but that an additional component, possibly Syk, is required for the transduction of downstream signals (47).

One model for the sequential activation of PTKs following BCR ligation, proposed by DeFranco (1995) (26), suggests that the phosphorylation of the ITAM motifs by Src kinases such as Lyn or Fyn results in the binding of Syk, which is already associated with the resting receptor complex, to the phosphorylated ITAM motifs (via its

two SH2 domains). Src kinases, which are also recruited to the phosphorylated ITAM motifs, then phosphorylate and activate Syk, which in turn tyrosine phosphorylates downstream target proteins. The association of Syk with the resting receptor complex has been demonstrated using immunoprecipitation of sIg. It was also found that if the receptor complex was stripped of Ig-α and Ig-β using a nonionic detergent (eg. NP-40) then Syk remained associated with sIg, indicating that Syk associates with this component of the resting BCR, possibly via the polar region in the cytoplasmic tail of μm (24, 38) (Syk lacks both hydrophobic sequences and a myristoylation site, indicating that it probably cannot associate independently with the plasma membrane or with the transmembrane portion of sIg). Recent studies have sugggested that tyrosine kinases, such as Srcs and Syk, recognise substrate peptides differentially and hence are likely to recruit different effector molecules. Each PTK could therefore activate distinct downstream pathways, and it is possible that the relative levels of the various PTKs could dictate the responses of B cells to BCR ligation, possibly in a maturation stage-specific manner (24).

There are a number of signalling pathways activated downstream of PTK activity following ligation of the BCR. One of the these involves the tyrosine phosphorylation and activation of $PtdInsP_2$-specific PLC-γ, which appears to require Syk in cooperation with Lyn (24, 34). However, G-protein antagonist (GDPβS) and reconstitution studies indicate that this regulation is additionally and proximally regulated by a pertussis toxin-sensitive G protein(s) (reviewed by Harnett & Rigley, 1992) (48). The precise mechanism of PLC-γ activation, and the respective roles of PTK activities and heterotrimeric G proteins, remains unclear. The PLC-γ-mediated hydrolysis of $PtdInsP_2$ results in the generation of inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilises intracellular calcium stores, resulting in a rise in the concentration of Ca^{2+} in the cytosol and activation of calcium-calmodulin kinase II (CamKII). There is evidence to suggest that CamKII phosphorylates the Ets-1 DNA binding protein (24), possibly modifying its transcription regulating activity. DAG is an activator of various protein

kinase C (PKC) isoforms. The PKC isoforms which are expressed in B cells include α, β, δ, ζ, and η of which α, β, and δ are DAG regulated (49). PKC has been found to modify the activity of the transcription factor AP-1, which is implicated in the early response of lymphocytes to activation through the antigen receptor (50).

Another cytoplasmic signalling pathway activated by BCR ligation involves phosphatidylinositol 3-kinase, which phosphorylates the 3 position on the inositol ring of inositol phosholipids (reviewed by Cantley, 1991) (51). It has been demonstrated that ligation of the BCR leads to the tyrosine phosphorylation and subsequent activation of PI3-K, and to an increase in its association with Lyn (52). One of the products of PI3-K, $PtdInsP_3$, activates PKCζ, a PKC isoform which is not regulated by DAG. The activation of PKCζ leads to phosphorylation and inactivation of IκB which regulates the activity of the transcription factor NFκB (53).

Finally, BCR ligation is followed by activation of the low molecular weight G protein $p21^{ras}$ within 1-2 min (54). Ras activation appears to be regulated not only by the ubiquitous Grb2-Sos system, but also by Vav, a haemotopoietic GTP-exchange factor which is rapidly tyrosine phosphorylated, and presumably activated, following ligation of the BCR (24, 55). Ras activity is also regulated by Ras GTPase activating protein (GAP) which is also rapidly phosphorylated following sIg crosslinking (56). Ras activation leads to modulation of c-raf activity and initiation of the sequential phosphorylation of mitogen-activated protein (MAP)-kinase kinase, and MAP-kinase (57, 58). This pathway has been implicated in the regulation of a variety of cellular responses in many cell types by its targeting of protein kinases (eg. ribosomal S6 kinase), other signal transducers (eg. phospholipase A_2 (PLA_2)) and regulators of gene expression such as c-*jun*, c-*fos*, and c-*myc*, which have been implicated in the control of mitogenesis, apoptosis and differentiation in a number of cell types.

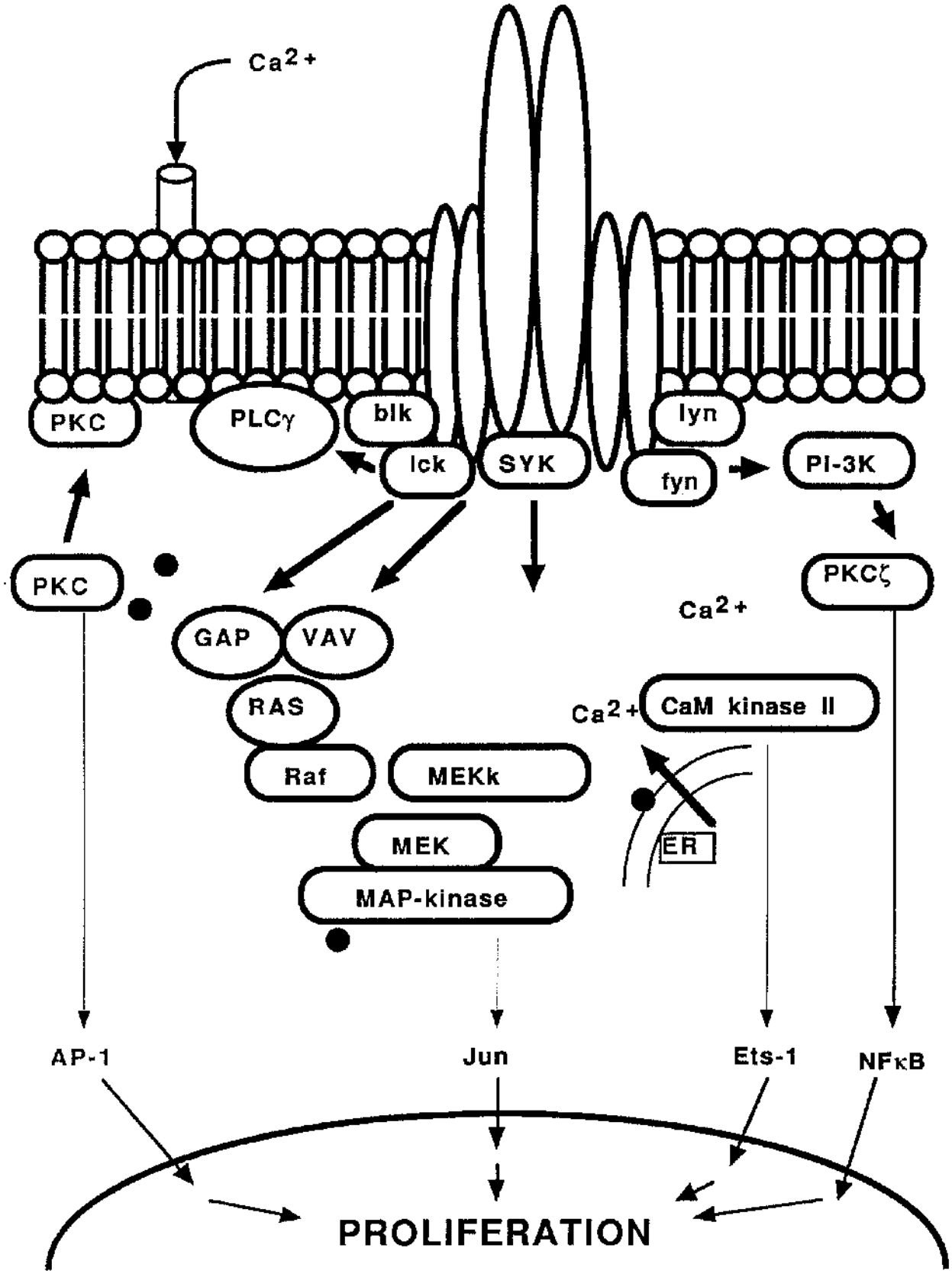

Ca2+
PKC
PLCγ
blk
lck
SYK
lyn
fyn
PI-3K
PKC
PKCζ
Ca2+
GAP
VAV
RAS
Raf
MEKk
Ca2+
CaM kinase II
MEK
MAP-kinase
ER
AP-1
Jun
Ets-1
NFκB
PROLIFERATION

Figure 6. Signal transduction from the B cell antigen receptor.

1.4 Coreceptors and the modulation of BCR signalling.

The biological consequences of modulation of B cell activation by T cell-derived stimuli was discussed in section 1.2.2.2. Here, the signalling mechanisms initiated and modulated by these stimuli and B cell counterstructures are described.

1.4.1 The role of CD22 in BCR signalling.

CD22 is a B cell-restricted glycoprotein of Mr 135 kDa, which has been implicated as an intrinsic component of the BCR (22): only $CD22^+$ B cells are capable of signalling through the antigen receptor and many studies have demonstrated the co-immunoprecipitation of sIg and CD22. Moreover, crosslinking of sIg induces the rapid tyrosine phosphorylation of CD22, which contains a number of YXXL sequences similar to ITAM motifs (24, 59, 60). Furthermore, ligation of CD22 enhances and lowers the threshold of sIg-mediated signalling. However, CD22 does not cocap with sIg and only 1-2% of the available CD22 co-immunoprecipitates with the BCR, suggesting CD22 may also have BCR-independent signalling functions. CD22 is therefore probably better described as a co-receptor for the BCR. Although the precise details of how CD22 signalling modulates BCR responses are unclear, tyrosine phosphorylation of CD22 has been observed to induce recruitment of PI-3-K, Lyn, Syk, PLC-γ, and p120. p120 has not yet been identified, but it is found in association with Syk and PLC-γ, and may therefore serve as a "bridge" for BCR-CD22 crosstalk (23, 62). CD22 has been identified as a lectin, which binds glycoconjugates containing α2,6-linked sialic acid on lymphocytes. The sIg-mediated tyrosine phosphorylation of CD22 also induces the recruitment and activation of SHP (protein tyrosine phosphatase (PTP)-1C) which suppresses signalling via sIg (63). Furthermore, the ligation of CD22 to prevent its coaggregation with sIg, as may occur when B cells are in a lymphoid environment, lowers by 100-fold the threshold at which sIg activates the B cell. Thus, CD22 may act as a represser of B cell activation, which is alleviated following interaction with α2,6-linked sialic acid on T cells, generating a bias for the sIg-mediated activation of B cells in secondary lymphoid organs (63).

1.4.2 The role of CD19/ CD21 in augmenting B cell stimulation via the BCR.

The CD19/CD21 complex is a multimeric receptor consisting of CD19, CD21, TAPA and Leu13, and its ligation can reduce the threshold of sIg-mediated B cell activation by up to two orders of magnitude (20, 21, 24, 64). CD21, which is the complement receptor 2 (CR2), is a 140 kDa protein consisting of an extracellular domain comprising 15 short consensus repeats, a single transmembrane spanning domain, and a 34 amino acid cytoplasmic tail. CD21 is also a receptor for iC3b, C3dg, CD23, IFN-α and EBV, suggesting that this complex may sense immune complexes bound to Ag or cells expressing CD23. Thus, concomitant crosslinking of the BCR by Ag, and CD21 by iC3b bound to the Ab in an immune complex, will lead to enhanced activation of B cells through the antigen receptor (24). This co-crosslinking could serve to inform the B cell that the antigen being bound via its sIg has already elicited an humoral immune response, in the form of secreted Ab. CD21 itself does not appear to transduce signals directly, and this function is thought to be carried out by two other components of the complex: CD19 and TAPA (24, 65, 66). CD19 is a 95 kDa glycoprotein of the Ig superfamily, and has a cytoplasmic domain of 243 amino acids; TAPA (CD81) is a 20 kDa serpentine protein containing 4 transmembrane spanning domains. CD19 contains nine tyrosine residues within its cytoplasmic domain, six of which are flanked by acidic residues, suggesting that they are likely to be tyrosine kinase phosphorylation sites (24). Several of these potential phosphorylation sites are characteristic of binding sites for the SH2 domains of cytoplasmic signal transducers. Indeed, the likely importance of these sites is demonstrated by the fact that CD19 is highly tyrosine phosphorylated following sIg ligation, facilitating recruitment and possibly activation of the Src-PTKs Lyn and Yes, as well as PI-3-K and GAP. Fyn and PLC-γ can also associate with CD19, although this may not be direct, as CD19 does not contain consensus binding sites for either effector (20). Although these findings highlight the role of BCR-CD19/CD22 crosstalk, the signalling capacity of CD19 is not dependent on the co-expression of sIg, and ligation of CD19 induces signals such as tyrosine phosphorylation and calcium mobilisation in the

absence of BCR crosslinking (20). Moreover, CD19 is functionally active on B cell precursors which lack cell surface expression of sIg, and it is tyrosine phosphorylated following ligation of CD40 or CD72.

In contrast to the role for CD19/CD22 in the amplification of signals via the antigen receptor, ligation of this complex prior to sIg-ligation abrogates signalling via the BCR, which could be due to the desensitisation of sIg-coupled signals (67). Furthermore, this negative signalling does not appear to be mediated by the CD19 cytoplasmic tail, but via its transmembrane domain, which is important for interactions with TAPA. These findings are consistent with a role for TAPA in mediating negative signals which was suggested by the fact that TAPA was first identified as the target of an anti-proliferative antibody, TAPA-1 (20).

1.4.3 The role of CD20 in regulation of cell cycle progression.

CD20 is a 33k Da cell surface molecule with four membrane-spanning domains as well as cytoplasmic N- and C-terminal domains. CD20 is expressed as a homodimer or homotetramer on the cell surface and generally associates with several other membrane proteins, possibly as a component of a multimeric receptor complex (68). The expression of CD20 is restricted to B cell precursors and mature B cells, and is lost following B cell differentiation to plasma cells. It has been suggested that CD20 is a component of a signalling complex involved in the regulation of B cell growth. Transfection studies have demonstrated that CD20 forms a calcium channel, which can be regulated by PKC and CamKII, and it is thought that CD20 may modulate BCR-mediated cell cycle progression by altering calcium-dependent homeostasis. Ligation of CD20 can also induce activation of ser/thr and tyr-kinases, possibly indicating other intrinsic functions for this protein, or the activation of these kinases following calcium mobilisation. Thus, CD20 may play an important role in the modulation of BCR-mediated B cell activation (68).

1.4.4 The role of CD45 in co-ordinating tyrosine phosphorylation in B cell activation.

CD45 is the major lymphocyte membrane tyrosine phosphatase, and some evidence suggests that it may be a component of the BCR complex (62). CD45 expression is required for full BCR signalling, and a number of proteins, incuding Ig-α/Ig-β, CD19/CD21 and CD22, are substrates for this phosphatase. Moreover, B cells purified from CD45 knockout mice were not stimulated to proliferate following incubation with a mitogenic concentration of anti-Ig (24, 69). These findings indicate that a pool of nonphosphorylated effector molecules is required for the transduction of signals following stimulation via the BCR. In contrast, prior ligation of CD45, which could inactivate this protein phosphatase, abrogates sIg signalling, suggesting that CD45 may also be involved in positive signalling. Indeed, recent evidence has shown that CD45 dephosphoylates Lyn kinase (at a C-terminal tyrosine), inducing its activation. Thus, CD45 may be involved in both positive and negative regulation of BCR-signalling (62).

1.4.5 MHC class II molecules in T cell-dependent B cell activation.

As discussed previously in section 1.2.2.2, the crosslinking of MHC class II molecules following cognate recognition of bound antigen fragments by the antigen receptors on $CD4^+$ T cells, plays a role in the T cell-dependent activation of B cells, and augments the activation of B cells induced by sIg crosslinking and T cell-derived cytokines such as IL-4. MHC class II molecules have been shown to be differentially coupled to different intracellular signals depending on the activation state of the B cell (70, 71). Thus, whereas the crosslinking of MHC class II molecules on naive mature B cells induces the generation of cAMP, and the sequestration of PKC in a detergent-insoluble compartment (events which are antagonistic to B cell activation), ligation of class II molecules on B cells, preactivated by anti-Ig and IL-4, induces PTK-mediated activation of PLC-γ, mobilisation of Ca^{2+}, activation of PKC and proliferation (71, 72). The cognate recognition of presented antigen fragments, and resultant MHC class II

crosslinking, therefore only induces activation of B cells if they have previously received signals via the antigen receptor, as the result of cognate interaction with specific antigen.

1.4.6 The role of IL-4 in modulating signalling via the BCR.

IL-4 elicits various responses from a range of B cell maturation stages including i) sustained growth of pre-B cells, ii) the promotion of the early activation of quiescent B lymphocytes, iii) an increase in the expression of MHC class II molecules and low-affinity Fc receptors and iv) directed isotype switching in activated B cell blasts (1, 73). Interestingly, IL-4 has been found to induce different signals in human and murine B lymphocytes. Thus, in human B cells the IL-4 receptor (IL-4R) is coupled to transient PLC activation followed by sustained generation of cAMP (74), whereas these signals are not oberved following stimulation of murine B cells with IL-4. However, IL-4 stimulation of murine B cells can reduce the threshold of sIg-mediated PKC activation by promoting its translocation to the plasma membrane (75), and the IL-4R is coupled to the PTK-mediated activation of PI-3-K, possibly via the novel docking protein, 4PS (76). Moreover, an unknown protein (Mr 42 kDa) is phosphorylated in murine membrane preparations in the presence of IL-4. (77, 78)

The IL-4R has been cloned and identified as a multimeric HRS (haemopoietin receptor signalling) receptor, comprising an IL-4-specific 140 kDa glycoprotein ligand binding receptor and the common HRS signal transducing subunits, γc, which are shared by the IL-2, IL-4, IL-7, IL-9, IL-13, and IL-15 receptor complexes (79). IL-4 regulation of gene transcription appears to involve the Janus kinases (Jak) 1 and 3. These phosphorylate STATS (signal transducers and activators of transcription), such as IL-4NAF and STF-IL-4. The Jak-STAT pathway of HRS-mediated regulation of gene transcription is reviewed by Darnell, 1994 (80). In addition, the IL-4 up-regulation of MHC class II expression appears to be regulated by a specific DNA binding protein, NF-BRE (81).

1.4.7 CD40 as a signal transducer.

As has already been discussed in sections 1.2.1 and 1.2.2.2, the ligation of CD40 on B cells can i) drive resting cells into cycle; ii) reduce their threshold for sIg signalling; iii) rescue germinal centre B cells (centrocytes) from apoptosis and iv) induce growth arrest or apoptosis in transformed cell lines and lymphomas (reviewed by Durie *et al.*, 1994 (82)). Some preliminary investigations have examined the molecular mechanisms underlying these effects. Lyn (PTK) activation appears to be important for all CD40-mediated signals (83); and CD40 is coupled to cAMP generation in resting B cells, and to PLC-γ and PI-3-K in activated B cells (82). These findings suggest that this receptor may mediate activation state-dependent responses of B cells to T cell help.

1.5 Signal transduction in sIg-mediated growth arrest and apoptosis in B cells, and differences between sIgM- and sIgD-mediated signals.

Crosslinking of sIg on immature B cells can induce clonal anergy or deletion. This has been found to depend on the degree of antigen receptor crosslinking. Thus, high doses of antigen or crosslinking antibodies induces the loss of B cells through apoptosis, whereas lower doses merely induce a state of unresponsiveness (84). Immature B cells express only the IgM isotype of surface immunoglobulin, whereas mature B cells, which proliferate in response to antigen receptor crosslinking, express both sIgM and sIgD. Furthermore, sIgD expression is down-regulated following receptor crosslinking and although all classes of Ig can be expressed in a secretory form, secretory IgD is virtually absent from serum (85),. These observations have led to the prediction that sIgD plays a unique role in the differentiation and activation of mature B cells. Studies involving the differential crosslinking of sIgM and sIgD on immature B lymphoma cells using specific antibodies have supported this hypothesis: sIgM crosslinking was found to induce growth arrest and apoptosis, whereas sIgD crosslinking did not (86-89). These findings

have led to a search for differential signals generated following crosslinking of sIgM and sIgD.

Early investigations did not identify any differences in the signals generated immediately following crosslinking of the antigen receptors on mature and immature B cells. Thus, BCR crosslinking on either mature or immature B cells was found to induce tyrosine phosphorylation of target proteins (90-92), the activation of PLC-γ, and the resultant generation of DAG, IP_3 and calcium influx. However, further studies found that, whereas BCR crosslinking on mature B cells resulted in the activation of PKC following its recruitment to the plasma membrane, this was greatly reduced in immature B cells (93). Furthermore, immature B cells were found to be protected from sIgM-mediated growth arrest and apoptosis by stimulation with PMA, a PKC activator (94), indicating that PKC activation is antagonistic to the antigen-driven negative selection of lymphocytes.

Initial findings therefore suggested that the differential responses of immature and mature B cells to antigen receptor crosslinking could be due to "rewiring" of sIg coupling to downstream signalling elements in maturation stage-specific manner, rather than the generation of different immediate signals following sIg crosslinking. However, recent studies conducted by Reth and coworkers on immature murine B lymphoma cells have identified proteins specifically associated with sIgM or sIgD (85, 95), perhaps providing a basis for the differential responses to sIgM and sIgD crosslinking. It was found that the sIgM antigen receptors of B cells are associated with three proteins of Mr 32, 37, and 41 kDa, which were termed BAP (BCR associated protein) 32, 37, and 41, respectively (95). These proteins were not found to be associated with sIgD. Amino acid sequencing identified BAP 32 as prohibitin, a finding which was corroborated by western blotting, and also demonstrated a high degree of sequence homology between BAP 37 and BAP 32. Prohibitin has been strongly implicated in the inhibition of proliferation, which would be consistent with a role in sIgM-mediated signalling in immature B cells. Both BAP 32/prohibitin and BAP 37 were found to possess ser/ thr kinase phosphorylation sites and a motif, NPXY, close to the carboxy terminus. This motif is often found in the

cyoplasmic domains of transmembrane proteins and is required for the coated pit-mediated internalization of the low density lipoprotein receptor, suggesting that BAP 32 and BAP 37 may be involved in the internalization of sIgM-BCR. BAP 42, only low levels of which were associated with sIgM-BCR, was not further characterised.

Two novel transmembrane proteins which associate preferentially with sIgD in B cells: BAP 29 and BAP 31 have also recently been identified (85). Although these proteins had no significant sequence homology with BAP 32 and BAP 37, they were predicted to have a similar structure. Interestingly, studies using the expression of sIgM chimeras in pre-B cells, and sIgD chimeras in myeloma cells, found that the specificity of sIgM interaction with BAP 32/prohibitin and BAP 37, or sIgD interaction with BAP 29 and BAP 31, was determined by the transmembrane domain of each sIg isotype. Both sIgM and sIgD have also been found to associate with the Ig-α/Ig-β heterodimer via their transmembrane domains. These findings are therefore consistent with the proposal by Reth that the α-helix, which constitutes the transmembrane domain of sIgM or sIgD, has two faces: one which is conserved between the two sIg isotypes and is responsible for the interaction with Ig-α/Ig-β; and the other face which is class-specific and is responsible for the interaction of sIgM or sIgD with specific effector molecules such as BAPs. Recent reports have also demonstrated considerable differences between the tyrosine phosphorylation events following sIgM and sIgD crosslinking. Crosslinking of sIg on sIgD-expressing B cells was found to induce much more prolonged protein tyrosine phosphorylation than that observed following sIg crosslinking on $sIgM^+$ B cells (both sIgM and sIgD were of identical antigen specificity, on a transfected B cell line of mature phenotype) (96). Thus, anti-IgM-mediated protein tyrosine phosphorylation was found to be maximal at 1 min following receptor crosslinking, and declined after 60 min; whereas anti-IgD-mediated protein tyrosine phosphorylation was found to be maximal at 60 min following receptor crosslinking, and declined after 240 min (96). Further investigation, employing chimeric sIg molecules, demonstrated that the membrane-proximal and/or transmembrane regions of sIgD were required for induction of sustained protein tyrosine phosphorylation. This finding suggested that the difference in signals

generated via sIgM and sIgD could be dependent on the direct interaction of the two sIg isotypes with different effector molecules such as BAPs.

Recently, several investigators have reported significant differences in a number of signalling pathways activated following antigen receptor crosslinking on mature and immature lymphocytes. Many investigators have studied B lymphomas of immature phenotype, such as WEHI 231 and CH31 which undergo growth arrest and apoptosis in response to sIg crosslinking, as a model for antigen-mediated negative selection of immature B cells. The study of BCR-mediated signalling in these cells has provided considerable insight into the molecular events underlying sIgM-mediated growth arrest and apoptosis.

Pharmacological studies employing specific inhibitors of protein tyrosine kinases, such as tyrphostin and genistein, have indicated that sIgM-mediated growth arrest and apoptosis of immature B cells requires protein tyrosine kinase activity. Further studies have investigated the roles of specific PTK activities. Firstly, the ability of CH31 B-lymphoma clones to undergo anti-IgM-induced apoptosis correlated with the activation of blk kinase (97). Moreover, antisense oligonucleotides specific for blk were able to block sIgM-mediated cell cycle arrest and apoptosis in these cells (91). Another study, using Fyn-specific antisense oligonucleotides in the BCL_1 lymphoma, has implicated a role for Fyn in sIgM-mediated growth arrest, but not apoptosis (92).Secondly, a p160 protein, which co-precipitates with kinase activity(ies) acting on serine, threonine and tyrosine residues, has been isolated from B cells. Crosslinking of the BCR on BAL17 human mature B lymphocytes was found to induce an increase in the kinase activity associated with p160 (98). In contrast, crosslinking of sIg on neonatal murine splenic B cells or WEHI 231 immature B cells, was found to induce the transient loss of kinase activity associated with p160. Moreover, the reduction in p160-associated kinase activity was found to be dependent on an increase in tyrosine phosphatase activity induced by the crosslinking of sIg on WEHI 231 cells. Finally, another investigation analysed the signalling events following crosslinking of the antigen receptors on a WEHI 231 subline resistant to anti-IgM-mediated growth arrest and apoptosis (99). It was found that the

expression of p75HS1, a substrate for $p53/p56^{lyn}$, was reduced in the resistant WEHI 231 cells, possibly indicating a role for this protein (and for lyn) in sIgM-mediated apoptosis.

WEHI 231 sublines have also been used to investigate the role of lipid signals in the regulation of apoptosis in B lymphocytes. One lipid second messenger-generating pathway implicated in the regulation of apoptosis in a number of cell types involves the hydrolysis of sphingomyelin by sphingomyelinase and the resultant release of ceramide. This pathway has been found to be activated following the crosslinking of sIgM on WEHI 231 cells (100). The level of ceramide in cells is also regulated by the hydrolytic enzyme ceramidase. An inhibitor of this enzyme, *n*-oleoyl-ethanolamine, was used to select WEHI 231 sublines which were low ceramide producers and resistant to anti-Ig. Thus, WEHI 231 cells resistant to *n*-oleoyl-ethanolamine did not produce ceramide in response to sIg crosslinking and did not undergo apoptosis. This strongly implicates the ceramide pathway in the control of apoptosis in immature lymphocytes, and this is discussed further in chapter 4.

Studies have also strongly suggested a role for the mobilisation of Ca^{2+} in the induction of apoptosis in human immature B cells (101). Incubation of group 1 Burkitt's lymphoma cells or B104 lymphoma cells with anti-Ig or ionomycin (a calcium ionophore) was found to induce apoptosis in these cells. This indicated that stimulated calcium influx could be involved in the sIg-mediated induction of apoptosis. Furthermore, blockade of calcineurin, a ser/thr phosphoprotein phosphatase, or inhibition of phosphatase 2B by cyclosporin A, was found to protect these immature B cells against Ca^{2+}-mediated apoptosis. These findings implicate sustained mobilisation of calcium and the Ca^{2+}-dependent activation of downstream effectors in the control of apoptosis in immature B cells.

The above investigations have therefore demonstrated that a number of intracellular signals, including tyrosine phosphorylation, calcium mobilisation and ceramide production, are required for sIgM-mediated growth arrest and apoptosis in B cells. Several of these signals were found to be qualitatively and quantitatively different in

cells which undergo apoptosis in response to sIg crosslinking and those which do not. Furthermore, studies on the association of sIgM and sIgD with potential signal transducers have suggested a possible molecular basis for the generation of different signal via these two sIg isotypes.

Several studies have suggested a role for the transcription factor Myc in the downstream regulation of growth arrest and apoptosis in immature B lymphomas. Crosslinking of sIgM on WEHI 231 cells has been found to induce a transient increase in c-*myc* transcription, followed by a drop to below detectable levels of c-*myc* message within a few hours (102). Interestingly, stimulation of $sIgM^+sIgD^+$ transfected immature B lymphomas with anti-IgM Ab was found to induce a similar pattern of changes in c-*myc* expression (103). In contrast, although anti-IgD Ab also induced a transient increase in c-*myc* message levels, the decline in Myc levels did not fall below basal levels. Moreover, whilst anti-IgM antibodies induced apoptosis in these cells, anti-IgD antibodies did not (89, 103, 104). These findings indicate that it is not likely to be the transient increase, but rather the dramatic suppression to below basal levels of Myc which may possibly lead to the induction of apoptosis. Indeed, addition of antisense oligonucleotides against c-*myc* which were found to stabilize Myc levels, rather than abolish expression, prevented the induction of growth arrest and apoptosis induced by anti-IgM Ab, suggesting that maintenance or elevation of Myc levels protects against induction of apoptosis.

WEHI 231 and CH31 immature B lymphomas are constitutively proliferating cell lines. Analysis of the cell cycle-dependence of the induction of growth arrest and apoptosis conducted by Scott and coworkers has provided further insight into the molecular mechanisms underlying these processes (2, 87, 88, 105). sIgM-mediated growth arrest and apoptosis in these cells only occurs when the cells are in early G_1 phase (2). Thus, treatment of CH31 or WEHI 231 cells with anti-IgM antibodies was found to lead to a loss of cells in S phase and an accumulation of cells in G_1 phase, prior to apoptosis (2, 87, 88, 105). This indicates that crosslinking of sIgM in G_1 phase prevents the further cell cycle progression of immature B lymphoma cells, and initiates

the sequence of events leading to apoptosis. These findings therefore suggested that cellular effectors whose activity is regulated in a cell cycle-dependent manner may be involved in the sIgM-mediated induction of apoptosis in B cells. This possibility has been supported by investigation of the role of the retinoblastoma gene product, pRB, in the regulation of apoptosis in B cells. pRB is a nuclear phosphoprotein whose phosphorylation state is regulated in a cell cycle-dependent manner. The hypophosphorylated form of pRB suppresses growth and is found in the G_1 phase of the cell cycle, when it is tightly associated with the nuclear membrane (106). The hypophosphorylated form of pRB binds to the transcription factor E2F, thereby inhibiting transcription of cellular genes containing E2F binding sites, and preventing S-phase entry (107). Phosphorylation of pRB on ser/thr residues, probably via cdc2 or related kinases, then occurs in mid-late G_1 (108, 109). The phosphorylated form of pRB persists until mitosis when it is dephosphorylated to the growth suppressive form (110). A recent report established that pRB phosphorylation is inhibited in B lymphomas following addition of anti-IgM in early G_1, but not at later points in the cell cycle (111). This finding clearly implicates pRB in the mediation of sIgM-mediated growth arrest and possibly apoptosis.

1.5.1 Gene expression in sIgM-mediated apoptosis in B cells.

A number of genes have been identified which are expressed in various cell types, following apoptotic stimulation. These genes were identified using subtractive hybridisation and include *RP-8, PD-1, bad, bax, nur77, ICE*-related genes and *p53* (112, 113). Indeed, the appearance of *nur77* message has been detected in WEHI 231 cells, following treatment with anti-IgM, indicating that the transcription (and presumably translation of mRNA) of specific genes is induced by apoptotic stimuli (113). In contrast, Ishida *et al.* found that RNA synthesis was not required for apoptosis in WEHI 231 (114), although it was required for T cell apoptosis. This finding suggests that message, and possibly the proteins, necessary for the induction of apoptosis are already present in

WEHI 231 cells. This is not necessarily imcompatable with the finding that *nur77* transcription is activated following sIgM crosslinking, as expression of this gene may not be required for apoptosis in WEHI 231 cells.

1.5.2 Protection of B cells from sIgM-mediated apoptosis.

It has been found that a number of stimuli inhibit the anti-IgM-induced apoptosis of immature B cells. These include T cell-derived stimuli (115): i) contact with T_H2 membranes (3, 101, 102), ii) IL-4 or IL-5 (3), and iii) CD40 crosslinking have been reported to inhibit anti-IgM-induced growth arrest and apoptosis of CH31 and WEHI 231 cells (116-119). Also, PMA has been found to inhibit induced apoptosis in these cells (94), possibly indicating a role for PKC activation in the protection of B cells from apoptosis by specific stimuli. Moreover, LPS, a polyclonal B cell activator, has been found to markedly inhibit the induction of apoptosis in murine B cells (120).

A novel family of proteins, which protect B cells from apoptosis, has recently been identified. The first member of this family to be isolated was Bcl-2 (reviewed by Nunez *et a.*, 1994 (121)), an integral membrane protein that has been located to the outer mitochondrial membrane, perinuclear membrane and smooth endoplasmic reticulum (ER). The pattern of Bcl-2 expression by B cells appears to be tightly regulated (121). Bcl-2 is highly expressed at the earliest stages of B cell development (pro-B cells) and in mature B cells. In contrast, Bcl-2 expression is downregulated at the pre-B/IgM^+IgD^- stages and in germinal centre B cells. Thus, B cells express Bcl-2 at stages of maturation where they are relatively resistant to apoptosis, and downregulate Bcl-2 expression at stages where they are prone to apoptosis. Furthermore, the susceptibility to glucocorticoid-induced cell death in B cells has been found to correlate with the level of Bcl-2 expression. This suggests that the maturation stage-specific expression of Bcl-2 may play a role in modulating the susceptibility of B cells to apoptosis and facilitate the appropriate selection events during B lymphocyte development. This hypothesis has been supported by the finding that Bcl-2 expression is upregulated in GC B cells following the

crosslinking of sIg and ligation of CD40 (122). However, studies in $sIgM^{+}sIgD^{-}$ immature B lymphocytes and lymphomas have found that overexpression of Bcl-2 does not protect these cells from anti-IgM-induced apoptosis(123, 124), suggesting that sIgM-mediated apoptosis may involve signalling through a pathway not regulated by Bcl-2. However, the activity of Bcl-2 (which is an anti-oxidant) is regulated by an inhibitor, bax, and it is therefore possible that high levels of bax expression in immature B cells may prevent the inhibition of anti-IgM-induced apoptosis (125).

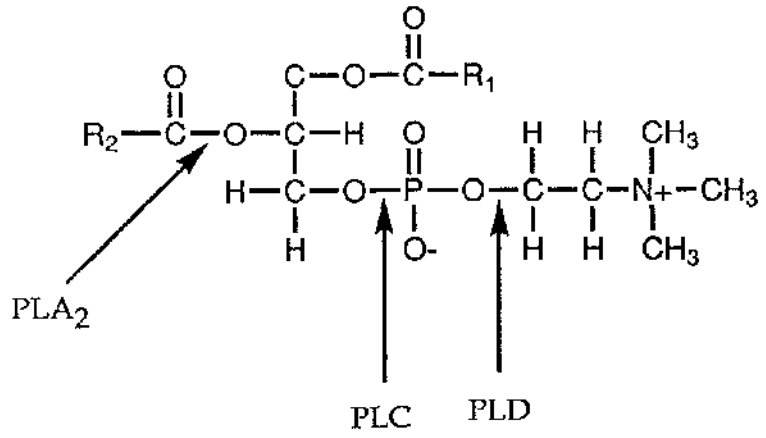
Further investigation has recently identified another gene involved in the regulation of apoptosis: Bcl-x (121). This gene has been found to have a high degree of sequence homology with Bcl-2, and gives rise to two gene products by alternative mRNA splicing: Bcl-x_L and Bcl-x_S, the long and short forms of Bcl-x, respectively. Bcl-x_L was found to inhibit apoptosis in IL-3-dependent cells following growth factor deprivation. In contrast, Bcl-x_S, which differs from Bcl-x_L in that it lacks an internal sequence of very high sequence homology to Bcl-2, has been found to facilitate programmed cell death, and is implicated as an inhibitor of both Bcl-2 and bcl-x_L (121). The mechanism of this inhibition is unknown, but the sequence homology of Bcl-x_S with Bcl-2 and Bcl-x_L suggests that it may inhibit their interaction with regulator and/or effector molecules, possibly via some sort of competition mechanism. A recent study, investigating the role of Bcl-x_L in WEHI 231 immature B lymphoma (126), found that overexpression of this protein rendered these cells refractory to anti-Ig-mediated apoptosis. Furthermore, CD40 crosslinking, which has been found to protect WEHI 231 from sIgM-mediated apoptosis, was found to induce the expression of Bcl-x_L in these cells, strongly indicating a role for Bcl-x_L in the T cell-mediated protection of immature B cells from antigen-driven apoptosis. While the precise mechanism(s) underlying the effects of Bcl-2 and Bcl-x_L remains unknown, it has been postulated that these proteins may regulate the mobilisation of calcium from intracellular stores. This would be consistent with the role established for these proteins in the inhibition of apoptosis, as a prolonged increase in cytosolic Ca^{2+} levels has been strongly implicated as a signal for the induction of apoptosis. Investigation of this possibility involved the stimulation of

WEHI 231 cells with thapsigargin, which is an inhibitor of the Ca^{2+}-ATPase located on the ER membrane, and a potent inducer of apoptosis. Ligation of CD40, or the overexpression of Bcl-x_L, was found to protect WEHI 231 cells from thapsigargin-mediated apoptosis (126). This finding strongly suggests a role for the Bcl-x_L, and possibly Bcl-2, in the regulation of Ca^{2+} mobilisation from the ER and possibly other intracellular Ca^{2+} stores.

These findings indicate that the regulation of apoptosis in B cells is a complex process, involving a number of stimuli which can induce or abrogate the induction of growth arrest and/or cell death, and integrating specific intracellular signals and molecular events and the regulated expression of specific genes.

1.6 Investigation of the role of lipid signalling in lymphocyte activation, maturation and cell death.

Many of the downstream signals involved in apoptosis such as Bcl-x and pRb are currently being elucidated in other laboratories. However, the signalling elements responsible for transducing early receptor events are not. Investigation of signals generated via B cell antigen receptors has found that sIg crosslinking induces a much greater activation of PKC in mature B cells than in immature B cells. Together with the finding that PMA, a pharmacological activator of PKC, protects immature B cells from sIgM-mediated growth arrest and apoptosis (94), these results have therefore suggested that differential PKC activation may play a key role in the differential responses to sIg crosslinking observed in mature and immature B cells. The mechanistic basis for the differential activation of PKC in mature and immature B cells is at present poorly understand but could lie at the level of PKC itself: for example the maturation-dependent expression of specific PKC isoforms (49). Alternatively, the differences observed in PKC activation could be due to differences in the production of PKC activating signals, which are usually lipid second messengers. These are typically generated by the



Products	
Phospholipase A_2	lyso PC (1-acyl) + fatty acid
Phospholipase C	sn-1,2-diacylglycerol + choline phosphate
Phospholipase D	phosphatidic acid + choline

Figure 7. Enzymatic hydrolysis of Phosphatidylcholine.

stimulated hydrolysis of specific lipid species by hydrolytic enzymes, such as phospholipases.

The earliest signalling events detected following B cell receptor crosslinking include the activation of PtdInsP$_2$-PLC-γ and the resultant generation of InsP$_3$ and diacylglycerol (DAG), an activator of certain PKC isoforms. This pathway appears to similarly activated in both mature and immature B cells, suggesting that the antigen receptors at one (or both) of these maturation stages may be coupled to additional lipid signalling pathways in order to transduce differential responses. It has recently become apparent that other phospholipid classes besides inositol phospholipids, such as phosphatidylcholine (PtdCho), are hydrolysed and may give rise to DAG and other biologically active lipids in response to a wide range of agonists (127-130). Two candidate pathways for a central role in cellular responses involve the hydrolysis of phosphatidylcholine (or phosphatidylethanolamine) by phospholipase D (PLD) or phospholipase A$_2$ (PLA$_2$) (127-130).. The products of the hydrolytic cleavage of PtdCho by these enzymes are summarised in Figure 7. PLD-catalysed PtdCho cleavage gives rise to phosphatidic acid (PtdCho) and choline. PtdOH is a putative second messenger which has been implicated in the regulation of a number of cellular processes such as the respiratory burst in neutrophils and mitogenesis in a number of cell types (127-130).It has also been found to modulate a number of intracellular signals such as PKC and ras activation. These functions are discussed more fully in the introduction to Chapter 3. The hydrolysis of PtdCho by PLA$_2$ generates lysophosphatidylcholine (LPC) and fatty acid, particularly arachidonate. A role for LPC as an intracellular second messenger is not well-established, however, LPC has been shown to modulate the activation of PKC by DAG and may play a role in cellular differentiation(128, 131, 132). Arachidonate has a well-established role as a precursor for eicosanoid, intercellular inflammatory mediators, and is strongly implicated as an intracellular second messenger in the regulation of signal transducing elements such as PKC and sphingomyelinase (61, 128, 133), and may be involved in the regulation of mitogenesis or apoptosis in a number

of cell types. The occurrence and functions of stimulated PLA_2 activation are discussed in the introduction to Chapter 4.

1.7 Aims and objectives

The aims and objectives of this investigation were to:

i) identify PLD and PLA_2 activity in B cells;

ii) determine whether either phospholipase is coupled to the antigen receptors or other receptors on B cells;

iii) determine whether PLD or PLA_2 plays a role in the transduction of maturation stage-specific signals, leading to either proliferation or anergy/apoptosis.

2 Materials and Methods

2.1 Materials

Amersham International, Buckinghamshire	[5,6,8,9,11,12,14,15-^{3}H] arachidonic acid (213 Ci/mmol); myo-[^{3}H]inositol (87 Ci/ mmol); [9,10(n)-^{3}H] myristic acid (49 Ci/mmol); [9,10(n)-^{3}H] oleic acid (10 Ci/mmol); [6-^{3}H]thymidine (5 Ci/ mmol); [^{32}P]phosphoric acid (200 mCi/ mmol); [1-^{14}°C]stearic acid (54 mCi/ mmol); L-3-phosphatidylcholine,1,2,-D1(1-^{14}°C); [methyl-^{3}H]choline chloride (82 Ci/ mmol); (1(3)-^{3}H)glycerol (3.1 Ci/ mmol); Phosphoryl [methyl-14°C] choline, ammonium salt (56mCi/ mmol); [1-^{3}H]ethan-1-ol-2-amine hydrochloride (34 Ci/ mmol); ECL wetern Blotting Reagents; HRP-linked antibodies.
Amicon Ltd., Upper Mill, Stonehouse, Gloucester GL10 2BJ	Centricon filters

University of Glasgow, University Ave, Glasgow G12 8QQ	Male BALB/c mice were bred and housed in the animal facility and used at 4 (immature) or 12-16 (mature) weeks of age.
Dr. R. Callard, Institute of Child Health, London	human pre-B cell lines, 207, 697 and REH
Du Pont (UK) Ltd, Diagnostics and Biotechnoloty Systems, Wedgwood Way, Stevenage, Hertfordshire SG1 4QN	[9,10(n)-^{3}H]-palmitic acid (39 Ci/ mmol)
ECACC, Porton Down	Human B cell lines: Daudi and Ramos Human T cell line: Jurkat Murine B cell line: WEHI 231 Pertussis toxin

Fisons Scientific Equipment, Bishops Meadow Road, Loughborough, Leicestershire LE11 ORG	rabbit anti-human cPLA2 peptide Ab raised against a C-terminal peptide (amino acids 727-749) coupled to KLH
Gibco, Paisley, Scotland,	RPMI-1640 F-10 (HAM) Fetal Calf Serum HEPES penicillin strepomycin MEM non-essential amino acids glutamine
Immunex Corp., 51 University St., Seattle, Washington 98101	Murine rIL-4
Jackson Immunoresearch Laboratories, inc., Stratech Scientific Ltd, Bedfordshire	Rabbit anti-human Ig Ab ($F(ab')_2$ fragments) Rabbit anti-mouse Ig Ab ($F(ab')_2$ fragments)

Lipid products, Nutfield Nurseries, Crabhill Lane, S. Nutfield, Surrey RH1 5PG	phosphatidylbutanol
Packard, Ulgersmaweg 47, 9731 BK, Groningen, The Netherlands	Ultima-Flo scintillant
Dr. R.M.E. Parkhouse Institute for Animal Health, Ash Road, Pirbright, Surrey GU24 0NF	anti-Thy-1 Ab (NIMR-1)
Pharmingen, Cambridge Bioscience, Cambridge, United Kingdom	anti-CD3 Ab FITC-labelled anti-CD4 Ab PE-labelled anti-CD8α Ab FITC-labelled anti-human CD19 Ab PE-labelled anti-human CD3 mAb

Pierce and Warriner, 44 Upper Northgate St., Chester CH1 4EF	NHS-Biotin protein G
Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Lanarkshire, Scotland ML8 5ES	sheep red blood cells
Sera-Lab, Crawley Down, Sussex RH10 4FF	Guinea Pig Complement
Sigma Chemical Company, Poole, Dorset, United Kingdom	ammonium persulphate arachidonate avidin bovine serum albumin (BSA) ceramide Dowex (8 x 100) ficoll-hypaque FITC-labelled anti-mouse Ig Ab goat anti-mouse Ig antibodies (polyvalent)

	ionomycin
	lipopolysaccharide (LPS)
	pepstatin
	percoll
	Phorbol 12-myristate 13-acetate
	phospholipase D (from cabbage)
	phytohaemagluttinin (PHA)
	protein A-sepharose
	protein G-sepharose
	Quantum Red Streptavidin
	sphingosine
	TEMED
	trypan blue
	unlabelled lipid standards
	orthovanadate

Whatman LabSales Ltd,	t.l.c. plates:
St Leonard's Rd,	LKD5F
20/20 Maidstone,	LKD6F
Kent ME16 0LS	Silica gel-60

Yorkhill Children's Hospital,	Human tonsils
Glasgow	

All other reagents were supplied by Fisons or BDH and were of the highest grade available.

2.2 Purification and Culture of Lymphocytes

2.2.1 Preparation of Murine Splenic B lymphocytes

Small, dense B cells were prepared from the spleens of 12-16 week old (mature;>90% sIg^+) or 4 week old (immature) male Balb/C mice (134, 135). Spleens were excised from the mice, and the cells dispersed by pressing through a gauze mesh (Sigma) into RPMI-1640 medium. Cell debris was removed from the suspension by centrifugation (30g, room temp) for 5 min. The cell suspension was then layered onto a cushion of Ficoll-Hypaque (3 ml) in 13 ml polystyrene tubes (3 spleens/ tube) and centrifuged (440g, room temp) for 15 min. Mononuclear cells were recovered from the ficoll-medium interface and washed twice in RPMI-1640 by centrifugation (630g, room temp) for 10 min. T cells were depleted by incubation with anti-Thy-1 Ab (NIMR-1) and guinea pig complement at 37°C for 45 min. NIMR-1 (working stock: 1 in 300 dilution of 1 ml ascites) was added at 0.1 ml/ spleen; lyophilised GPC was reconstituted in distilled water and added at 0.25 ml/ spleen; and the suspension was made up to a total volume of 3 ml/ spleen with RPMI-1640. The cells were then pelleted by centrifugation (630g, room temp) for 10 min, resuspended in RPMI-1640 and washed twice by centrifugation (630g, room temp) for 10 min. They were then separated by percoll gradient centrifugation. Percoll (100%) was prepared by the addition of 2ml x 10 strength PBS to 18 ml percoll, and dilutions (85%, 65%, 50%) were prepared by addition of 1 x PBS. These were then layered (2.5 ml aliquots) in 13 ml polystyrene tubes to form a discontinuous gradient onto which the B cells were loaded (approx. 3 spleens/ tube in 2 ml medium), and the tubes were centrifuged (1230g, room temp) for 20 min. High-density "resting" B cells were harvested from the 85%-65% interface and low-density "in vivo" activated B cells from the 65%-50% interface. Cells were then resuspended in RPMI-1640, washed twice by centrifugation (630g, room temp) for 10 min, and then once by centrifugation (270g, room temp) for 10 min. During the final wash an aliquot (100 µl) of cells was removed, an equal volume of trypan blue (0.4%) was added and the cells were counted using an haemocytometer.

2.2.2 Preparation of Murine Thymocytes

4 week old male Balb/c mice were sacrificed by sagital overdose, and thymic tissue was removed from the mice and pressed through steel gauze to obtain a single cell suspension in RPMI-1640. The suspension was then layered onto Ficoll-Hypaque cushions (3 ml) in 13 ml polystyrene tubes (3 thymi/ tube) and centrifuged (440g, room temp) for 15 min. Thymocytes were recovered from the interface, washed twice in RPMI-1640 by centrifugation (630g, room temp) for 10 min, and then separated on a discontinuous percoll gradient (gradient 85%-65%-50%) as described above in section 2.2.1. Thymocytes were recovered from the 85%-65% interface and washed three times in RPMI-1640. During the final wash the cells were counted using an haemocytometer (136).

2.2.3 Preparation of Murine Splenocyte PHA-blasts

Spleens were removed from mice as described in section 2.2.1, and the cells dispersed and depleted of erythrocytes by centrifugation through Ficoll-Hypaque. The splenocytes recovered were then resuspended (10^7/ ml) in RPMI-1640 supplemented with glutamine (2 μM), 2-mercaptoethanol (50 μM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FCS, and cultured in the presence of PHA (50 μg/ml) overnight at 37°C in a 5% (v/v) CO_2 atmosphere at 95% humidity. Following incubation the cells were separated on a discontinuous percoll gradient of 85%-50% as described above. Cells were recovered from the 85%-50% interface, resuspended in RPMI-1640 and washed twice by centrifugation (630g, room temp) for 10 min. They were counted as described above using an haemocytometer during the second wash. The purified splenocyte-blasts were then resuspended and fixed by incubation with paraformaldehyde (0.4%, final concentration) for 5 min at room temperature. The reaction was quenched by the addition of 2 volumes of L-lysine (0.2M) and the fixed splenocyte-blasts were pelleted by centrifugation (440g, room temp) for 10 min. They were then washed twice by centrifugation and resuspended (10^7/ ml) in the appropriate medium.

2.2.4 Preparation of Human Tonsillar B Lymphocytes

Human tonsils were obtained from Yorkhill Childrens' Hospital, Glasgow. Each tonsil was rinsed twice and diced in 15 ml RPMI-1640 in a petri dish (137). The diced lumps were then pressed through gauze using a syringe plunger. The spill was transferred to a 50 ml Falcon tube and the debris allowed to settle out under gravity for about 5 min. The supernatant was then transferred to a fresh tube and the cells pelleted by centrifugation (440g, room temp) for 10 min. Cells were resuspended in RPMI-1640 (20 ml/ tonsil), then layered onto cushions of Ficoll-Hypaque (3 ml) and centrifuged (440g, room temp) for 15 min. Lymphocytes were removed from the interface and washed twice in RPMI-1640. Human tonsillar B cells were separated from tonsillar T cells using a technique known as "rosetting". This involved incubation of the tonsillar cells with AET-coated sheep red blood cells to which human T cells adhere, forming a "rosette" or clump. Rossetted T cells were then depleted by centrifugation. In brief, cells were counted using an haemocytometer, resuspended (at 10^7/ ml) in RPMI-1640 and 10% volume of AET-SRBC (prepared as described below in section 2.2.4.1) was added. The suspension was pelleted (120g, room temp) for 5 min and 1 ml FCS was pipetted gently onto the pellet. This was incubated on ice for 30 min. The pellet was then resuspended by gentle rocking and the entire suspension was transferred to a 10 ml cushion of Ficoll, before centrifugation (440g, room temp) for 20 min at room temperature. B cells were harvested from the interface and washed three times in RPMI-1640. (T cells could be recovered from the rosette by the addition of a few drops of distilled water to lyse the red blood cells, followed by rapid washing in media). They were then layered onto a three-step percoll gradient (85%-65%-50%) and spun (1230g, room temp) for 20 min. The cells were washed twice in RPMI-1640 and resuspended in RPMI-1640 plus 10% FCS. B cells were counted during the final wash (137).

2.2.4.1 AET treatment of sheep red blood cells

Sheep erythrocytes were coated with 2-aminoethylisothiouronium bromide (AET). 5 ml of sheep erythrocytes (SAPU) were centrifuged (440g, room temp) for 10 min, then resuspended in 10 ml RPMI-1640 and washed twice again. At the same time 102 mg of AET

was dissolved in 10 ml distilled H_2O, adjusted to pH 9.0 with 5M NaOH and filter sterilised. AET solution (4 ml) was added to the erythrocytes and the suspension was incubated at 37°C for 20 min. The cells were then washed 5 times in RPMI-1640 by centrifugation (630g, room temp) for 10 min and resuspended in a final volume of 9 ml. The treated SRBCs could be stored for up to a week at 4°C, and before use they were diluted 1:5.

2.2.5 Cell Culture

The human B cell lines Ramos, Daudi and EDR, human tonsillar B cells, and Jurkat human T cells were cultured (2 x 10^5 - 10^6/ml) in RPMI-1640 supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FCS. WEHI 231 murine B cells (2 x 10^5 - 10^6/ ml), murine splenic B cells (10^7/ ml) and murine thymocytes (10^7/ ml) were cultured in RPMI-1640 supplemented with glutamine (2 mM), 2-mercaptocthanol (50 µM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 8% FCS. All were incubated at 37°C in 5% humidified CO_2.

2.2.6 Measurement of DNA synthesis.

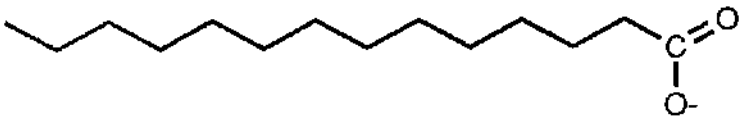
For measurement of DNA synthesis, murine splenic B cells (2 x 10^5/ well), WEHI 231 cells (10^4/ well) or EDR cells (3 x 10^4/ well) were cultured in triplicate in round bottom microtitre plates in RPMI-1640 medium supplemented with glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids, 2-mercaptoethanol (50 µM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 5% FCS, in the presence of the appropriate agonist in a total volume of 200µl. Cells were cultured at 37°C in a 5% (v/v) CO_2 atmosphere at 95% humidity for 24, 48 or 72 hours. [^{3}H]thymidine (0.5 µCi/ well) was added 4 hours prior to harvesting of the cells with an automated cell harvester (Skatron). Incorporated label was estimated by liquid scintillation counting (135).

2.3 Phospholipid Signalling Assays

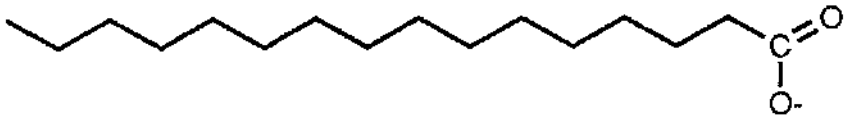
2.3.1 Analysis of Phospholipid Labelling by [^{3}H]fatty acids

Radiolabelling of cellular phospholipids can be achieved by the incubation of cells with radiolabelled fatty acids which are incorporated as the acyl chains of phospholipids. There is variation between the acyl chains of different phospholipid species, and specific fatty acids will be preferentially incorporated into particular phospholipids. The labelling of cellular phospholipids by different fatty acids was studied to provide information for the design of signalling assays involving radiolabelling of phospholipids (138).

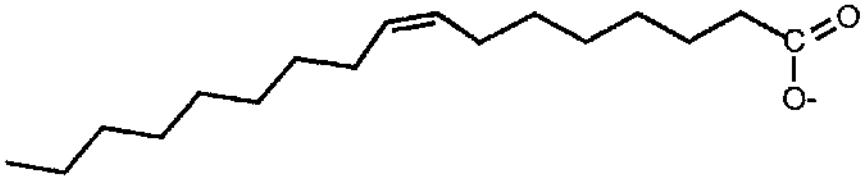
Murine splenic B cells were resuspended (1.5 x 10^7/ml) in RPMI-1640 supplemented with glutamine (2 mM), 2-mercaptoethanol (50 μM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FCS. Cells (0.3 ml aliquots) were cultured in 24-well plates and labelled with [^{3}H]palmitate, [^{3}H]arachidonate, [^{3}H]myristate, or [^{3}H]oleate (3 μCi/ well) and incubated at 37°C for either 4 or 18 hours at 37°C in a 5% (v/v) CO_2 atmosphere at 95% humidity. Methanol (500 μl) was then added to each well and the disrupted cell suspension removed to glass vials. Chloroform (500 μl) and methanol (500 μl) were added to each tube, which was vortexed, and the samples were left to extract on ice for 10 minutes. Phase separation was achieved by the addition of 500 μl chloroform and 600 μl water followed by vortexing and centrifugation (270g, 4°C) for 5 min. Aliquots (900 μl) of the lower organic phase were removed to glass vials and dried *in vacuo*. Each sample was redissolved in chloroform: methanol (25 μl; 2: 1, by vol.), supplemented with the non-radioactive standards PtdIns, PtdCho, PtdEth, and PtdOH (20 μg of each/ tube) and applied to a pre-activated Silica-gel 60 plates. Prior to use, silica-gel 60 plates (20 x 20cm, 250mm thick; Merck, Darmstadt, Germany) were activated by soaking in 1 mM EDTA, air-drying and heating at 120°C for 1 hour. Plates were developed in a paper-lined tank, pre-equilibrated with the solvent chloroform: acetone: methanol: glacial acetic acid: water (80: 30: 26: 24: 14, by vol.). The phospholipids were detected by iodine vapour, and the radioactivity associated with the



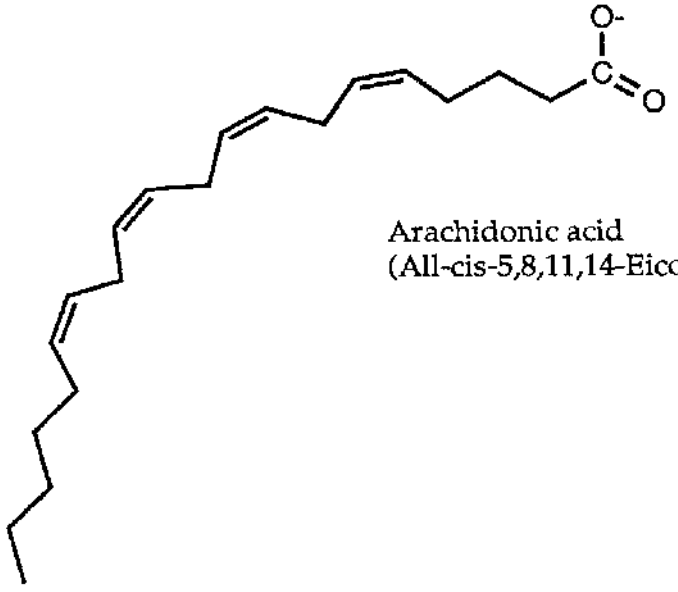
Myristic acid
(n-Tetradecanoic acid)



Palmitic acid
(n-Hexadecanoic acid)



Oleic acid
(cis-9-Octadecenoic acid)



Arachidonic acid
(All-cis-5,8,11,14-Eicosatetraenoic acid)

Figure 8. The structures of myristate, palmitate, oleate and arachidonate.

spots corresponding to PtdIns, PtdCho, PtdEth, and PtdOH determined by scraping and liquid scintillation counting (138).

2.3.2 Phospholipase D Transphosphatidylation Assay

Hydrolysis of phospholipids by PLD results in the release of a water-soluble polar head group and the lipid, phosphatidic acid. However, in the presence of primary alcohols such as butanol, phospholipase D catalyzes a transphosphatidylation reaction in which the phosphatidyl moiety of the susbstrate is transferred to the alcohol, producing the corresponding phosphatidylalcohol (Figure 9). This reaction serves as a definitive accumulation assay for PLD activity as phosphatidylaclohols are not readily metabolised by cells and are not formed by alternative pathways (139, 140).

Detection of PtdBut formation upon cellular stimulation is achieved by radiolabelling either the alcohol or phospholipid substrate of the transphosphatidylation reaction. Radiolabelled primary alcohols, such as [^{3}H]butan-1-ol, are used to study total cellular PLD activity because [^{3}H]phosphatidylalcohols are formed irrespective of the PLD substrate specificity. The use of radiolabelled phospholipids often gives an indication as to the substrate specificity of PLD. The majority of such studies have focused on the PLD-mediated hydrolysis of PtdCho (generally labelled by incubation with the fatty acid [^{3}H]palmitate, unless otherwise stated). However, a number of investigators have also demonstrated the PLD-mediated hydrolysis of other phosopholipids, such as PtdIns and PtdEth, and have identified these as separate, independently regulated PLD activities.

Murine splenic B cells or human tonsillar B cells (5 x 10^7cells/ ml) were labelled in RPMI-1640 medium containing 10% dialysed FCS and [^{3}H]-palmitate, [^{3}H]arachidonate, [^{3}H]myristate, [^{3}H]stearate or [^{3}H]oleate (1 µCi/ 10^6 cells) for 4 hours. Alternatively, murine B cells (10^7/ ml), prepared under asceptic conditions, were pre-incubated overnight in RPMI-1640 medium supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), 2-mercaptoethanol (50 µM) and 10% FCS in the presence of [^{3}H]-palmitate (1 µCi/ 10^6 cells) and the indicated agonist, as

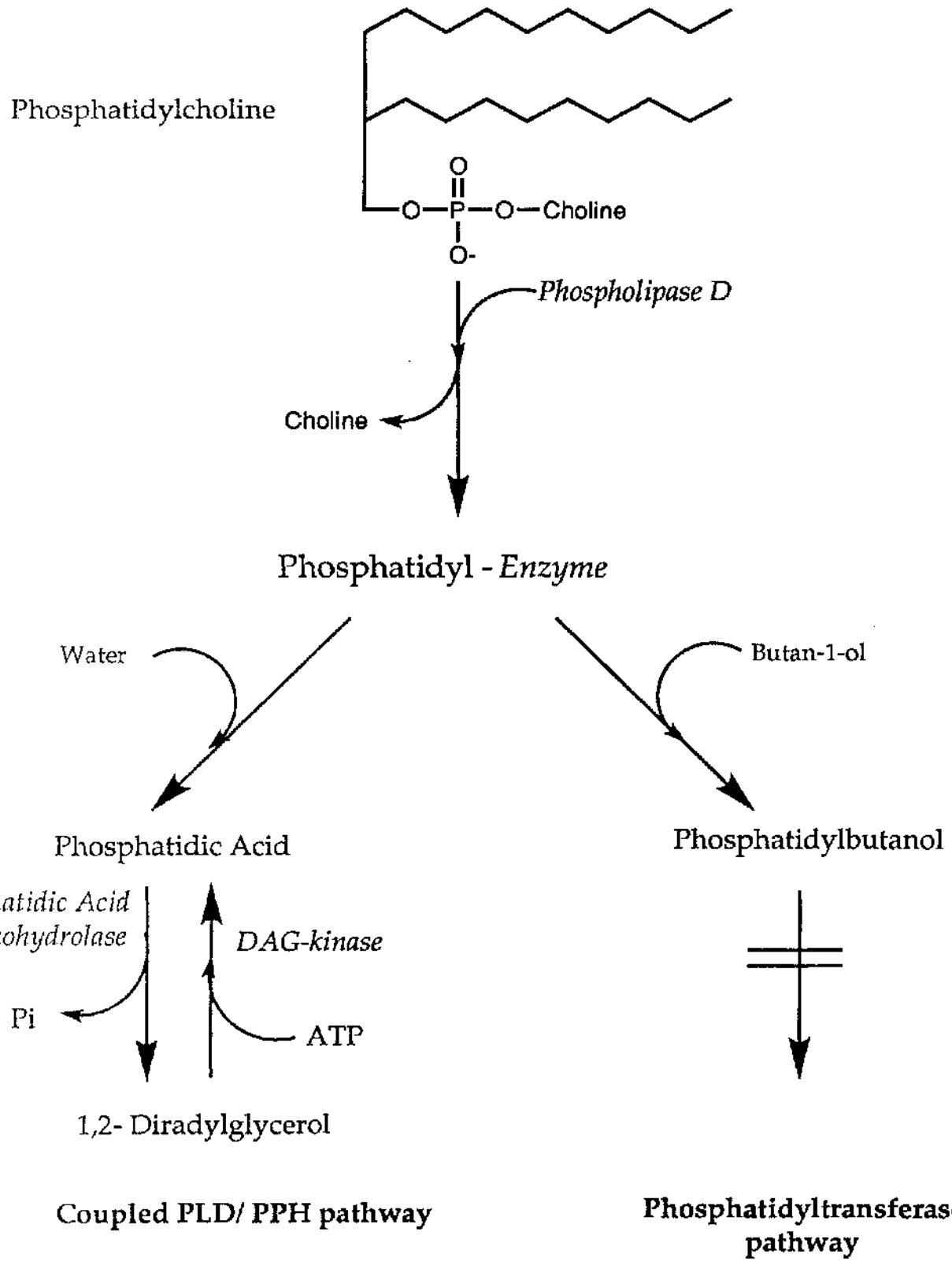

Phosphatidylcholine
O
O—P—O—Choline
O-
Phospholipase D
Choline
Phosphatidyl - Enzyme
Water
Butan-1-ol
Phosphatidic Acid
Phosphatidylbutanol
Phosphatidic Acid Phosphohydrolase
DAG-kinase
Pi
ATP
1,2- Diradylglycerol
Coupled PLD/ PPH pathway
Phosphatidyltransferase pathway

Figure 9. Phosphatidyltransferase Assay for phospholipase D activity. Under physiological conditions, phospholipase D catalyses the hydrolysis of PtdCho and other phospholipids. However, primary alcohols, such as butan-1-ol, are preferential nucleophilic acceptors over water for the phosphatidyl group. Therefore, in the presence of primary alcohols, phospholipase D catalyses the transphosphatidylation of phospholipids. These products are not formed by any other pathway in whole cells, and their formation can therefore be used as a marker for phospholipase D activity.

cells were then washed and resuspended (3 x 10^7 cells/ ml) in RPMI-1640 containing 20 mM described in the appropriate Figure legend. Labelled HEPES, 0.1% BSA, and 0.3% butan-1-ol and equilibrated at 37°C for 15 min prior to stimulation. Alternatively, in order to measure total phospholipid-PLD activity, unlabelled cells were resuspended (3 x 10^7/ ml) in RPMI-1640 containing 20 HEPES, 0.1% BSA, and [^{3}H]butan-1-ol (20 μCi/ ml) and then incubated at 37°C for 15 min prior to stimulation.

Cellular stimulation was initiated by the addition of cells (100 μl; 3 x 10^7/ ml) to glass vials containing 50 μl stimulus (x 3 concentration), followed by incubation in a waterbath at 37°C. After the indicated time, incubations were terminated by the addition of chloroform: methanol (750 μl; 1: 2, by vol.) and the samples left to extract on ice for approximately 10 min. Phase separation was achieved by the addition of chloroform (250 μl) and water (300 μl), followed by vortexing and centrifugation (270g, 4°C) for 5 min. An aliquot (450 μl) of the lower chloroform phase was then removed and dried down in glass vials *in vacuo*. Whatman LK5DF tlc plates were activated at 120C for 30 min and allowed to cool on the bench. Each dried sample was redissolved in chloroform: methanol (25 μl; 19: 1, by vol) and applied to an individual lane of the tlc plate, followed by a further 25 μl wash of each vial. Unlabelled PtdBut (30 μg) was also spotted on each lane. The plate was then developed in the organic phase of the solvent 2,2,4-trimethylpentane: ethyl acetate: acetic acid: water (5: 11: 2: 10, by vol.), using an unlined, non-equilibrated chromatography tank. The position of PtdBut was identified by exposure of the plate to iodine vapour and the appropriate area was scraped from each lane. The radioactivity associated with [^{3}H]PtdBut was determined by liquid scintillation counting (141).

2.3.2.1 Preparation of [^{14}C]phosphatidylbutanol Standard

For some PLD assays, [^{3}H]PtdBut was located by its co-migration with a [^{14}C]PtdBut standard run on a separate lane of the tlc plate. The [^{14}C]PtdBut standard was prepared by incubating [^{14}C]PtdCho-containing micelles with a PLD activity extracted from cabbage.

Micelles were prepared as follows: [^{14}C]PtdCho was dried down under N_2 in a clean glass tube. SDS (5 mM) in buffer A (100 µl; 0.1M sodium acetate, pH 5.6) was then added and the tube was sonicated in a waterbath for 10 min. $CaCl_2$ (375 mM) in buffer A (100 µl), butan-1-ol (75 µl) and buffer A (625 µl) were then added to the micelle preparation, and the PLD reaction initiated by the addition of cabbage PLD (100 units). After incubation at 30C for 3 hours, the reaction was terminated by the addition of chloroform: methanol (3 ml; 2: 1, by vol) and the sample was left on ice to extract for 10 min. Phase separation was achieved by the addition of chloroform (1 ml) and water (1 ml), vortexing and centrifugation (270g, 4°C) for 5 min. The entire lower organic phase was then removed to a clean glass tube and dried down under N_2 and redissolved in chloroform: methanol (200 µl; 19: 1, by vol). This was then applied to two lanes of an activated LK5DF tlc plate: 10 µl on lane 1 and 190 µl on lane 2; and the plate was developed in the upper organic phase of the solvent 2,2,4-trimethylpentane: ethyl acetate: acetic acid: water (5: 11: 2: 10, by vol.), using an unlined, non-equilibrated chromatography tank. The position of [^{14}C]PtdBut was then determined by scraping and liquid scintillation counting individual fractions of lane 1. The appropriate fractions were then scraped from lane 2 and the silica removed to a clean glass tube. Chloroform: methanol (1 ml; 19: 1, by vol) was added to the silica, and the tube was shaken and left to stand on the bench for 5 min. The entire contents of the tube were then removed to a scintered glass funnel under vacuum which was draining into a fresh tube. The silica was washed with chloroform: methanol (1 ml; 19:1, by vol) and again with chloroform (1 ml). The collected [^{14}C]PtdBut solution was then aliquotted to smaller glass vials, dried down under N_2 and stored at –20°C.

2.3.3 Analysis of the water-soluble products of phosphatidylcholine and phosphatidylethanolamine breakdown.

The hydrolysis of PtdCho by PLC produces diacylglycerol (DAG) and choline phosphate (ChoP), whereas PtdCho-specific PLD activity generates phosphatidic acid (PtdOH) and choline (Cho). ChoP and Cho are water-soluble products which can be separated

by ion-exchange chromatography, and their release in [^{3}H]choline-labelled cells can therefore be used as a marker for the activation of PtdCho-specific PLC or PLD activity (139, 140).

The PLD-catalysed hydrolysis of phosphatidylethanolamine (PtdEtn), which has been demonstrated in some cells, results in the generation of PtdOH, and ethanolamine. Ethanolamine (Etn) which can be separated from ethanolamine phosphate (EtnP) on ion-exchange columns in a manner identical to that described for choline and choline phosphate. The release of Etn of can thus serve as a marker for PtdEtn-specific PLD activity.

Murine splenic B cells or human tonsillar B cells were resuspended (5 x 10^7/ ml) in RPMI-1640 supplemented with 10% dialysed FCS and labelled with [^{3}H]methylcholine at 37°C for 4 hours. Human Jurkat T cells were resuspended (10^7/ml) in RPMI-1640 supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FCS and labelled with [^{3}H]methylcholine or [^{3}H]ethanolamine (1 µCi/ 10^6 cells) overnight. Cells were then washed three times by centrifugation (630g, room temp) for 10 min, and resuspended (3 x 10^7/ ml) in RPMI-1640 plus 0.1% BSA and 10 mM HEPES. In pulse-chase experiments cells were washed by centrifugation (630g, room temp) for 10 min and resuspended in (10^6/ml) in RPMI-1640 supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FCS, and incubated at 37°C for 1 hour. They were then washed and resuspended prior to stimulation as described above.

Cellular stimulation was initiated by the addition of cells (100 µl; 3 x 10^7/ ml) to glass vials containing 50 µl of stimulus (x 3 concentration) and vials were incubated in a waterbath at 37°C for the appropriate time. Reactions were terminated by the addition of chloroform: methanol (750 µl, 1:2, by vol.) and the samples were extracted on ice for approximately 10 min. Phase separation was achieved by the addition of chloroform (250 µl) and distilled water (300 µl). Vials were then capped, vortexed and centrifuged (270g, 4°C) for 5 min. Samples could be stored overnight at –20°C.

An aliquot (750 µl) was removed from the upper methanolic/ aqueous phase of each sample and diluted to 5 µl with distilled water. The samples were then loaded onto Dowex columns (prepared as described below) which were washed with a further 2 ml distilled water.

The run-through was collected as the glycerophosphocholine (GroPCho) (or glycerophosphoethanolamine, GroPEth) fraction. The ChoP (or EthP) fraction was eluted with 150 mM KCl (10 ml), and the Cho (or Eth) fraction was eluted with 1M KCl (4 ml). The radioactivity associated with these fractions was then determined by liquid scintillation counting (139, 140).

2.3.3.1 Preparation of Dowex Columns.

Dowex-50-WH+ (100-200 ml) was washed three times in 3-4 volumes of distilled water, and then three times in 5 volumes of 1M HCl. Finally, the Dowex was washed with several volumes of distilled water until the washings were of constant pH (between 4.5 and 5.5). The Dowex was then stored in an equal volume of distilled water at 4°C.

Columns (1ml Dowex) were prepared in glass wool plugged Pasteur pipettes. Elution profiles of PtdCho- and PtdEth-specific PLC and PLD products were validated by analysis of the elution of radiolabelled standards from identical columns. These standards were prepared in the same way as samples (i.e. phases were split by achieving the ratio 1: 1: 0.9 of chloroform: methanol: aqueous and the methanolic/aqueous phase was used), and comprised [^{3}H]choline (or [^{3}H]ethanolamine) (10 µCi), [^{14}C]choline phosphate (2 µCi), or a mixture of both standards. Percentage recovery was estimated by expressing the level of eluted Cho and ChoP as a percentage of the radiolabel found in identically prepared samples (750 µl).

2.3.4 Analysis of Inositol Phospholipid hydrolysis

The hydrolysis of inositol phospholipids by phospholipase C results in the release of diacylglycerol (DAG) and inositol phosphates (IPs). Preincubation of cells with LiCl prevents degradation of IP_1 to inositol and hence causes IPs to accumulate, providing a measure of total IPs formation (142). Inositol phosphates can be purified on Dowex-formate columns, and this technique was used to determine their release upon stimulation of [^{3}H]inositol-labelled cells in the presence of lithium. In addition, [^{3}H]inositol phospholipids (PtdIns, $PtdInsP_1$, and $PtdInsP_2$) were separated and quantified by thin-layer chromatography (tlc) (142).

2.3.4.1 Determination of Inositol Phosphates formation

B cells (10^8/ ml) were resuspended in F-10 (HAM) medium containing 20 mM HEPES and 1% dialysed serum, and labelled with [^{3}H]inositol (100 μCi/ ml) at 37°C for 4 hours. The labelled cells were then washed twice by centrifugation (630g, room temp) for 10 min, resuspended (3 x 10^7/ ml) in RPMI-1640 containing 20 mM HEPES, 0.1% BSA, and 10 mM LiCl and incubated at 37°C for 15 min.

Cellular stimulation was initiated by the addition of cells (100 μl; 3 x 10^7/ ml) to glass vials containing 50 μl stimulus (x 3 concentration) in the above medium. Incubations were terminated by the addition of chloroform: methanol (750 μl; 1: 2, by vol.) and the samples left to extract on ice for approximately 10 min. Phase separation was achieved by the addition of chloroform (250 μl) and water (300 μl). Samples were then capped, vortexed and centrifuged (270g, 4°C) for 5 min. Samples could be stored overnight at –20°C.

An aliquot (750 μl) was removed from the upper aqueous/ methanol phase of each sample and loaded onto a Dowex-formate column (prepared as described below). The column was then washed with 6 ml of distilled water to elute free inositol and this was discarded. Glycerophosphoinositides (GPIs) were eluted using 60 mM sodium formate/ 5 mM sodium tetraborate (6 ml), and this fraction was also discarded. Total inositol phosphates (IP_1, IP_2, and IP_3) were then eluted with 1M ammonium formate/ 0.1M formic acid (3 ml), and the associated radioactivity was determined by liquid scintillation counting. [^{3}H]Inositol phosphate released was expressed as a percentage of the total [^{3}H]inositol incorporated into cellular lipids. Total incorporation or [^{3}H]inositol was approximated by counting aliquots of the lower lipid/ organic phase of unstimulated cells.

2.3.4.1.1 Preparation of Dowex-formate and separation columns

Dowex-chloride 8 x 100 (100 g) was weighed out into a large beaker, washed twice in 5 volumes of distilled water to remove broken beads and then with 20 volumes of 2M NaOH. The treated Dowex was then washed with 10 volumes of distilled water, followed by 5 volumes of 1M formic acid. The Dowex-formate was washed with 50 volumes of distilled

water (until pH was constant at around 5.5), resuspended in an equal volume of distilled water and stored at 0-4°C.

Separation columns were prepared by plugging pasteur pipettes with glass wool and adding 1 ml of Dowex slurry. These dowex columns were washed with 4 ml of distilled water prior to use.

2.3.4.2 Determination of [^{3}H]inositol phospholipid levels

Resting B cells were labelled with inositol (2.5 μCi/ 10^6 cells) for 4 hours at 37°C, stimulated with the appropriate ligand and the reactions quenched by two-phase separation as outlined above. An aliquot (450 μl) was removed from the lower chloroform phase of each sample and dried down in a glass vial *in vacuo*. Silica-gel 60 plates (20 x 20 cm, 250 mm thick; Merck, Darmstadt, Germany) were activated by soaking in a solution of 1.2% oxalic acid in methanol: water (3: 2), air-drying and heating at 120°C for 1 hour. Each dried sample was then dissolved in chloroform: methanol (25 μl; 2: 1, by vol.) and applied to an activated Silica-gel 60 tlc plate, along with 20 μg each of unlabelled PtdIns, PtdInsP, and $PtdInsP_2$. The plates were developed in a paper-lined tank, pre-equilibrated with the solvent chloroform: acetone: methanol: glacial acetic acid: water (80: 30: 26: 24: 14, by vol.). They were then exposed to iodine vapour and [^{3}H]PtdIns, [^{3}H]PtdInsP and [^{3}H]$PtdInsP_2$ were located by visualisation of the unlabelled standards. The radioactivity associated with these fractions was then determined by scraping and liquid scintillation counting.

2.3.5 Phospholipase A_2 Assay

PLA_2 catalyses the hydrolysis of the ester bond at the 2-position of the glycerol backbone of a phospholipid, generating a fatty acid and a lysophospholipid (Figure 7). A number of phospholipids are substrates for PLA_2, including PtdCho and PtdIns, and their hydrolysis usually results in the release of arachidonic acid. Exogenously added arachidonic acid is readily incorporated into phospholipids by whole cells; and the detection of [^{3}H]arachidonate release by tlc analysis upon stimulation of [^{3}H]arachidonate-labelled cells is the method by which PLA_2 activation is determined in this study.

Murine splenic B cells or thymocytes (5 x 10^7/ ml) were resuspended in RPMI-1640 supplemented with 10% dialysed fetal calf serum and cultured in the presence of [^{3}H]arachidonate (0.5 μCi/ 10^6 cells) at 37°C for 4 hours. WEHI 231 cells (2 x 10^6/ ml), grown in RPMI-1640 supplemented with glutamine (2 mM), 2-mercaptoethanol (50 μM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FCS, were labelled overnight with [^{3}H]arachidonate (0.25 μCi/ 10^6 cells). Cells were then washed twice and resuspended (3 x 10^7/ ml) in RPMI-1640 plus 0.1% BSA and 10 mM HEPES.

Cellular stimulation was immediately initiated by the addition of cells (100 μl) to glass vials containing 50 μl of stimulus (x 3 concentration) and vials were incubated in a waterbath at 37°C for the appropriate time. Reactions were terminated by the addition of ice cold methanol: glacial acetic acid (500 μl; 100: 1.5, by vol.) and the vials were incubated on ice for approximately 10 min. Chloroform (250 μl) was added to each vial and the samples incubated on ice for a further 10 min. Phase separation was then achieved by the addition of chloroform (250 μl) and water (300 μl), vortexing and centrifugation (270g, 4°C) for 5 min.

Whatman LK5DF tlc plates were activated by heating at 120°C for 15 min and allowed to cool on the bench. An aliquot (450 μl) of the lower chloroform phase was removed from each sample to a clean glass vial, spiked with unlabelled arachidonate (30 mg) and dried down *in vacuo*. Each dried sample was redissolved in chloroform: methanol (25 μl; 2: 1, by vol) and applied to a pre-absorbant strip of the t.l.c. plate along with a further wash of the vial. The plate was then developed in a paper-lined tank pre-equilibrated with the solvent hexane: diethyl ether: formic acid (80: 20: 2, by vol.). Arachidonate was located by exposure of the plate to iodine vapour and the radioactivity associated with each sample was then determined by scraping and liquid scintillation counting (138, 143).

2.4 Purification and preparation of antibodies

2.4.1 Purification of antibody from cellular supernatant

Bet-2 (rat anti-mouse chain, IgM H chain) and anti-murine IA^d antibodies were purified from tissue culture supernatant, using the following procedure:

A column, consisting of a glass wool-stoppered Pasteur pipette containing 1 ml of or Protein G-speharose (capacity: 35 mg/ml)) was prepared, and this was connected to a reservoir made from a 50 ml syringe. PBS (5 ml) was then run through to wash away the ethanol in the protein buffer. Tissue culture supernatant (<500 ml,assuming a maximum of 20 μg/ml of antibody) was then run through the column, and the flow-through retained. The columns were washed with PBS until the level of eluted protein (as measured at A_{280}) had returned to baseline.The bound antibody was eluted using 0.5 ml aliquots of 0.2M acetic acid which were collected in vials containing an equal volume of 1M Tris buffer. The elution was followed and the amount of protein estimated by measuring A_{280} (an OD of 1.4 being approximately equivalent to 1 mg/ml of protein). The appropriate fractions were then pooled and dialysed in PBS overnight, filtered sterilised and stored in 1 ml aliquots at 4°C (134, 144).

2.4.2 Biotinylation of purified antibody with NHS-biotin

Antibody solution was diluted with sodium bicarbonate solution (minimal dilution, final conc. 50 mM) (pH 8.5) in a clean test tube. NHS-biotin (0.3 mg) was then dissolved in DMSO (10 μl) and added to the solution of Ig which was incubated at room temperature for 30 min. Excess biotin was then removed by centrifugation of the solution at 1,000g for 20 min in a Centricon-30 Microconcentrator (Amicon). After centrifugation the sample was diluted in 0.1 M sodium phosphate (pH 7.0). This process was repeated twice. Final protein concentration was determined by A_{280}.

2.4.3 Purification of FGK anti-CD40 antibody using ammonium sulphate precipitation and S-sepharose.

Equal volumes of saturated ammonium sulphate solution and tissue culture supernatant were mixed and the immunoglobulin fraction allowed to precipitate overnight at 4°C. The precipitated immunoglobulin was harvested by centrifugation at 10,000 rpm and then dialysed exhaustively against malonate buffer (per litre: malonic acid sodium salt (6.5 g), malonic acid (1.2 g), betaine (1 g)). A column consisting of a glass wool-stoppered 20

ml syringe was then filled with 5 ml S sepharose gel. The antibody solution was loaded onto the column which was then washed with malonate buffer until the eluant A_{280} had reached a basal level. The antibody was then eluted using the malonate buffer containing 0.5M NaCl, the profile of the elution being followed using A_{280}. The antibody solution was then extensively dialysed against PBS, filter-sterilised, and stored at 0-4°C.

2.5 SDS-PAGE and Western Blot analysis of $cPLA_2$ expression.

In order to detect the expression of $cPLA_2$ in lymphocytes, cellular extracts were separated by SDS-PAGE, then transferred to nitrocellulose and probed using antibodies raised against specific peptides to C-terminal or internal sequence of $cPLA_2$.

2.5.1 Preparation of cell lysates.

Cell were washed three times in modified RIPA buffer (50 mM Tris-HCL buffer, pH 7.4 containing 150 mM NaCl; 1 mM EGTA; 1 mM PMSF; 1 mM Na_3VO_4 and 1 mM NaF) before resuspension and solubilisation in RIPA lysis buffer containing 1% (v/v) NP-40; 0.25% (w/v) sodium deoxycholate and 1g/ ml each of antipain, aprotinin, chymostatin, leupeptin and pepstatin for 15 min on ice. Following solubilisation, cells were microfuged AT 16,000 rpm for 30 min at 4°C and resulting supernatants stored at –20°C for analysis of $cPLA_2$ expression.

2.5.2 SDS-PAGE

The following solutions were prepared prior to the preparation of SDS-polyacrylamide gels:

i) Acrylamide (30% solution):30 g acrylamide, 0.8 g methylene bisacrylamide, made up to 100 ml with distilled H_2O

ii) 1.5M Tris-HCl, pH 8.8

iii) 0.5M Tris-HCl, pH 6.8

iv) 10% SDS (or sodium lauryl sulphate)

v) 10% Ammonium persulphate:prepared fresh

vi) Tank buffer:(per litre) 3 g Tris (free base), 14.4 g glycine, 10 ml 10% SDS.

(Made up at x 5 stock)

vii) Sample buffer (x 3 stock): 10 ml 0.5M TrisHCl (pH 6.8), 10 ml 10% SDS, 10 ml Glycerol, 2 ml 0.5% Bromophenol Blue, 1% (v/v) mercaptoethanol

7.5% polyacrylamide gels were prepared as follows:
30% acrylamide (7.5 ml), H_2O (14.3 ml) and TrisHCl (pH 8.8) (7.5 ml) were mixed in a side arm conical flask and degassed. This was decanted to a beaker with a stirrer, and SDS (0.3 ml), 10% ammonium persulphate (150 µl) and TEMED (10 µl) were added to initiate polymerisation. Gels were poured, overlayed with H_2O and allowed to set for approximately 30 mins. The layer of water was removed and the stacking gel (3% polyacrylamide) prepared and poured as follows. 30% acrylamide solution (1.5 ml), H_2O (9.45 ml) and 0.5M TrisHCl (pH 6.8) (3.75 ml) were mixed and degassed. Then 10% SDS (150 µl) , 10% ammonium persulphate (150 µl) were added and polymerisation initiated with TEMED (10 µl). The stacking gel was poured, a well comb was inserted and the gel allowed to set for approximately 30 minutes. The comb was removed and the wells overlayed with H_2O. The positions of the wells were identified with a marker pen.

Samples were denatured in the appropriate volume of sample buffer by heating for 2 min in a boiling water bath. Then 100 µg protein was loaded into each well. Molecular weight Rainbow markers (10 µl) were also loaded onto one or two wells. The samples were electrophoresed into the stacking gel at 50V and then resolved in the separating gel at 200V for 2 hours in an ice-cooled PAGE apparatus.

2.5.3 Transfer of resolved proteins to nitrocellulose

SDS-PAGE-resolved proteins were transferred to nitrocellulose using a semi-dry blotter (Sartorius). Graphite plates were rinsed with distilled H_2O. Three sheets of filter paper were cut to the size of the gel, soaked in buffer (pH 9.2): 48 mM Tris; 39 mM Glycine; 1.3 mM SDS and 20% methanol, and placed on the cathode (lower) plate. The gel was placed on the filters and any air bubbles were removed with a buffer-wetted gloved finger. Nitrocellulose was soaked in the above buffer and placed on the gel, and any air

bubbles were removed. Three sheets of buffer-soaked filter paper were placed on top of the gel/ filter sandwich. The anode plate was replaced and the transfer run at up to 4mA/ cm^2 (constant current) for 30 min at room temperature. 60V was not exceeded, in order not to blow the plates.

2.5.4 Western Blot analysis of $cPLA_2$ expression

Samples were resolved by 7.5% SDS-PAGE (as described in section 2.5.1), and then transferred to nitrocellulose filters by semi-dry blotting (as described in section 2.5.2). Following blocking with 10% non-fat milk overnight, the filters were incubated for 2 hours with a rabbit anti-human $cPLA_2$ peptide antibody ($^1/_{1000}$ dilution of serum) raised against a C-terminal peptide (amino acids 727-749) coupled to KLH. All antibody solutions were diluted in 10% marvel/PBS/Tween-20 (0.1%). The filters were then washed in marvel/PBS/Tween-20 five times for 20 min. Immunoreactivate $cPLA_2$ was visualised by developing with a donkey anti-rabbit Ig-HRP antibody ($^1/_{1000}$ dilution) and the ECL system (Amersham Internation plc).

3 Investigation of the role of phospholipase D in B cell signalling.

Phospholipase D (PLD) activities catalyse the hydrolysis of phosphodiester bonds distal to the glycerol backbone of phospholipids, generating phosphatidic acid (PtdOH) and a free polar head group (Figure 7) (128). PtdOH has been identified as a putative lipid second messenger and has been implicated in the modulation of a range of biological responses such as the respiratory burst in neutrophils, granule secretion by mast cells, insulin secretion in the pancreas, and mitogenic responses in BALB/c fibroblasts and natural killer cells (128). PLD activation is therefore involved in a diverse array of cellular responses and this is reflected in the variety of cellular signalling elements which PtdOH has been reported to modulate. These include activation of certain Protein kinase C (PKC) isoforms, Ras-like G-proteins (including Ras, Rho, and Rac), the mobilisation of calcium, and expression of the proto-oncogenes *c-fos* and *c-myc*. (128, 145).

PtdOH may also play a role in cellular responses via its conversion by PtdOH phosphohydrolase (PPH) to diacylglycerol (DAG) (139, 140), an activator of most PKC isoforms (Figure 7). In many cells, the PPH-mediated hydrolysis of PtdOH is responsible for the sustained production of DAG and prolonged activation of PKC needed for a mitogenic response. PtdOH may also act via its lysoderivative, lysoPtdOH (LPA) (146-148) which has been shown to be a potent mitogen for several cell systems and mediates its effects via pertussis toxin-sensitive G-protein receptors coupled to the mobilisation of calcium and downstream activation of the Ras-MAP kinase cascade (148). The effects of PLD activation can therefore be mediated not only through the direct effects of PtdOH on downstream signalling events but also via its subsequent metabolism and resultant generation of other lipid messengers such as LPA and DAG.

3.1 Phospholipid specificity and subcellular localisation of phospholipase D activities.

In the vast majority of studies PtdCho has been identified as the preferred substrate for receptor-coupled PLD signalling (reviewed by Exton, 1994) (128). However, in several systems PLD has been demonstrated to hydrolyse phospholipids other than PtdCho. For example, stimulation of NIH 3T3 fibroblasts with platelet-derived growth factor (PDGF) results in the PLD-catalysed hydrolysis of both PtdCho and phosphatidylethanolamine (PtdEtn) (149, 150). Moreover, exclusive breakdown of PtdEtn by PLD is observed in glial cells following stimulation with fetal calf serum and PMA (151). Yet another study has provided evidence for the existence of two, independently regulated PLD activities in Madin-Darby Canine Kidney (MDCK) cells (152): in this system, bradykinin stimulation results in the PLD-catalysed hydrolysis of PtdIns, whereas stimulation with PMA induces the activation of PtdCho-PLD. These types of study provide strong evidence for the existence of distinct, differentially regulated PLD isozymes within a single cell type. Moreover, mammalian PLD activities have been found to exist in both membrane-bound and cytosolic forms: whilst the membrane-bound forms appear to exhibit strict specificity for PtdCho whereas the cytosolic forms have been shown to hydrolyse PtdCho, PtdEtn or PtdIns (128, 145, 153-161).

Interestingly, no lipid second messenger functions have, as yet, been demonstrated for the free polar head groups choline, ethanolamine and inositol which are released upon the PLD-mediated hydrolysis of phospholipids. It therefore appears that their generation is not the basis of any differential effects induced by the PLD-catalysed hydrolysis of specific phospholipids. However, since PtdCho, PtdEtn and PtdIns differ in their acyl chain composition, their hydrolysis results in the production of distinct PtdOH species, each potentially performing a unique second messenger function. For example, only the stearoyl-arachidonyl species of PtdOH has been postulated to activate ras proteins (162). This activation of ras is the result of a combination of PtdOH inhibiting GTPase activating protein (GAP) and activating GTPase inhibiting protein

(GIP). In summary, the downstream effect of the activation of a particular PLD isozyme may therefore depend on its subcellular localisation and its phospholipid substrate specificity.

3.2 The regulation of receptor coupling to phospholipase D.

Activation of PLD can be mediated via both G protein-coupled receptors (GREs) and receptor tyrosine kinases (RTKs) (128, 129, 145). However, the precise mechanisms underlying the coupling of receptors to distinct phospholipase D activities have not been elucidated. Studies to date using pharmacological activators and inhibitors indicate that receptor-PLD coupling may be mediated by multiple intracellular signals including protein phosphorylation, calcium mobilisation, and G-protein activation. Very little information is available concerning the regulation of PtdEtn- and PtdIns-specific PLD activities. Therefore, unless specified most of the discussion below refers only to PtdCho-specific PLD activities.

3.2.1 Role of protein tyrosine phosphorylation

Phospholipase D activation has been demonstrated in a number of cell types in response to stimulation with growth factors such as PDGF and EGF. Growth factors bind to and activate intrinsic receptor tyrosine kinases (RTKs) and this raises the question of the role of tyrosine phosphorylation in the regulation of PLD activity. It would appear that in several cases receptor-mediated activation of PLD is dependent on tyrosine kinase activity (128, 129, 145, 163, 164). For example, activation of PLD in EGF-stimulated fibroblasts is abrogated by PTK-inhibitors but unaffected by PKC inhibitors (165). Furthermore, pervanadate, an inhibitor of protein tyrosine phosphatases (PTPs), will activate PLD in human neutrophils and other cell types (145). At present it is not clear if these effects are due to direct interaction between RTKs and PLDs or occur as a result of other signals more proximal to the receptor. Tyrosine phosphorylation is also reported to be involved in the fMLP-mediated activation of PLD in neutrophils (166). As the fMLP

receptor does not possess intrinsic tyrosine kinase activity, this suggests that both RTKs and non-receptor PTKs may be involved in the regulation of PLD activity.

3.2.2 Role of G proteins in PLD regulation

G protein-coupled receptors (GREs), such as α_2 receptors on rat-1 fibroblasts and endothelin receptors on mesangial cells, are also found to be coupled to PLD activity (167). Activation of PLD upon stimulation of rat-1 fibroblasts with α_2-agonists is inhibited by pertussis toxin, which ADP-ribosylates Gi-like G proteins, suggesting the involvement of heterotrimeric G proteins in the regulation of PLD activity (167). This is supported by the finding that the non-hydrolysable GTP analogue guanosine 5'[γ thio] triphosphate (GTPγS) will stimulate PLD activation in permeabilized cells such as neutrophils (145). However, several laboratories have recently reported the involvement of small GTP-binding proteins in the regulation of PLD activity. These include members of the ADP Ribosylation Factor (ARF) family(157-159, 168) (136-138, 168), which are required for the ADP-ribosylation of G-type G proteins by cholera toxin, and a membrane-associated member of the Rho family (156). However, the precise mechanism by which these G proteins regulate PLD activation is still unclear.

G proteins have also been shown to interact with other intracellular signals in the regulation of PLD activity: pervanadate and GTPγS were reported to activate PLD synergistically in phagocytic leucocytes, indicating crosstalk between G proteins and protein tyrosine phosphorylation to generate maximal PLD activation (163). It is not clear, however, whether G proteins couple to PLD directly or indirectly through a mechanism such as calcium mobilisation.

3.2.3 The role of phosphoinositide breakdown and protein kinase C activation in the activation of PLD.

In many systems, agonist-mediated stimulation of PLD activity is preceded by the activation of $PtdInsP_2$-PLC-γ, leading to the generation of DAG and IP_3, and the resultant mobilisation of Ca^{2+} and activation of PKC. For example, stimulation of rat

fibroblasts with α-thrombin or endothelin is reported to induce PLD activation, which is preceded by the activation of PLC-γ (145). This suggests that receptor-mediated PLD activation may be dependent on signals generated by the hydrolysis of phosphoinositides, such as the activation of PKC. Indeed, stimulation of most cells with phorbol esters, which are pharmacological activators of PKC, will induce activation of PLD, indicating that PLD may be regulated either directly or indirectly by PKC (128, 129, 145). This is supported by findings that PKC inhibitors, or chronic phorbol ester treatment to downregulate PKC levels, will inhibit both PMA-mediated and endothelin-induced PLD activation in rat-1 fibroblasts. More direct evidence of PKC involvement in PLD activation was provided by a study in which over-expression of PKC-α1 in rat-1 fibroblasts was found to enhance the activation of PLD in response to PMA, endothelin or α-thrombin (145, 169).

In contrast, in receptor systems such as EGF stimulation of Swiss 3T3 cells (via a RTK) (165) and angiotensin stimulation of vascular smooth muscle cells (via a GRE) (145), agonists will induce the activation PLD in the absence of phosphoinositide hydrolysis. Moreover, in other receptor systems, including α-thrombin stimulation of human platelets (145), PLD activation is reported to be insensitive to both PKC inhibition and chronic phorbol ester treament. In addition, the evidence that PKC is the sole target for PMA is weakening, and it has been suggested that PMA interacts directly with phospholipase D. Indeed, addition of PMA to membrane preparations from HL60 cells has been reported to directly stimulate PLD activity. Therefore, although PLC-γ-mediated signals may play a role in the coupling of some receptors to PLD activation, there are clearly receptors which couple to PLD through mechanisms other than the hydrolysis of phosphoinositides and PKC activation.

3.2.4 The role of Ca^{2+} mobilisation in the regulation of PLD activity.

In a variety of receptor systems PLD activation can be blocked by chelation of extracellular calcium (152, 170-172). Moreover, in many cells Ca^{2+} ionophores such as ionomycin are good activators of PLD (128, 170), suggesting a possible role for calcium influx in the receptor-mediated activation of PLD. In neutrophils, for example, phorbol esters and calcium ionophores act synergistically to activate PLD (173, 174). This may be consistent with the finding that cytochalasin B is required for the fMet-Leu-Phe-mediated activation of PLD in these cells, as in cytochalasin B-treated neutrophils, the chemotactic peptide, fMet-Leu-Phe induces a prolonged influx of calcium from the external medium, rather than the transient mobilisation of calcium from intracellular stores observed in untreated cells. However, it should be noted that cytochalasin B is also found to disrupt the cytoskeletal network, and its role in the fMLP-mediated activation of PLD may therefore be more complex than the priming of neutrophils for calcium influx (173-175). Nevertheless, such findings may suggest a role for the influx of extracellular calcium, as opposed to the mobilisation of calcium from intracellular stores, in the activation of PLD. Studies in partially purified PLD preparations have also provided evidence for the involvement of calcium in the regulation of PLD. For example, PLD activity in a preparation from rat brain has been found to be enhanced by the addition of Ca^{2+} (176). Ca^{2+} has also been found to be required for GTPγS-mediated stimulation of PLD in granulocyte homogenates (168)

In contrast, there are a number of PLD activities which do not appear to require calcium for activity (128, 129, 145). Receptor systems in which PLD activation occurs in the absence of Ca^{2+} mobilisation include stimulation of rat tail artery with norepinephrine and stimulation of mesangial cells with vasopressin (177). Also, in several partially purified preparations, including hepatocyte membranes and spermatozoal extracts, exogenous Ca^{2+} is not required for PLD activity. Moreover, PMA-mediated activation of PLD in intact cells is usually unaffected by the chelation of extracellular and intracellular Ca^{2+} with EGTA (165).

There is also evidence, however, for the inhibition of PLD by Ca^{2+}. For example, a PLD preparation from rat brain synaptosomes, which was found to function optimally at a pH of 7.2 and was stimulated by oleate (4 mM) and Mg^{2+} (<1 mM), was also stimulated by "low" concentrations of Ca^{2+} (<0.4 mM) (176). This PLD activity, however, was totally inhibited by higher concentrations of Ca^{2+} (2 mM). However, it is very unlikely that the enzyme would encounter such a high concentration of Ca^{2+} in the cytosol, and so the physiological relevance of this finding is debatable. Nevertheless, other preparations of PLD have exhibited inhibition by calcium: a preparation of a membrane-bound PLD activity from rat brain, which required the detergent Triton X-100 for activity, was inhibited by Ca^{2+} and Mg^{2+} at all concentrations tested (178); furthermore, a PLD activity inhibited by Ca^{2+} and Mg^{2+} has also been identified in amniotic membranes (179).

These reports from intact cells, membranes and partially purified preparations of PLD therefore indicate the possible existence of multiple PLD activities which can be differentially regulated by Ca^{2+}: ie. calcium-dependent, calcium-independent and calcium-inhibited forms of PLD.

3.2.5 Role of sphingolipid second messengers

The lipid second messengers (LSMs) sphingosine and ceramide, which are formed as a result of sphingomyelin hyrolysis by sphingomyelinase (Figure 10), are implicated in the regulation of a number of cellular processes including differentiation, mitogenesis, and particularly, the induction of apoptosis (61, 180-182). Ceramide has been reported to induce the activation of a novel ceramide-activated protein kinase (CAPK) and the protein phosphatase 2A (PP2A) (181). Sphingosine is thought to mediate its effects, at least in part, through its conversion to sphingosine-1-phosphate (SPP) (183, 184). Both ceramides and sphingosine/SPP are reported to modulate the activity of phospholipase D (149, 180, 184-192) .

Pre-incubation of rat fibroblasts with cell-permeable ceramides was found to inhibit the activation of PtdCho-PLD by PMA or GTPγS, as well as the induction of

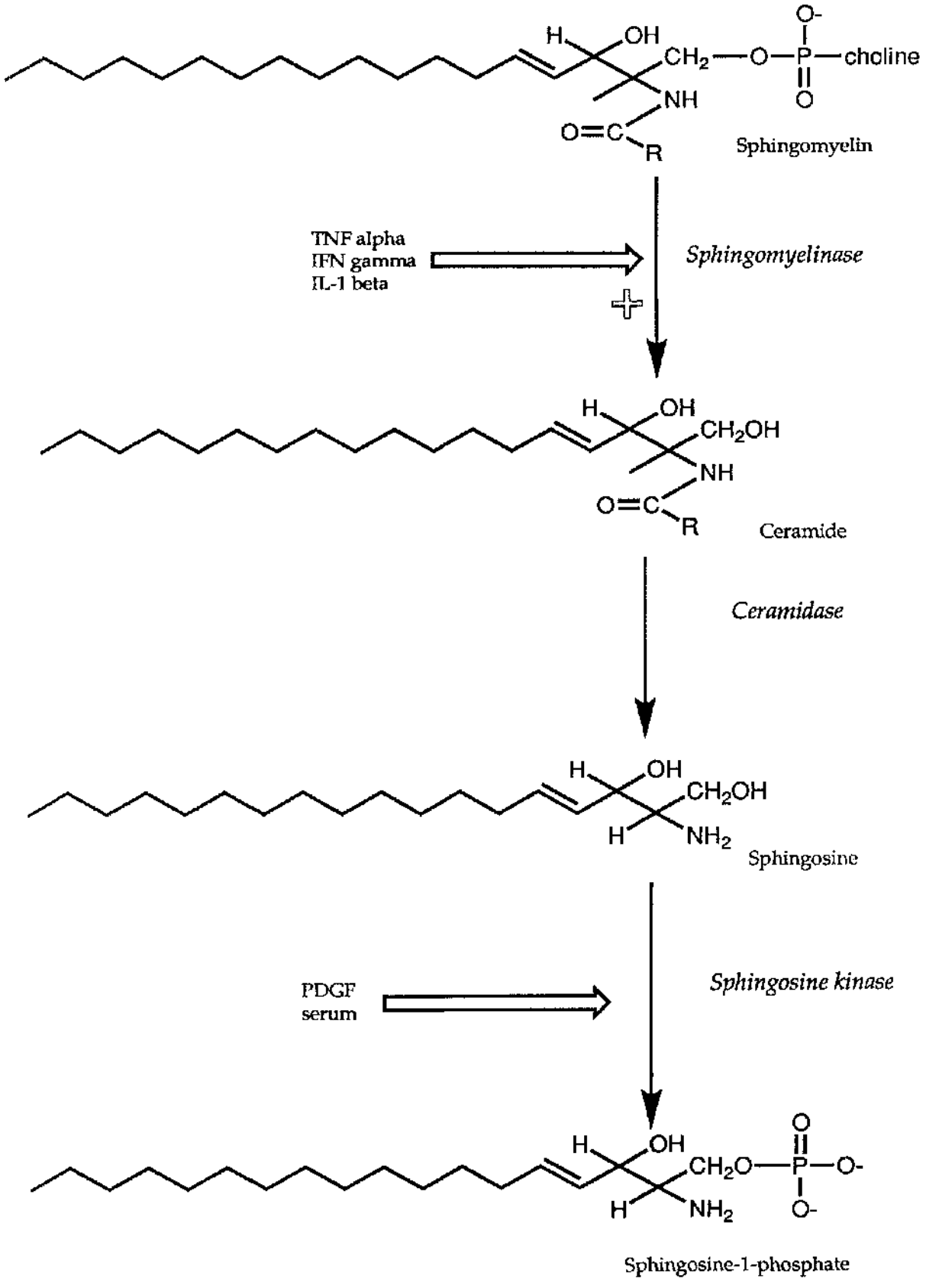

Sphingomyelin
TNF alpha
IFN gamma
IL-1 beta
Sphingomyelinase
Ceramide
Ceramidase
Sphingosine
PDGF
serum
Sphingosine kinase
Sphingosine-1-phosphate

Figure 10. The sphingomyelinase pathway.

DNA synthesis induced by PtdOH or LPA (180, 190, 193). In contrast, exogenously added sphingosine or sphingosine-1-phosphate has been found to induce activation of PtdCho-PLD in a variety of cell types (128, 194), in addition to other reported effects such as the mobilisation of calcium. Moreover, the activation of a PtdEtn-selective phospholipase D has also been reported in multidrug-resistant MCF-7 human breast carcinoma cells upon their stimulation by sphingosine, PMA or H_2O_2 (191).

These findings indicate that sphingolipid-derived second messengers may play a physiological role in the receptor-coupled activation of PLD activity, although the mechanisms of this regulation are, as yet, unknown.

3.2.6 Four major classes of PtdCho-PLD activity.

The precise details of receptor coupling of these various phospholipase D activities, indicated by pharmacological manipulation, have not as yet been delineated. Investigations have been hampered by the fact that no PLD species have been cloned, and by the lack of defined, as opposed to partially purified, PLD preparations available: currently, there is only a single report of purification to homogeneity of a PLD activity (190 kDa) (195). However, recent evidence obtained in *in vitro* reconstitution studies of partially purified PLD preparations, has substantially rationalised the wealth of conflicting pharmacological data and has suggested the existence of four major classes of PtdCho-PLD activity differing in their cofactor requirements:

(i) oleate-dependent PLD(s): a PLD activity, solubilized from rat brain membranes by Triton X-100 and reconstituted in lipid vesicles, was found to be optimally active at neutral pH and absolutely dependent on sodium oleate for activity (optimal conc. 4 mM). This PLA activity could be further activated by Mg^{2+} and Ca^{2+} (158, 176).

(ii) $PtdInsP_2$ and ARF -dependent PLD enzymes: another PLD activity, solubilized from rat brain and separated from the oleate-dependent species by HPLC, was found to require $PtdInsP_2$ for activity (optimal conc. 8μM) (157). This $PtdInsP_2$-

dependent PLD was dramatically activated by the addition of the low molecular weight G protein, ADP ribosylation factor (ARF) 1 and GTPγS. Moreover, in contrast to the oleate-dependent PLD isolated from rat brain, this PLD activity was completely inhibited by the addition of oleate (0.3 mM). The $PtdInsP_2$/ ARF-dependent PLD could be activated by recombinant ARFs 1, 5 or 6 and, interestingly, this activation was found to be enhanced if myristoylated ARFs were used, suggesting that membrane association of these proteins is important for activation of PLD (158). Furthermore, similarly regulated PLD activities have also been found in HL60 cells (activated by ARFs 1 and 3) (159). ARFs have been strongly implicated in the process of vesicular budding from the Golgi membrane, suggesting that PLD activation may also be involved in this process, and the identification of ARF-activated PLD activity in Golgi-enriched membranes from CHO cells, lends further weight to this hypothesis (161). Furthermore, PLD activation by ARF and GTPγS in these cells was blocked by brefeldin A, a drug which blocks ARF binding to Golgi membranes. As a result of these findings, it has been proposed that alterations in lipid content by such ARF-regulated PLDs may play an important role in vesicular dynamics.

(iii) Rho-activated PLD(s): reconstitution of a GTPγS-activated PLD in human neutrophil lysates was found to require protein factors from the plasma membrane and the cytoplasm (156). The GTP binding protein which activates this PLD activity was located in the plasma membrane, and was found to have a low Mg^{2+} requirement and to be insenstive to aluminium fluoride treatment. This suggested the PLD activity was likely to be regulated by a low Mr G protein, and this was supported by the finding that a GDP dissociation stimulator, which stimulates the exchange of GDP for GTP on such proteins (enhancing their activation), stimulated GTP-dependent PLD activation. Furthermore, Rho GDP dissociation inhibitor, which binds to and inhibits Rho family G proteins, was found to inhibit GTP-mediated PLD activation, indicating that this neutrophil PLD activity may be regulated by a membrane-associated Rho family G protein.

(iv) PLD(s) dependent on the levels and activation status of PLC-γ: PDGF-mediated activation of $PtdInsP_2$-PLC and PLD was studied in TRMP canine kidney epithelial cells, which were overexpressing wild type, or one of several mutant, PDGF receptors (154). These receptors had specific mutations abolishing their ability to bind various intracellular signalling proteins. This investigation established that PDGF-mediated activation of PLD is dependent on the ability of the PDGF receptor to bind $PtdInsP_2$-PLC-1 but not other signalling proteins including Ras GAP, PI3-kinase, and Syp, an SH2-containing phosphotyrosine phosphatase. Furthermore, overexpression of PLC-γ1 in NIH 3T3 fibroblasts has been shown to lead to a 10-fold increase in the activation of PLD upon stimulation of the cells with PDGF (155). Down-regulation of PKC by prolonged treatment with phorbol ester was found to completely inhibit the PDGF-mediated activation of PLD in these cells, suggesting that PLD activation may occur downstream of the RTK-mediated phosphorylation and activation of PLC-γ1, and subsequent $PtdInsP_2$ hydrolysis and PKC activation.

Overall, these reports establish the existence of a number of distinct PLD subtypes in mammalian cells which appear to differ in their coupling to cell surface receptors, subcellular localisation and phospholipid substrate specificities. Differential activation of these PLD subtypes may therefore generate specific PtdOH species in distinct subcellular compartments, eliciting unique downstream effects.

3.3 Phospholipase D in lymphocytes

3.3.1 Phospholipase D in T lymphocytes

There is very little data on the role of PLD in B and T cells. However, investigation of PLD activation in T cells has primarily involved the use of Jurkat human T cells, a proliferating leukemic T cell line. Phospholipase D activity can be detected using a number of techniques: 1) the release of phospholipid head groups such as choline and ethanolamine (Figure 7); 2) the release of PtdOH (this is readily interconverted with

DAG by the enzymes PPH and DAG kinase (Figure 9), complicating the interpretation of changes in its levels) and 3) the transphosphatidylation reaction by which PLD catalyses exchange of the phospholipid head group for a primary alcohol, generating a phosphatidylalcohol (relatively stable and not formed by any other process in whole cells) (Figure 9). This final assay has been used by a couple of groups, who have identified one or more PLD activities in T cells.

Stewart and coworkers (1991) (196) were the first to demonstrate TCR-coupled PLD activity in T cells: stimulation of [^{32}P]orthophosphate-labelled Jurkat cells with anti-CD3 antibodies or PMA resulted in the generation of [^{32}P]PtdOH or [^{32}P]phosphatidylethanol (PtdEth) in the presence of ethanol. They also showed that addition of exogenous PtdOH to Jurkat cells induced the mobilisation of [Ca^{2+}], in the absence of phosphoinositide hydrolysis. Interestingly, an unrelated study has reported that T cell receptor-mediated Ca^{2+} mobilisation involves both inositol-1,4,5-trisphosphate-dependent and inositol-1,4,5-trisphosphate-independent effector mechanisms, cosistent with a role for TCR-PLD in calcium mobilisation during T cell activation. However, preparations of PtdOH have been shown to be contaminated with trace amounts of LPA (148, 197), which appear, at least in some cases, to be sufficient to induce the responses previously ascribed to exogenously added PtdOH, and may cast doubt on the validity of a proposed role for PtdOH in the mobilisation of calcium.

Further work by Stewart et al. suggested a role for PKC in antigen receptor-PLD coupling, as down-regulation of PKC by prolonged phorbol ester treatment abrogated anti-CD3-induced PLD activation (188). However, it is difficult to draw concrete conclusions from this finding as there is evidence that PLD activity itself may be compromised by prolonged exposure to phorbol ester. These researchers also reported the activation of PLD upon incubation of Jurkat cells with sphingosine (169, 188) .

Investigation of such PLD activation in [^{32}P]orthophosphate-labelled Jurkat T cells did not give any indication as to the phospholipid substrate specificity of the TCR-coupled PLD. However, studies in [^{3}H]palmitate-labelled Jurkat T cells have indicated that the antigen receptor is coupled to a PtdCho-hydrolysing PLD activity (146). (Studies

on [^{3}H]palmitate-labelling of cells have found that it is primarily incorporated into PtdCho). This finding is consistent with reports on other cell types that calcium-mobilising receptors are usually coupled to the activation of PtdCho-PLD.

PLD has also been implicated in the coupling of the T cell antigen receptor to activation of the transcription factor AP-1 (146), one of the key early events in T lymphocyte activation. AP-1 activation was found to be induced upon stimulation of Jurkat cells with anti-CD3, or upon addition of exogenous PtdOH. Moreover, anti-CD3-mediated activation of AP-1 was inhibited by ethanol, which inhibits the PLD-dependent formation of PtdOH and DAG by promoting transphosphatidylation, and by wortmannin, which has been reported to inhibit receptor-PLD coupling. Interestingly, the activation of AP-1 by anti-CD3 was not inhibited by neomycin, an inhibitor of phosphoinositide hydrolysis, indicating that both PLD activation and AP-1 activation may be independent of the activation of PLC-γ. However, both PtdOH- and anti-CD3-mediated activation of AP-1 were blocked by PKC inhibition or PKC down-regulation. These results may therefore indicate that antigen receptor-mediated activation of AP-1 is dependent on the activation of PKC downstream of PLD activity.

Characterisation of the TCR-coupled PtdCho-PLD activity in Jurkat cells, and primary murine and human T cells has also been carried out in this laboratory (M.M. Harnett and P. Reid, unpublished results). In agreement with the previous reports, crosslinking of the antigen receptors on [^{3}H]palmitate-labelled Jurkat cells, primary murine splenic T cells, or human tonsillar T cells, resulted in the activation of PtdCho-PLD. Moreover, investigation of the dose-dependence of anti-CD3-mediated PLD activation indicated that antigen receptor coupling to PLD is not dependent on, and indeed bears an inverse relationship to, the induction of phosphoinositide hydrolysis: PLD activation was found to be induced by concentrations of anti-CD3 significantly below those required for the induction of inositol phosphates formation, and was found to be desensitised at anti-CD3 concentrations which induce maximal production of IPs. Studies involving PTK/PTPase inhibitors, Jurkat cell lines overexpressing lck and the reconstitution of CD3-coupled PtdCho-PLD activity in streptolysin-O permeabilised

Jurkat cells suggested that, although T cells contained G protein regulated PLD activity, coupling of the T cell receptor to PtdCho-PLD(s) is likely to be predominantly mediated by protein tyrosine kinase- and Ca^{2+}-dependent mechanisms.

These characterisations of PLD activity in T cells therefore indicate that PtdCho-PLD may play a role in the TCR-mediated activation of T cells. However, investigation of PtdCho-PLD signalling in thymocytes found that crosslinking of the antigen receptors or incubation of thymocytes with the calcium ionophore ionomycin, stimuli which can induce apoptosis in these cells, led to the activation of PtdCho-PLD in these cells (M.M. Harnett and P. Reid, unpublished results). This indicates that PtdCho-PLD is unlikely to play a pivotal role in T cell activation, but may suggest that PtdCho-PLD is involved in cell cycle progression leading either to activation or activation-induced cell death.

3.3.2 Phospholipase D in B lymphocytes

Valentine et al. (1992) reported the involvement of phosphatidic acid and diacylglycerols in the sIg-mediated activation of protein kinase C in human B lymphocytes (198). Crosslinking of sIg on resting tonsillar B cells was found to induce production of PtdOH and DAG, accompanied by the translocation and activation of PKC. Interestingly, these authors reported that several proliferating B cell lines had reduced PKC activation following crosslinking of sIgM, compared to the level of activation observed in primary quiescent cells. This reduction in PKC activation correlated with the absence or reduced production of PtdOH or DAG following stimulation of the antigen receptor: PKC activation was only observed under conditions in which sIg crosslinking induced both PtdOH and DAG generation. These results may indicate desensitisation of DAG/ PtdOH production and PKC activation in proliferating cell.

In a related study, Valentine and coworkers (1992) reported that ligation of surface IgM activates a phospholipase D activity to form PtdOH (198). These findings may therefore indicate that the induction of PLD activity upon sIg crosslinking is involved in the activation of PKC for the initiation, rather than the maintenance, of B cell proliferation. Consistent with this, PLD stimulation was observed in [^{3}H]butanol-

labelled EBV-transformed human B cells upon stimulation with PMA, anti-Ig, or the calcium ionophore A23187 (199). This evidence supported the coupling the antigen receptors on human B cells to PLD activation.

These studies have demonstrated the existence of phospholipase D in lymphocytes and the coupling the antigen receptors to PLD activities in both B and T cells. It was therefore decided to investigate the role of PLD activity in B lymphocyte signalling and its coupling to B cell antigen receptors.

3.4 Aims and objectives.

This investigation focused on the identification, characterisation and coupling of phospholipase D activities in murine B cells. In particular, the aims of this investigation were:

i) to identify and characterise phospholipase D activity(ies) in murine B cells

ii) to investigate the coupling of PLD to the antigen receptors and other receptors on murine B cells

iii) to determine the mechanism(s) of receptor-PLD coupling and the regulation of PLD activity in murine B cells

iv) to assess whether PLD(s) play a maturation stage-dependent role(s) in B cell biology.

3.5 Identification of PtdCho-PLD activity in murine B cells.

The initial aims of this study were to 1) identify PLD activity in murine B cells and 2) investigate the coupling of this activity to the antigen receptors on B cells. PtdCho-PLD activity is the most studied, and probably the easiest studied PLD activity, so the early experiments focused on the role of PtdCho-PLD in B cell activation.

3.5.1 Measurement of phospholipase D activity.

The most direct method for the detection of PLD activity is to measure the products of PLD-catalysed phospholipid hydrolysis: polar head groups and PtdOH (Figure 7). The generation of polar head groups such as choline and ethanolamine from their respective phospholipids can be taken as a fairly direct measure of PLD activity. However, PtdOH is readily interconverted with diacylglycerol (DAG) by the action of phosphatidate phosphohydrolase (PPH) and DAG kinase, thus complicating the interpretation of changes in the levels of these two second messengers. Fortunately, PLD activity can be definitively monitored using its ability to catalyse a transphosphatidylation reaction in the presence of a primary alcohol (Figure 9). This exchanges the headgroup of the phospholipid for the alcohol, generating phosphatidylalcohols which are relatively stable to degradation, and are not produced by any other mechanism in whole cells.

3.5.2 Analysis of B cell phospholipid labelling by [^{3}H]fatty acids.

Many phospholipid signalling assays, including the phospholipase D transphosphatidylation assay, involve the labelling of cellular phospholipids by incubation of cells with radiolabelled fatty acids. With a view to the design of PtdCho-specific assays, the relative labelling of different phospholipid species by specific fatty acids was investigated in murine B cells. Resting murine splenic B cells were incubated with [^{3}H]palmitate, [^{3}H]myristate, [^{3}H]oleate or [^{3}H]arachidonate (1 μCi/ml) for 4 or 18 hours, and [^{3}H]-incorporation into PtdCho, PtdIns, PtdEtn and PtdOH was

determined (Figure 11). This study demonstrated that all four fatty acids used were preferentially incorporated into PtdCho which is the predominant fatty acid in B cell membranes. There was, however, low but significant incorporation of [^{3}H]fatty acids into PtdEtn. In addition, both [^{3}H]palmitate and [^{3}H]arachidonate achieved a much higher degree of incorporation into cellular phospholipids than [^{3}H]myristate or [^{3}H]oleate, indicating that these fatty acids would be most appropriate for studying the PLD-catalysed hydrolysis of PtdCho. [^{3}H]palmitate was used in the majority of such experiments because it is considerably cheaper than [^{3}H]arachidonate and because it had already been used by a number of researchers to study PtdCho-specific PLD in other cells.

3.5.3 PMA or pervanadate stimulate PtdCho-PLD activity in murine B cells.

In most cell types studied, activation of PtdCho-PLD has been observed following stimulation of cells with PMA, a pharmacological activator of PKC, and this was investigated in B lymphocytes. Stimulation of [^{3}H]palmitate-labelled resting murine B cells with PMA (0-500 ng/ml) in the presence of 0.3% butan-1-ol strongly stimulated [^{3}H]PtdBut formation (Figure 12). Stimulation of [^{3}H]palmitate-labelled B cells with PMA thus constitutes a reliable positive control for the induction of PtdCho-PLD activity and was used in all of the experiments described below. In addition, the tyrosine phosphatase inhibitor, pervanadate (pV) (0.5 mM) stimulated PtdCho-PLD activity in B cells, presumably via a PTK-dependent mechanism (Figure 12). Interestingly, incubation of B cells with saturating amounts of PMA (100 ng/ml) and pV (0.5 mM) led to the additive stimulation of [^{3}H]PtdBut generation (Figure 12), indicating the activation of two distinct PtdCho-PLD activities under independent control.

3.5.4 sIg on murine B cells is not coupled to the activation of PtdCho-PLD.

The preliminary findings detailed above demonstrate the existence of PtdCho-PLD activities in murine B cells and support their regulation by PKC- and PTK-dependent mechanisms. Stimulation of B cells via the antigen receptor has been shown to induce the activation of several PKC isoforms and the tyrosine phosphorylation of target proteins (1, 24). These results, in conjunction with the findings in T lymphocytes, therefore suggested that PtdCho-PLD activation in B cells might be coupled to signalling through the antigen receptor. However, crosslinking of sIg on [^{3}H]palmitate- or [^{3}H]arachidonate-labelled resting murine splenic B cells, using a mitogenic concentration (50 μg/ml) of anti-Ig antibodies, did not stimulate the formation of [^{3}H]PtdBut (Figure 13), under conditions in which PMA did activate PLD. [^{3}H]palmitate-labelled murine B cells were stimulated with anti-Ig (50 μg/ml) for periods of 15 sec - 60 min, but no activation of PtdCho-PLD was observed (data not shown). It was possible that antigen receptor coupling to PtdCho-PLD was desensitised by the high concentrations (50 μg/ml) of crosslinking antibodies used to mimic optimal stimulation (as in T lymphocytes), or that PtdCho-PLD could be differentially coupled to the two sIg isoforms present on the mature B lymphocyte: sIgM and sIgD. However, stimulation of [^{3}H]palmitate-labelled resting B cells with a range of anti-Ig concentrations (0.05-50g/ ml), or with anti- or anti-mAbs (50 μg/ml), did not stimulate PtdCho-PLD activity (Figure 13). Moreover, lipopolysaccharide (LPS) (50 μg/ml), which induces polyclonal B cell activation and proliferation, also failed to stimulate PtdCho-PLD activity (Figure 13). Similar results were obtained following stimulation of *in vivo* activated murine splenic B cells (Figure 14), and the human lymphoblastoid cells lines EDR (Figure 14), Daudi and Ramos (Figure 15). These investigations were made under conditions in which anti-Ig induced release of [^{3}H]InsPs from [^{3}H]inositol-labelled Daudi or Ramos cells (Figure 15), indicating that they were still responsive to stimulation via sIg. Taken together, these findings suggest it is unlikely that (i) the antigen receptors on B cells are coupled to

PtdCho-PLD in a cell cycle-dependent manner or (ii) PtdCho-PLD plays a role in the maintenance of antigen-driven B cell proliferation.

3.5.5 Analysis of PtdCho breakdown in lymphocytes.

Since it was rather surprising that sIg, a calcium-mobilising receptor, was not found to be coupled to PtdCho-PLD under the above conditions, it was important to rule out the possibility that the absence of detectable [^{3}H]PtdBut formation by B cells in response to stimulation with anti-Ig might be because [^{3}H]palmitate and [^{3}H]arachidonate are not incorporated into the appropriate pool of PtdCho. It was therefore decided to use other techniques to examine potential PtdCho hydrolysis. These techniques should also detect hydrolysis of PtdCho by phospholipase C as well as PLD (139, 140).

As discussed previously, the hydrolysis of PtdCho by PLD or PLC results in the release of the polar head groups choline (Cho) or choline phosphate (ChoP), respectively (Figure 7). Therefore, levels of Cho and ChoP were analysed by ion-exchange chromatography in [^{3}H]methylcholine-labelled murine splenic or human tonsillar B cells, stimulated with anti-Ig or PMA for 10 min. No significant changes in either [^{3}H]Cho or [^{3}H]ChoP were observed in response to anti-Ig or PMA (data not shown). This was puzzling because PMA stimulates PtdCho-PLD activity in B cells, and should therefore induce the release of choline. Analysis of basal levels of Cho and ChoP revealed large cellular pools of both metabolites that were likely to prevent detection of the relatively small PLD- or PLC-mediated changes in Cho or ChoP concentrations. In an attempt to circumvent this problem, the intracellular and extracellular levels of [^{3}H]Cho and [^{3}H]ChoP were analysed, using pulse-chased labelled B cells. However, no significant, reproducible changes were observed in the intracellular levels of [^{3}H]Cho or [^{3}H]ChoP (Figure 16) (although PMA did induce the formation of choline very weakly and inconsistently), and a rapid rise in the extracellular levels of both metabolites (Figure 16) in all samples (including control) perhaps indicated rapid non-specific metabolite release following addition of cells to the reaction vial. As no release of choline was detected upon

stimulation of B cells with PMA these results suggest insufficient labelling by [^{3}H]methylcholine of the hormone-sensitive pool of PtdCho.

To test this hypothesis Cho and ChoP levels were also determined in Jurkat cells - a proliferating human T leukemic cell line - as these cells should be labelled to equilibrium by incubation with [^{3}H]methylcholine overnight. Stimulation of Jurkat cells with PMA has been shown to induce PtdCho-PLD activation, and should therefore induce the release of choline. However, as in murine and human primary B cells, no significant rise in either [^{3}H]Cho or [^{3}H]ChoP was detected upon PMA stimulation, and high basal levels of both metabolites were observed (Figure 17). It was therefore concluded that the measurement of [^{3}H]Cho and [^{3}H]ChoP levels by ion-exchange chromatography is not appropriate for the study of PtdCho hydrolysis in lymphocytes. This is probably because the hormone-sensitive pools of PtdCho label poorly with [^{3}H]methylcholine, leaving high intracellular pools of choline and choline phosphate. Similarly, PtdCho levels were then examined in [^{32}P]orthophosphate-labelled EDR human B cells, in an attempt to detect any agonist-mediated [^{32}P]PtdCho hydrolysis. Unfortunately, there was no detectable decrease in [^{32}P]PtdCho upon stimulation of EDR cells with anti-Ig or PMA (Figure 18).

PtdCho constitutes approximately 40% of the B cell membrane phospholipids and a large proportion of tis PtdCho is likely to be hormone-insensitive; it thus appears likely that any PLD-catalysed hydrolysis of PtdCho in lymphocytes must be relatively small and cause undetectable changes in PtdCho levels - labelled via fatty acids, polar head groups or [^{32}P]orthophosphate.

Thus, although the analyses of PtdCho hydrolysis were inconclusive, no evidence was found to support coupling of the antigen receptor to PtdCho-PLD activity in murine B cells. In addition to these findings, no evidence for the existence of PtdCho-PLC was detected in either [^{3}H]methylcholine- or [^{32}P]orthophosphate-labelled B cells. However, although the lack of reproducibility in the ability to detect [^{3}H]choline release upon cellular stimulation with PMA could perhaps indicate that the PLD activity measured

in [^{3}H]palmitate-labelled cells is not a PtdCho-hydrolysing activity, this possibility is highly unlikely as the [^{3}H]fatty acid labelling studies in B cells indicate that [^{3}H]palmitate is very preferentially incorporated into PtdCho. Furthermore, no hydrolysis of any other phospholipid has been detected upon stimulation of [^{3}H]fatty acid- or [^{32}P]orthophosphate-labelled murine B cells with PMA (data not shown), and the considerable evidence for the PMA-mediated induction of PtdCho-PLD in other cells, suggests that PMA very probably activates PtdCho-PLD in B cells.

3.6 Investigation of non-PtdCho-hydrolysing phospholipase D in B cells.

3.6.1 The B cell antigen receptor is coupled to a non-PtdCho-specific PLD.

The findings detailed above indicate that the B cell antigen receptor is not coupled to PtdCho-PLD activity. However, in addition to PtdCho-specific PLD activity, both PtdEtn- and PtdIns-hydolysing PLD activities have been identified in other cell types (152, 184, 189, 191, 192, 200-203). In order to determine whether the antigen receptors on B cells were coupled to PLD activity, B cells were mitogenically stimulated in the presence of [^{3}H]butan-1-ol (20 μCi/ml) to "trap" PtdBut products of all PLD activities, irrespective of phospholipid substrate specificity. Interestingly, under these conditions, stimulation of B cells with anti-Ig (50 μg/ml) was found to induce a rapid accumulation of [^{3}H]PtdBut (Figure 19) which appeared to desensitise over a period of approximately 10 min. In addition, PMA stimulation of B cells also resulted in the accumulation of [^{3}H]PtdBut (Figure 19). Taken together with the data on [^{3}H]palmitate-labelled B cells, these findings suggest that the antigen receptors on B cells are coupled to one or more non-PtdCho-hydrolysing PLD activities. Therefore, in an attempt to identify the substrate specificity of sIg-coupled PLD, the hydrolysis of other phospholipids in B cells was investigated.

3.6.2 Crosslinking of sIg does not induce hydrolysis of PtdEtn or release of ethanolamine.

Phosphatidylethanolamine (PtdEtn) is a substrate for PLD in several other cell types (184, 189, 191, 192, 200-203), and its possible role in B cells as a substrate for sIg-coupled PLD was investigated. [^{3}H]ethanolamine-labelled resting B cells were stimulated with anti-Ig (50 µg/ml) or PMA (100 ng/ml) for 5 min. However, no significant hydrolysis of [^{3}H]PtdEtn was observed in response to either stimulus (Figure 20). This was consistent with the findings in [^{32}P]orthophosphate-labelled EDR human B cells, which showed no reduction in [^{32}P]PtdEtn levels in response to stimulation with anti-Ig or PMA (data not shown). In addition, levels of [^{3}H]Etn and [^{3}H]ethanolamine phosphate ([^{3}H]EtnP) were determined by ion-exchange chromatography (Figure 20). No release of either metabolite was detected upon stimulation of B cells with anti-Ig or PMA for up to 5 min. These results indicate that it is unlikely that PtdEtn is hydrolysed by PLD (or PLC) upon stimulation of B cells with anti-Ig or PMA.

3.6.3 Crosslinking of sIg induces PtdIns hydrolysis in murine B cells.

The PLD-catalysed hydrolysis of phosphatidylinositol has also been demonstrated in other cell types (152), and in order to investigate PtdIns as a potential substrate for sIg-coupled PLD, PtdIns levels were analysed in stimulated murine B cells. Preliminary experiments using the radiolabelled fatty acids [^{3}H]palmitate, [^{3}H]arachidonate, and [^{14}C]stearate to label PtdIns were unsuccessful (results not shown), probably due to a low level of fatty acid incorporation and combined with the low levels of inositol containing phospholipids in membranes (approximately 0.5% of the membrane phospholipid). Similar inconclusive results were obtained in cells in which PtdIns was labelled with [^{3}H]glycerol- or [^{32}P]orthophosphate (results not shown). However, studies in [^{3}H]inositol-labelled cells demonstrated that anti-Ig antibodies (50 µg/ml) induce a substantial hydrolysis of PtdIns (Figure 21) in B cells. This finding indicates that PtdIns is a potential candidate substrate for sIg-coupled PLD activity.

The identification of distinct phospholipase D activities within B cells is consistent with findings in other cell types. For example, Huang et al. demonstrated the existence of two, differentially regulated PLD activities in Madin-Darby canine kidney (MDCK) cells (152) a PtdIns-specific PLD activated rapidly and transiently upon stimulation of the cells with bradykinin and a PtdCho-specific PLD activated in a more prolonged manner upon stimulation of the cells with phorbol ester. Our findings concerning PLD activity therefore suggest a situation in B cells which may be analogous to that found in MDCK cells.

3.7 Investigation of PtdCho-PLD coupling in the murine B cell.

The finding that B cell antigen receptors are not coupled to PtdCho-PLD was surprising considering the results obtained in murine T cells, and because most calcium mobilising receptors will activate PtdCho-PLD (128, 129, 145). However, these findings highlight a significant difference between signalling through the T and B cell receptors, as well as the possibility of investigating a novel role for PtdCho-PLD in B cells. In other cell types PtdCho-PLD has typically been implicated in positive and/ or mitogenic signalling events such as Bombesin stimulation of Swiss 3T3 cells and the induction of the respiratory burst in neutrophils (128, 129, 145). Thus, investigation of PtdCho-PLD coupling in B cells focused initially on positive immunostimuli which are co-stimulatory with the ligation of antigen receptors in the induction of B cell activation.

3.7.1 A role for PtdCho-PLD in T cell-dependent B cell activation?

Stimulation of B cells with anti-Ig antibodies alone is probably analogous to polyclonal activation of B cells by type-2 T cell-independent antigens (TI-2 Ag), which are generally large molecules of repeating epitopes capable of effectively crosslinking sIg. However, the vast majority of soluble antigens are T cell-dependent and hence, both cytokine-directed (eg. IL-4) and B-T cell contact-mediated (eg. MHC class II, CD40) signals are required, in addition to those via the antigen receptors, for full B cell activation (1). It was therefore decided to determine whether sIg, or any of these

additionally recruited receptor-mediated signals, were coupled to PtdCho-PLD activity during T cell-dependent B cell activation.

The T cell-derived cytokine IL-4 augments the activation of B cells via the antigen receptor (1, 204). For instance, sub-mitogenic concentrations of anti-Ig will induce DNA synthesis if added to murine B cells in the presence of IL-4 (Figure 22). It was decided to investigate whether stimulation of cells with both anti-Ig and IL-4 would induce PtdCho-PLD activation. However, stimulation of murine B cells with IL-4 (1-1000U/ ml), either alone or in combination with anti-Ig (5 μg/ ml), failed to induce PtdCho-PLD activation over a time period of 60 min (Figure 23), whereas PMA (100 ng/ ml) did activate PLD.

The generation of antibody secreting cells from resting B cells not only requires signalling through the antigen receptor, lymphokines and direct Th cell contact, but also requires these signals in the correct sequence for full activation. For example, Cambier and coworkers (72), investigating the role of B-T cell cooperation in B cell activation, showed that ligation of MHC Class II molecules on quiescent B cells by anti-Ia antibodies induces the production of cyclic AMP, and the sequestration of PKC in a detergent-insoluble compartment within the cell: these events appear to be antagonistic to B cell proliferation. However, cells which had been primed with anti-Ig and IL-4 (for 4 h), responded to MHC Class II ligation with the generation of inositol phosphates, calcium mobilisation and proliferation. The "priming" of B cells with specific stimuli can therefore lead to "rewiring" of the coupling of cell surface receptors to second messenger systems, and it was decided to address whether it was possible to induce coupling of PtdCho-PLD to a number of different stimuli through the priming of B cells. Firstly, B cells were incubated overnight with a number of stimuli which had already been investigated: anti-Ig (50 μg/ml), IL-4 (100 U/ml), anti-Ig (5 μg/ml) plus IL-4 (100 U/ml), or LPS (50 μg/ml). Further stimulation of the primed cells with anti-Ig (50 μg/ml), IL-4 (100 U/ml), anti-Ig (5 μg/ml) plus IL-4 (100 U/ml) or LPS (50 μg/ml), however, did not induce PtdCho-PLD activation (Figure 24), although activation of PtdCho-PLD was observed in all cases upon stimulation with PMA (100 ng/ml). Secondly, the coupling of Class II molecules to PtdCho-PLD activity was investigated in

resting, *in vivo* activated, and primed cells: i) resting B cells were stimulated with anti-Ia^d; ii) *in vivo* activated B cells were stimulated with anti-Ia^d either alone or in combination with anti-Ig (50 μg/ml), IL-4 (100 U/ml) or anti-Ig (5 μg/ml) plus IL-4 (100 U/ml); and iii) anti-Ig/ IL-4-primed B cells were stimulated with anti-Ia^d either alone or in combination with anti-Ig (50 μg/ml) or IL-4 (100 U/ml). In these experiments, the Class II molecules on [^{3}H]palmitate-labelled B cells were stimulated, either by crosslinking of biotinylated anti-Ia^d antibodies (anti-Ia^d mAb's 39-10-8 and 34, 5-3, final conc. 100 μg/ml) by avidin (25 μg/ml) or by incubation on plates precoated with anti-Ia^d antibodies. No activation of PtdCho-PLD could be detected under any of these conditions (Figure 25), however, PLD activation was observed in all cases upon stimulation with PMA.

B-T cell cooperation is mediated through a number of B cell surface receptors and their T cell counterstructures, in addition to the interaction via MHC Class II molecules and the T cell receptor (1, 6, 24, 205, 206). Ligation of MHC Class II molecules alone may therefore be insufficient to generate some of the signals which are induced in B cells by cognate B-T cell interaction, ie. the ligation of other receptors, or simultaneous recruitment of several receptors, may be required. One preliminary study examined CD40-coupled signalling events, including the possibility of CD40-mediated PtdCho-PLD activation. CD40 is a B cell surface receptor which is ligated by the CD40 ligand on T cells. It has been shown to play an essential role in the development of antibody-secreting plasma cells, and its crosslinking is found to promote B cell proliferation, prevent apoptosis of germinal-centre B cells and promote immunoglobulin class switching. However, stimulation of resting murine B cells (Figure 26) or the human B cell line, EDR (results not shown), with appropriate anti-CD40 antibodies did not induce PtdCho-PLD activation in these cells. It was then decided that if PtdCho-PLD in B cells were activated by T cell-dependent signals, this might require the simultaneous ligation of a number of B cell surface molecules, such as might be mediated by activated T cell membranes. Snow and coworkers had showed that incubation with fixed activated T cell blasts will induce cAMP production and changes in gene expression in B cells (205). It

was therefore decided to study the effect of contact with intact fixed PHA-activated T cells on the activation of PtdCho-PLD in B cells. Resting B cells, or B cells primed with anti-Ig (50 μg/ml), IL-4 (100 U/ml), or anti-Ig (5 μg/ml) plus IL-4 (100 U/ml), were stimulated with PHA-primed T cell blasts in the presence or absence of IL-4 (100u/ ml). No activation of PtdCho by B-T cell contact was observed under conditions in which PMA (100 ng/ml) stimulated [^{3}H]PtdBut production (Figure 27).

Finally, a preliminary study investigated the effects of various growth factors or hormones, or cytokines which are known to be involved in T cell-dependent B cell differentiation and/or proliferation and have been shown to activate PLD in other cell types (128, 149, 155, 165, 186, 200, 207-215). However, PDGF (50 ng/ml), LPA (1 μM), IL-3 (25 or 250 U/ml), IL-13 (10 or 100 ng/ml), IL-10 (10 or 100 U/ml), IL-2 (20 or 2000 U/ml), TNF-α (0.1 or 10 μg/ml) and IL-1 (0.1 or 1 μg/ml) failed to induce activation of PtdCho-PLD in [^{3}H]palmitate-labelled resting murine B cells, under conditions in which PMA (100 ng/ml) did (results not shown).

The above findings indicate that PtdCho-PLD signalling does not play a role in the T cell-dependent activation of B cells. Taken together with the data on antigen receptor coupling, this suggests that PtdCho-PLD does not play a role in either the initiation or maintenance of antigen-driven mature B cell proliferation.

3.7.2 PtdCho-PLD does not play a role in transducing apoptotic or anergic signals via the antigen receptor in immature B cells.

In contrast to mature cells, immature B cells respond to antigen by becoming anergic or undergoing apoptosis, presumably resulting in the unresponsiveness or deletion of autoreactive clones (87, 88, 115). Thus, incubation of WEHI 231 cells (a proliferating immature B cell line) with anti-Ig will induce growth arrest (Figure 28) and apoptosis. The biochemical mechanisms underlying the contrasting responses of mature and immature lymphocytes to antigen receptor crosslinking have yet to be elucidated, and it was decided to investigate the possibility that PtdCho-PLD is involved in the transduction of signals from sIg on immature B cells under conditions which induce

apoptosis. However, ligation of the antigen receptors on [^{3}H]palmitate-labelled immature splenic (IS) B cells (4 week old mice) or WEHI 231 cells did not induce stimulation of PtdCho-PLD activity (Figure 29). In addition, no stimulation of PtdCho activity could be detected following stimulation of immature splenic B cells or WEHI 231 cells with LPS (Figure 29). PMA (100 ng/ml) induced PtdCho-PLD activation in both IS and WEHI 231 B cells (Figure 29), indicating the expression and activation of PtdCho-PLD(s) in these cells.

The findings in both immature and mature B cells therefore indicate that PtdCho-PLD does not appear to play a role in the transduction of signals from the B cell antigen receptor or upon polyclonal B cell stimulation by LPS. Furthermore, PtdCho-PLD activity does not appear to be involved in the T cell-mediated activation of B cells. In view of these findings, it was decided to examine the coupling of PtdCho-PLD to other stimuli, including those which negatively modulate B cell activation and proliferation.

3.7.3 A role for PtdCho-PLD in the transduction of anti-proliferative signals in mature B cells.

Nucleotides act as intercellular messengers and exert a widespread influence on cellular function through a variety of cell surface receptors (216). ATP, which exerts its effects through P_2-purinoceptors, has been reported to exhibit immunomodulatory effects on human B cell activation and proliferation (217-219). In view of these reports, the effect of ATP on murine B cell activation was examined, and it was found that ATP profoundly inhibits anti-Ig- and LPS-mediated DNA synthesis in resting murine B cells (Figure 30). P_2-purinoceptors regulate cellular function via G proteins and are coupled to PtdInsP_2-PLC and calcium mobilisation. P_2-purinoceptors have also been shown to be coupled to PtdCho-PLD activation in a number of cell types (216, 220). It was therefore decided to investigate the coupling of P_2-purinoceptors to PtdCho-PLD in murine B cells. Stimulation of [^{3}H]palmitate-labelled murine resting splenic B cells with ATP (1.6 nM-5 mM) induced generation of [^{3}H]PtdBut (Figure 31), with maximal stimulation at 200 μM ATP. Moreover, PMA appeared to stimulate PtdCho-PLD in an additive manner

with both suboptimal and saturating concentrations of ATP (Figure 31). This indicates that PMA and ATP may induce the activation of distinct PtdCho-PLD activities.

The coupling of P_2-purinoceptors to PtdCho-PLD via G proteins was also investigated in B cells. B cells were pretreated with pertussis toxin which catalyses the ADP-ribosylation of Gi-like G proteins, inactivating them. Interestingly, pretreatment of resting murine B cells (Figure 32), or Ramos and Daudi B cells (data not shown), with pertussis toxin led to an increased basal production of [^{3}H]PtdBut, suggesting that PtdCho-PLD activity may normally be under the negative control of a Gi-coupled receptor on B cells (Figure 32). In addition to the elevated level of [^{3}H]PtdBut generation resulting from pertussis toxin treatment, ATP further stimulated [^{3}H]PtdBut in an additive manner (Figure 32), indicating the activation of two distinct PtdCho-PLD activities under independent control. This does not preclude G protein coupling of P_2-purinoceptors to PtdCho-PLD activation, as P_2-purinoceptors have been shown to be coupled to G_i, G_0 or G_q-type G proteins (216), but appears to rule out the involvement of pertussis toxin-sensitive G proteins, although they clearly play a role in the regulation of another, distinct PtdCho-PLD activity in B cells.

The involvement of protein tyrosine phosphorylation in P_2-purinoceptor coupling to PtdCho-PLD was also investigated. Pretreatment of murine B cells with the selective tyrosine kinase inhibitors, genistein and tyrphostin led to an increase in basal [^{3}H]PtdBut production, and to a greatly enhanced ATP-mediated signal (Figure 32). The findings are consistent with the existence of several, differentially regulated PtdCho-PLD activities within murine B cells.

These data, together with the proliferation assay which showed that ATP inibits DNA synthesis in B cells (Figure 30), suggest that, in murine B cells, PtdCho-PLD activities may be involved in the transduction of negative signals which are antagonistic to stimulation via the antigen receptor. To further examine this hypothesis, the effects of the lipid second messengers (LSMs) sphingosine, ceramide and arachidonate on B cell activation and cellular signalling, were examined. Sphingosine and ceramide are metabolites of the Sphingomyelinase cycle which have been found to play a role in

growth arrest and the induction of apoptosis in a variety of cell types (221, 222). Both have also been implicated in the regulation of phospholipase D activity (149, 180, 184-192). Arachidonate, which is generated upon the hydrolysis of phospholipids, predominantly PtdCho, by phospholipase A_2, has also been implicated in the transduction of apoptotic signals via ceramide production in several cell types (61). Moreover, it has also been found to modulate the activity of a number of intracellular signalling elements, particularly protein kinase C (133). Ceramide, sphingosine and arachidonate, were indeed found to inhibit basal and anti-Ig-induced DNA synthesis in murine B cells (Figure 30), and thus, it was decided to investigate their effect on PtdCho-PLD activation. Incubation of [^{3}H]palmitate-labelled resting murine B cells with sphingosine or C2-ceramide (40 nM-25 μM) was found to induce significant production of [^{3}H]PtdBut (max stimulation was observed at 40 nM ceramide, 200 nM sphingosine), whereas arachidonate (40 nM-25 μM) induced only weak, but significant activation of PtdCho-PLD (Figure 33), perhaps reflecting its rapid turnover by cells. Interestingly, stimulation of B cells with both sphingosine (20 μM) and a saturating concentration of PMA (100 ng/ml), induced no activation of PtdCho-PLD above that induced by the PMA alone (Figure 21). This probably indicates that either both sphingosine and PMA induce activation of the same PtdCho-PLD enzyme, or that, in addition to stimulating at least one PtdCho-PLD, PMA is antagonistic to the sphingosine-mediated induction of PtdCho-PLD. These findings further support the hypothesis that a PtdCho-PLD activity or activities are involved in the transduction of negative, anti-proliferative signals in murine B lymphocytes.

3.8 The role of phospholipase D in B lymphocyte signalling.

One of the central problems yet to be resolved in B lymphocyte signalling is how the antigen receptors transduce differential signals in a maturation state-dependent manner. Whereas ligation of the antigen receptors on mature B cells can lead to proliferation and differentiation into antibody producing cells, the majority of immature B lymphocytes respond by becoming anergic or undergoing apoptosis (24). Many of the earliest signalling events detected following B cell receptor ligation appear to be similar in both mature and immature lymphocytes, suggesting that the antigen receptors on mature immunocompetent lymphocytes may be coupled to additional, as yet undefined, signalling pathways in order to transduce proliferative responses. Activation of phospholipase D isoforms, with the resultant generation of distinct PtdOH lipid second messengers, has been proposed to play a central role in the transduction of a variety of cellular responses including membrane trafficking, the respiratory burst, exocytosis and proliferation (128, 129, 139, 140, 145). It was therefore decided to investigate the role of phospholipase D activities in mitogenic signalling of B lymphocytes via the antigen receptors.

The initial studies of PLD in B cells focused on a phosphatidylcholine-hydrolysing PLD activity, because PtdCho-PLD is the most common, the best characterised, and probably the easiest to study of the various PLD activities identified in numerous cell types (128, 129, 145). Moreover, the antigen receptors on T cells had already been reported to be coupled to PtdCho-PLD (146, 188, 196) (M.M. Harnett and P. Reid, unpublished results). Preliminary pharmacological studies demonstrated that one or more PtdCho-PLD pathways can be activated by stimulation of murine B cells with PMA or pervanadate (Figure 12), suggesting the regulation of PtdCho-PLD activity by tyrosine phosphorylation- and protein kinase C-dependent pathways. As the antigen receptors on B cells are coupled to both the tyrosine phosphorylation of target proteins

and the activation of specific PKC isoforms, these findings indicated that PtdCho-PLD might well be activated via the antigen receptors on B cells. However, extensive investigation demonstrated that the antigen receptors on mature B cells are not coupled to PtdCho-PLD following mitogenic stimulation with anti-Ig antibodies (Figure 13). These findings were supported by studies on "*in vivo*" activated murine B cells (Figure 14), and the human B cell lines, EDR (Figure 14), Ramos (Figure 15) and Daudi (Figure 15), which showed that the antigen receptors on these cells are also not coupled to PtdCho-PLD. Further work involved the stimulation of B cells under conditions designed to mimic T cell-dependent activation of B cells, and examined the possibility of "rewiring" receptor coupling to PtdCho-PLD by the priming of B cells with a number of stimuli known to induce B cell activation. However, these investigations failed to demonstrate any activation of PtdCho-PLD upon stimulation of resting B cells, or B cells, variously primed with anti-Ig, IL-4, LPS, or anti-MHC class II antibodies, either alone or in appropriate combinations (Figures 23-25). Furthermore, crosslinking of CD40 on resting B cells (Figure 26), or treatment of resting or primed B cells with fixed activated T cells also failed to induce activation of PtdCho-PLD (Figure 26). Taken together, these findings suggest that PtdCho-PLD does not play a role in either the initiation or the maintenance of antigen-driven B cell proliferation.

Studies in immature splenic B cells, and WEHI 231 immature B cells, demonstrated that sIg is not coupled to PtdCho-PLD in immature B cells (Figure 29) and thus does not appear to transduce sIg-directed apoptotic signals in these cells. Taken together with the findings in mature B cells, these findings therefore indicate that PtdCho-PLD is not involved in maturation state-dependent signalling from sIg.

Contrasting with the reports on PtdCho-PLD activity in T lymphocytes, these findings highlight a potentially important difference between the signals generated via B and T cell antigen receptors, although the functional significance of this is not yet known. Thus, delineation of the precise details of the receptor coupling and regulation of specific PLD activities in B and T cells, might give into insight into the intracellular signals which

are required to be generated for activation of B and T cells, as well as the stimuli which are required to initiate them in a physiological immune response.

The investigation of phospholipase D in [^{3}H]fatty acid-labelled murine B cells thus failed to demonstrate the coupling of B cell antigen receptors to PtdCho-PLD activity. However, studies using [^{3}H]butanol for the transphosphatidylation assay revealed that the antigen receptors on resting murine B cells are coupled to a non-PtdCho-specific PLD following mitogenic stimulation with anti-Ig antibodies (Figure 19). A similar PLD activity was recently reported to be coupled to mitogenic stimulation of the antigen receptors on EBV-transformed human B cells (199). Although the specificity of the sIg-coupled PLD activity in murine B cells has not yet been defined, labelling studies have ruled out a role for a PtdEth-specific PLD (Figure 20) and suggest that PtdIns may be the PLD substrate in murine B cells (Figure 21). A precedent for such a role for PtdIns-PLD in cellular activation has been set by the report that Bradykinin similarly stimulates a rapid and transient PtdIns-PLD response in Madin-Darby canine kidney (MDCK) cells (152). This is interesting because Bradykinin, which is a vasoactive peptide, has been shown to stimulate a number of intracellular signals in its target cells which are also generated via antigen receptors on lymphocytes, such as phosphoinositide hydrolysis and calcium mobilisation. Bradykinin has also been shown to stimulate PLA_2-mediated arachidonate release, a signal which the investigation reported in Chapter 4 has shown to be stimulated by the crosslinking of antigen receptors on immature lymphocytes. These findings may therefore suggest similar mechanisms of receptor coupling to PtdIns-PLD in B cells and MDCK cells.

The study in MDCK cells also reported the late (2 min), sustained activation of PtdCho-PLD activity stimulation of these cells with Bradykinin, or PMA. Bradykinin thus induces the specific PLD-catalysed hydrolysis of PtdCho and PtdIns in MDCK cells, suggesting the existence of two, differentially regulated bradykinin-coupled PLD activities within a single cell type. This possibility was confirmed by further work

involving the use of cell-free PLD assay systems, which demonstrated that MDCK cells express two PLD subtypes, differing in a number of characteristics:

i) A PtdCho-PLD activity which was identified in cellular fractions representing the plasma membrane and nucleus, had no requirement for Ca^{2+}, and was substantially enhanced by detergents such as Triton X-100;

ii) A PtdIns-PLD activity located in the cytosol, which was activated by Ca^{2+} (optimal concentration 1μM), and was unaffected by detergent.

These results prompt some interesting questions regarding the activation and functions of PLD activities in B and T lymphocytes. The studies of PtdCho-PLD signalling in lympocytes have already indicated that different signals are generated via the antigen receptors on B and T cells (ie. a putative PtdIns-PLD in B cells, and PtdCho-PLD in T cells). However, it is possible that the antigen receptors on T cells are coupled to a PLD activity similar to the putative PtdIns-PLD which is activated via antigen receptors on B cells. Although, a TCR-coupled PtdIns-PLD activity might have been detected in the PLD studies involving [^{32}P]orthophosphate labelling of Jurkat T cells. The labelling studies described in section 3.5.2 suggest that PtdIns-PLD would probably not be detectable in [^{3}H]palmitate-labelled T cells. Moreover, the studies by Stewart and coworkers (188, 196) did not indicate the sustrate specificity(ies) of the PLD activity(ies) which was activated by TCR crosslinking. Thus, T cell receptors could be differentially coupled to distinct PLD activities, in a manner analogous to Bradykinin stimulation of MDCK cells. This suggestion is somewhat speculative, however the identification of a non-PtdCho-PLD which is activated via the antigen receptors on B cells does raise the possibility that a PtdIns-PLD may play a role in antigenic activation of T cells via the antigen receptor. Kinetic studies of PtdIns hydrolysis involving [^{3}H]inositol-labelling of T cells are required to resolve this question.

PtdCho-PLD has typically been found to be activated by mitogenic/ cell activating stimuli such TCR crosslinking (146, 188, 196) or stimulation of Swiss 3T3 fibroblasts with bombesin (223). However, this investigation has shown that ATP, and the lipid second messengers sphingosine and ceramide (and to a lesser extent arachidonate), which inhibit murine B cell activation (Figure 30), stimulate PtdCho-PLD activation in B cells (Figures 31-33). These findings therefore suggest that PtdCho-PLD(s) may play a role in transducing anti-proliferative signals via P_2-purinoceptors, and possibly other immunomodulatory receptors, in mature, immunocompetent B cells. This appears to be the first report that PtdCho-PLD pathways may play a role in the negative regulation of cellular proliferation.

At present there is no definitive information regarding the regulation of the ATP-stimulated PtdCho-PLD, or indeed the sIg-coupled non-PtdCho-PLD in B cells. However, the data on PtdCho-PLD regulation by pharmacologocial agents have highlighted some novel and interesting features of PtdCho-PLD regulation in B cells. Firstly, in apparent contrast to earlier studies reporting that pertussis toxin (167, 224) and tyrosine kinase inhibitors (225) inhibited agonist-stimulated PLD in neutrophils, fibroblasts and RBL cells, it was found that pretreatment of murine B cells with pertussis toxin (4h) and the tyrosine kinase selective inhibitors, genistein and tyrphostin (1 h) stimulate both basal and ATP (enhanced in an additive manner)-stimulated PtdCho-PLD activity (Figure 32). It was also found that pertussis toxin pretreatment stimulates basal PtdCho-PLD activity in Daudi and Ramos B cells (results not shown) suggesting that, in B cells, PtdCho-PLD may be subject to negative control in a manner analogous to the receptor/Gi-mediated supression of cAMP levels observed in many cell types.

The finding that the antigen receptors on B cells are not coupled to PtdCho-PLD was initially rather surprising as (i) many of the receptors known to couple to PtdCho-PLD are, like the antigen receptors, calcium-mobilising receptors and capable of transducing mitogenic signals (128, 129) and (ii) in common with many studies on a wide range of cell types (128, 163, 226), PMA (presumably acting via PKC) and the tyrosine kinase inhibitor, pV stimulate one or more PtdCho-PLD activities in B cells,

results which, at first sight, are perhaps inconsistent with sIg-coupling to PTK and PKC, but not PtdCho-PLD activation following mitogenic stimulation of B cells via the antigen receptors (24, 25, 34). However, since the data also suggest that PtdCho-PLD activity in B cells is likely to be under the negative control of a Gi-like G-protein and/or tyrosine kinase-mediated signals (Figure 32), regulatory elements which are rapidly activated following ligation of the antigen receptors on B cells (24, 25, 34, 48), these perhaps unexpected results presumably reflect the complex crosstalk between particular Gi-like G-proteins, src-related PTKs, PKC isoforms and PtdCho-PLD activities expressed in B cells. Taken together with the findings that ATP, sphingosine and ceramide, reagents which can all inhibit sIg-mediated DNA synthesis (Figure 30), can also stimulate PtdCho-PLD activity (Figures. 31-33), these data suggest that PtdCho-PLD may be deleterious for sIg-driven B cell activation and that antigen receptor-coupled G-protein and/or tyrosine kinase-mediated signals may indeed act to suppress PtdCho-PLD activity.

The immunoregulatory role of P_2-purinoceptors on B cells is, at present, unclear but it is well established that ATP suppresses lymphocyte cytoxicity and proliferation (217-219). A possible role for the response of B cells to exogenous ATP is suggested by the physiological sources of extracellular nucleotides which include damaged cells, as well as platelets, endothelial cells and neurons (216). Damaged, necrotic or apoptotic cells could release their internal components into the extracellular space, and this could result in the recognition of these antigens by B cells expressing the appropriate antigen receptors. The existence of mature B cells expressing antigen receptors which would recognise such self antigens is possible because immature B lymphocytes might not encounter such intracellular self proteins during the maturation stage involving induction of anergy and deletion of autoreactive lymphocyte clones, and these B cells might therefore survive and mature. The release of intracellular proteins into the extracellular space could therefore result in the generation of an B cell autoimmune response. The binding of released ATP to purinoceptors on B cells might therefore constitute an

important signal for the suppression of B cell responses to autoantigens upon their release from damaged cells.

Previous studies have demonstrated that ATP-specific P_2Z receptors are expressed on human B cells and coupled to PtdInsP_2-PLC and mobilisation of calcium (227). In addition, ATP-stimulation of P_2-purinoceptors has also been shown to induce the expression of c-*fos*, c-*myc*, and the IL-2R and transferrin receptors in human B cells (227). However, since sIg is coupled to the generation of similar signals in B cells these data suggested that the anti-proliferative effects of ATP were likely to be due to additional transduction events. Interestingly, P_2-purinoceptors have been shown to be additionally coupled to PtdCho-PLD in a number of cell types (216, 220). This study has demonstrated that ATP stimulates PtdCho-PLD in murine B cells and that such coupling of the P_2-purinoceptors to PtdCho-PLD may, at least in part, provide a biochemical role for the transduction of ATP-mediated anti-proliferative signals in murine B cells.

Interestingly, lipid second messengers, such as sphingosine and ceramide, which can inhibit murine B cell activation, also stimulate PtdCho-PLD activation (Figure 33). In contrast to these results, it had previously been reported that sphingosine, when used as an inhibitor of PKC, could block agonist-stimulated PtdCho-PLD in a number of cell types (128) and that the antiproliferative effects of ceramide may be due, at least in part, to the supression of PtdCho-PLD activation stimulated by mitogens or growth factors in fibroblast cells (180, 190, 193). However, these apparent discrepancies may be quite simply resolved as (i) in these earlier studies, cells had been pretreated for 2-24 h with much higher concentrations of ceramide (5-50 μM) and these results may simply reflect that such pretreatment inhibits PLD. Alternatively, since these pretreatments were carried out in the absence of primary alcohols, they may mask ceramide-mediated activation and subsequent desensitisation of PLD which would therefore occur without the resultant early accumulation of trapped [^{3}H]phosphatidylalcohol; ii) ceramide has also been proposed to target PKCζ (221), a PKC isoform which is expressed (49, 228) and may stimulate PtdCho-PLD in B cells and iii) in addition to the studies reporting that

sphingosine blocks agonist-stimulated PLD activity (128), sphingosine has also been shown to stimulate PLD activity in neutrophils [eg. (190)]: however, at present it is not clear whether these stimulatory effects are due to sphingosine or conversion to sphingosine-1-phosphate, a lipid second messenger which has previously been shown to stimulate PLD activity in fibroblasts (229).

The mechanisms underlying the contrasting biological responses to activation of PLD isoforms in B cells (and other cell types) are currently unknown. However, the studies outlined above highlight recent findings that PtdCho- and PtdIns-specific PLD activities generate distinct PtdOH species (differing in their fatty acid composition) in different cellular compartments and with potentially differential downstream effector mechanisms. For example, only the steroyl-arachidonyl- species of PtdOH has been shown to prolong Ras activation by inhibiting the conversion of activated GTP-bound Ras to its inactive GDP-bound form by stimulating GTPase inhibiting protein (GIP) and inhibiting GTPase activating protein (GAP)(128, 129, 139, 140, 145, 154-156, 158-161, 168, 176, 230, 231) (119, 120, 123, 124, 133-135, 137-140, 142, 156, 213, 214). This may provide a biochemical rationale for the observations that whilst the putative PtdIns-PLD plays a role in mitogenic signalling in B cells, PtdCho-specific PLD(s) may be involved in the transduction of anti-proliferative signals via P2-purinoceptors, and possibly other immunomodulatory receptors, in mature, immunocompetent B cells.

3.9 Summary.

This investigation has identified multiple differentially-regulated PLD activities which may play key roles in mediating the transduction of mitogenic and antiproliferative signals in B cells:

i) pharmacological studies identified multiple PtdCho-PLD activities in B cells: these PtdCho-PLD activitites were regulated by PKC-, tyrosine phosphorylation- and G protein-dependent mechanisms.

ii) anti-Ig antibodies do not induce activation of PtdCho-PLD in mature or immature B cells indicating that the BCR is not coupled to PtdCho-PLD in these cells. However, further investigation identified an as yet undefined PLD activity which could be stimulated via sIg on B cells. Phospholipid labelling studies indicated that this could be a PtdIns-specific PLD activity.

iii) stimulation of B cells with a number of stimuli to mimic T cell-dependent B cell activation failed to activate PtdCho-PLD.

iv) stimulation of B cells with ATP, presumably via P_2-purinoceptors, stimulated PtdCho-PLD activation. As ATP has been found to suppress B cell proliferation, this suggests that a PtdCho-PLD activity(ies) could be involved in the transduction of negative, anti-proliferative signals in B lymphocytes.

v) further investigation of a role for PtdCho-PLD in negative signalling in B cells found that the lipid second messengers sphingosine and ceramide, which are implicated in the regulation of apoptosis in many cell types, induced the activation of PtdCho-PLD in B cells. Arachidonate, a lipid second messenger implicated in the regulation of both mitogenesis and apoptosis, also induced significant, but weak activation of PtdCho-PLD.

In conclusion, these results indicated that PtdCho-PLD is not involved in the transduction of signals regulating the initiation or maintenance of antigen-driven B cell proliferative responses, has implicated a role for PtdCho-PLD in the negative modulation

of B cell activation. In contrast, a putative PtdIns-PLD activity has been identified which may mediate the sIg-dependent activation of intracellular signalling elements such as Ras.

3.10 Future Perspectives.

Further investigation of the receptor-coupling of PLD enzymes in B cells will give a better indication of the precise role of these activities in the generation of downstream events and the regulation of B lymphocyte activation. There are a number of methods by which this coupling could be investigated:

i) [^{3}H]butanol could be used to further examine the mechanisms (ie. G protein, PtdIns, etc) by which sIg couple to the putative PtdIns-PLD activity in B cells. Knowledge of the regulation of this PLD activity, and comparison with reports on PLD enzymes from other systems, might give clues as to its identity in terms of substrate specificity and subcellular localisation.

ii) The species of PtdOH generated by the stimulation of B cells with different agonists or pharmacological agents can be analysed. The particular species of PtdOH formed can be determined by the preparation and separation of dinitrobenzoylated derivatives of PtdOHs by reverse phase HPLC and analysis by GLC/ mass spectroscopy. Comparison with acyl chains of phospholipids should definitively identify the lipid source of receptor coupled PtdOH formation. This work is currently being carried out in collaboration with M. Wakelam, Birmingham.

iii) the activation of specific PKC isoforms can also be analysed, in order to give clues as to the specific PtdOH signals required for the activation of PKC enzymes, and also for the identification of PKC isoforms which may be activated downstream of PLD activities. This can be achieved by assessing

autophosphorylation of PKC isoform-specific immunoprecipitates (isoform specific antibodies are available).

iv) P_2-purinoceptors typically couple to downstream effectors via heterotrimeric G proteins. A role for pertussis toxin-sensitive (G_i-like) proteins in the coupling of P_2-purinoceptors to PtdCho-PLD has already been exluded. P_2-purinoceptor-PLD coupling could be further investigated by examining the sensitivity of ATP stimulation of PtdCho-PLD to cholera toxin, which would indicate whether it is regulated by a G_α-like protein or not. However, the failure of cholera toxin to block the ATP stimulation of PtdCho-PLD would not exclude the possibility that P2-purinoceptors are coupled to PtdCho-PLD via a Gq-like protein. This could be investigated by incubating permeabilised B cells with anti-G_q antibodies to block any coupling prior to stimulation with ATP.

v) the role of GTP-binding proteins in the regulation of PLD activity in B cells could be further investigated by the addition of recombinant ARF and Rho to permeabilised B cells.

iv) the potential physiological role of ceramides and sphingosine in the regulation of PtdCho-PLD and the transduction of receptor-mediated signals could be investigated by determining whether the generation of these lipid second messengers is stimulated by signals which also activate PtdCho-PLD.

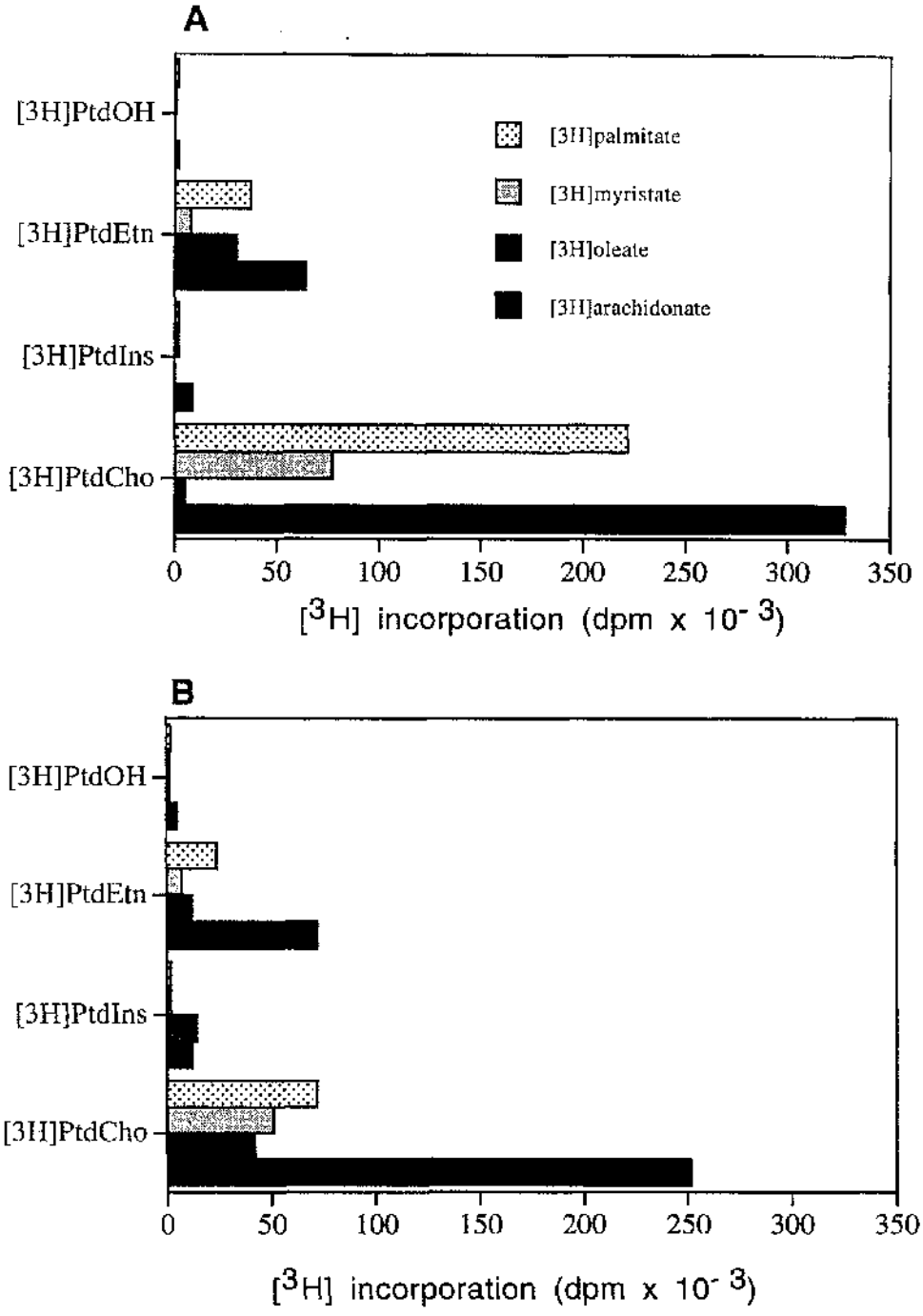

A
[3H]PtdOH
[3H]PtdEtn
[3H]PtdIns
[3H]PtdCho
[3H]palmitate
[3H]myristate
[3H]oleate
[3H]arachidonate
0
50
100
150
200
250
300
350
[3H] incorporation (dpm x 10- 3)
B
[3H]PtdOH
[3H]PtdEtn
[3H]PtdIns
[3H]PtdCho
0
50
100
150
200
250
300
350
[3H] incorporation (dpm x 10- 3)

Figure 11. [^{3}H]Fatty acids are preferentially incorporated into PtdCho in murine B cells. Murine splenic B cells were incubated with [^{3}H]arachidonate, [^{3}H]oleate, [^{3}H]myristate, or [^{3}H]palmitate for (A) 4 or (B) 18 hours and the incorporation of these [^{3}H]fatty acids into phospholipids was measured by tlc analysis as described in the "Materials and Methods" section. The results are expressed as means where n=3.

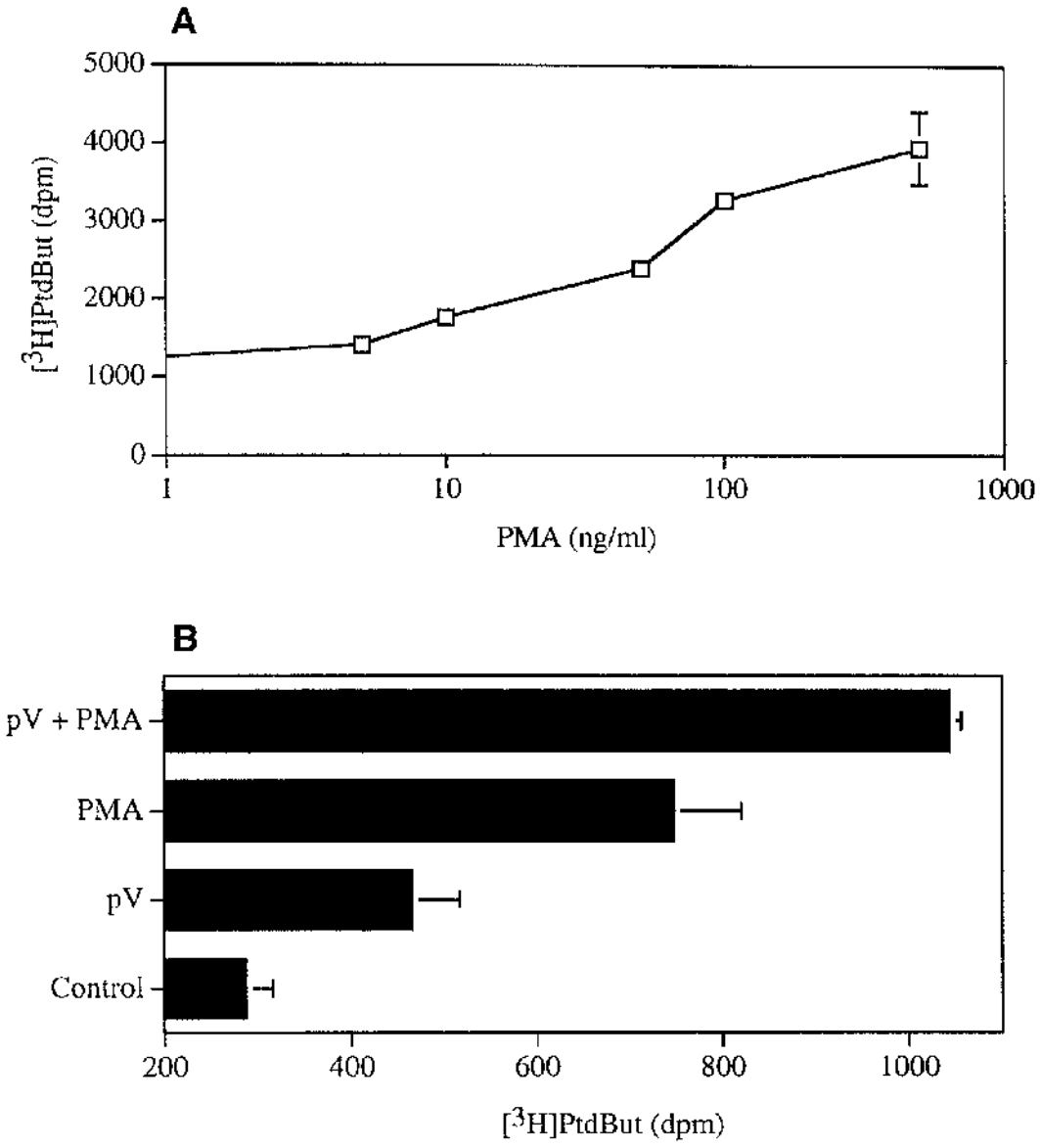
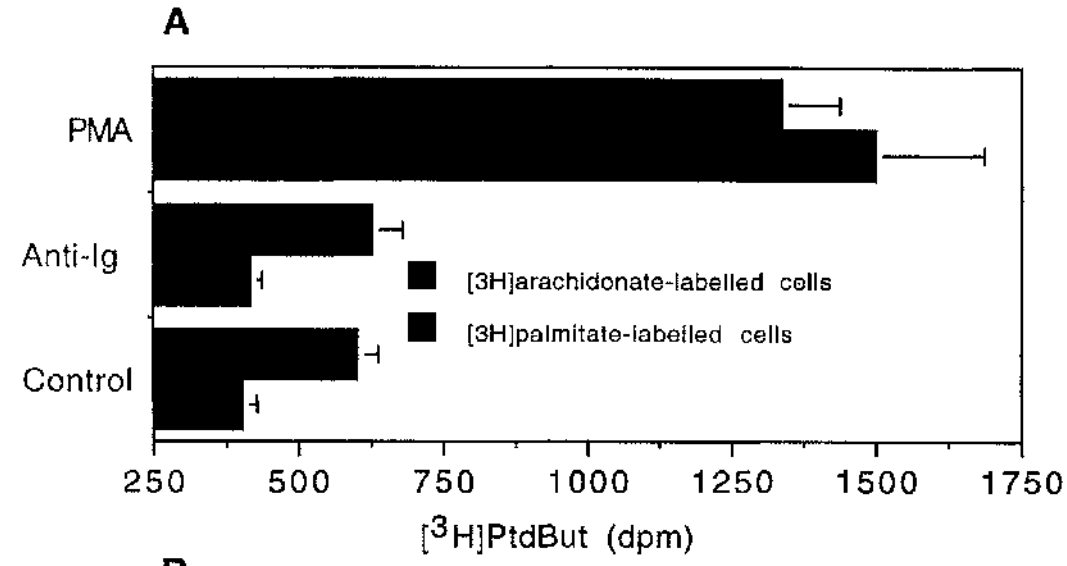
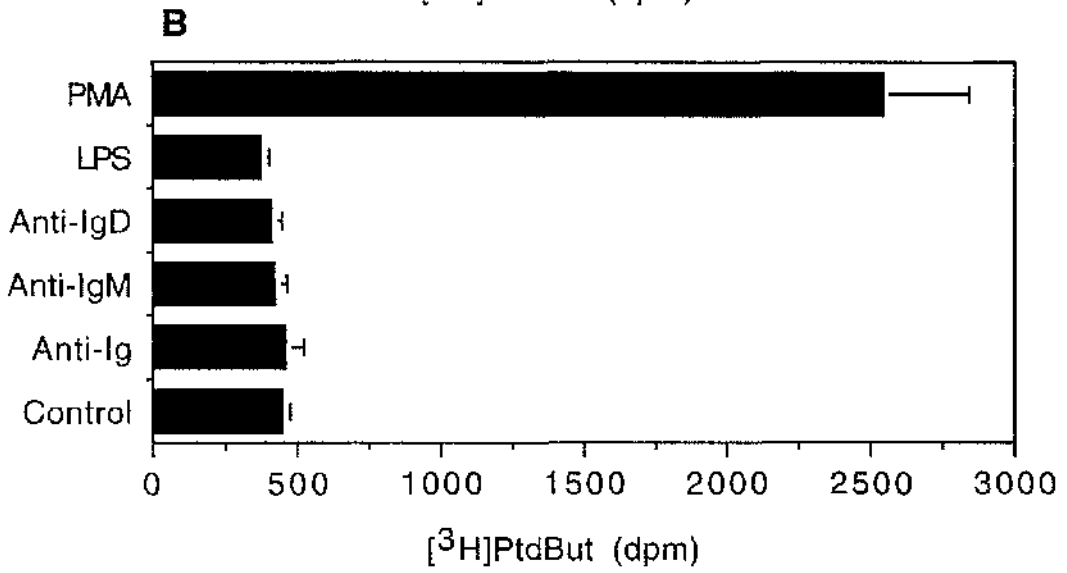

A
5000
4000
3000
2000
1000
0
[3H]PtdBut (dpm)
1
10
100
1000
PMA (ng/ml)
B
pV + PMA
PMA
pV
Control
200
400
600
800
1000
[3H]PtdBut (dpm)

Figure 12. PtdCho-PLD is activated by stimulation of B cells with PMA or pervanadate. Murine B cells, pre-labelled with [^{3}H]palmitate, were stimulated, in *panel A:* with PMA (0-500ng/ml); or in *panel B:* with pervanadate (pV; 0.5 mM), PMA (100 ng/ml), or pV (0.5 mM) plus PMA (100 ng/ml), for 30 min. [^{3}H]PtdBut was measured using tlc analysis as described in the methods section. The results are expressed as means ± SD, n=3.


A
PMA
Anti-Ig
Control
[3H]arachidonate-labelled cells
[3H]palmitate-labelled cells
250
500
750
1000
1250
1500
1750
[3H]PtdBut (dpm)


B
PMA
LPS
Anti-IgD
Anti-IgM
Anti-Ig
Control
0
500
1000
1500
2000
2500
3000
[3H]PtdBut (dpm)

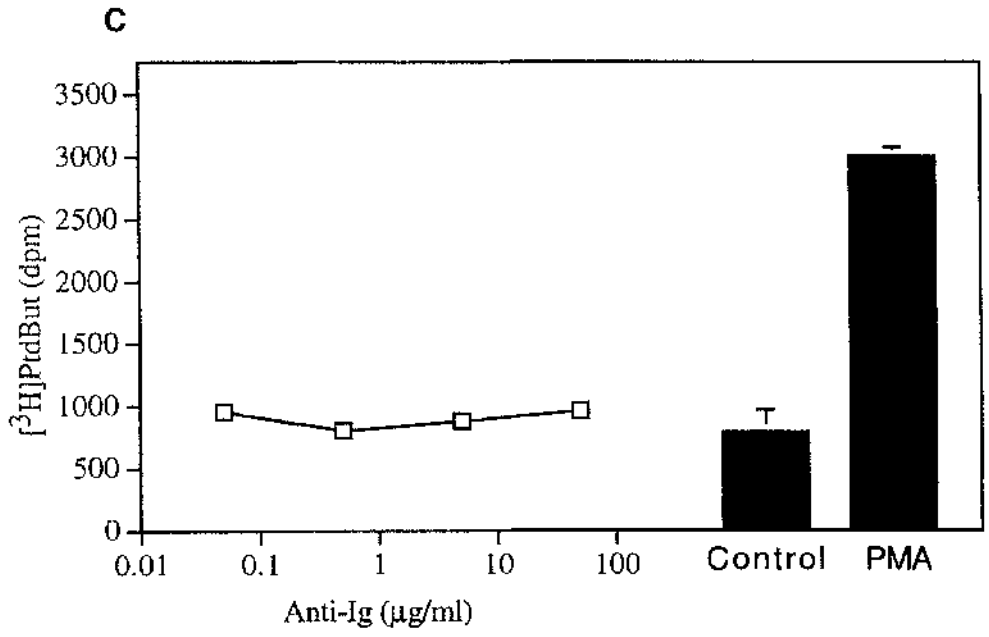
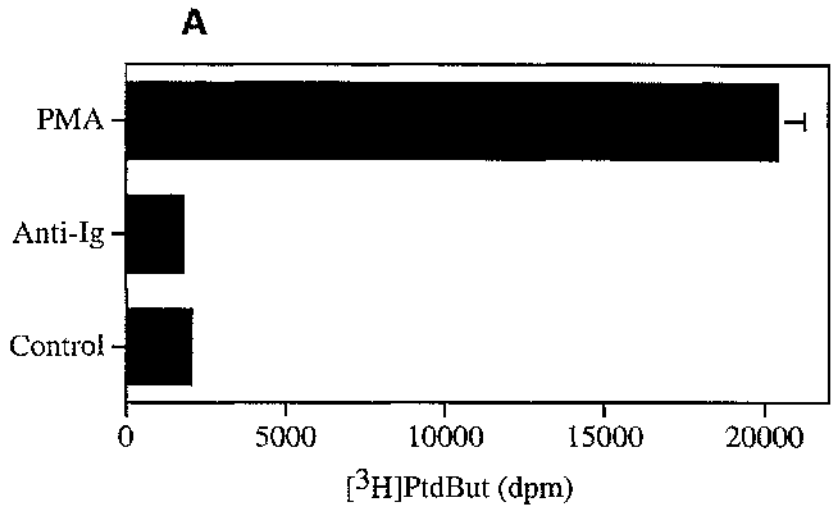

C
3500
3000
2500
2000
1500
1000
500
0
[3H]PtdBut (dpm)
0.01
0.1
1
10
100
Control
PMA
Anti-Ig (μg/ml)

Figure 13. The antigen receptors on murine B cells are not coupled to PtdCho-PLD. In *panel A:* murine B cells, prelabelled with [^{3}H]arachidonate or [^{3}H]palmitate, were stimulated with anti-Ig (50 μg/ml) or PMA (100 ng/ml); in *panels B and C:* [^{3}H]palmitate-labelled B cells were stimulated *(panel B)* with anti-Ig (50 μg/ml), anti-IgM (50 μg/ml), anti-IgD (50 μg/ml), LPS (50 μg/ml) or PMA (100 ng/ml); or *(panel C)* with anti-Ig (0.05-50 μg/ml) or PMA (100 ng/ml). Cells were stimulated for 30 min at 37C in the presence of 0.3% butanol and [^{3}H]PtdBut was measured using tlc analysis as described in the methods section. The results are expressed as means ± SD from single representative experiments where n=3.


A
PMA
Anti-Ig
Control
0
5000
10000
15000
20000
[3H]PtdBut (dpm)

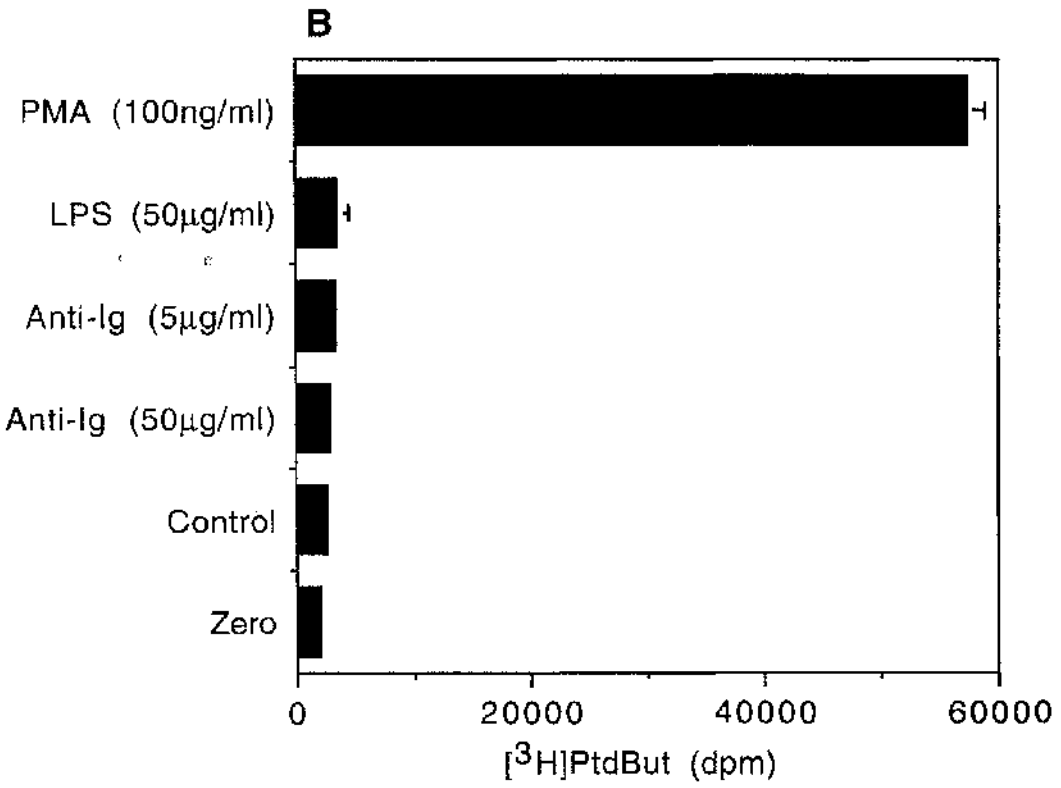

B
PMA (100ng/ml)
LPS (50µg/ml)
Anti-Ig (5µg/ml)
Anti-Ig (50µg/ml)
Control
Zero
0
20000
40000
60000
[3H]PtdBut (dpm)

Figure 14. Crosslinking of sIg on *in vivo* activated murine B cells or EDR human B cells does not induce activation of PtdCho-PLD. *Panel A: in vivo* activated murine splenic B cells were pre-labelled with [^{3}H]palmitate and stimulated with anti-Ig (50 μg/ml) or PMA (100 ng/ml) for 30 min. *Panel B:* EDR cells were pre-labelled with [^{3}H]palmitate overnight and stimulated with anti-Ig (5 or 50 μg/ml), LPS (50 μg/ml), or PMA (100 ng/ml) for 30 min. [^{3}H]PtdBut was measured as described in "Materials and Methods". The results are expressed as means ± SD, n=3.

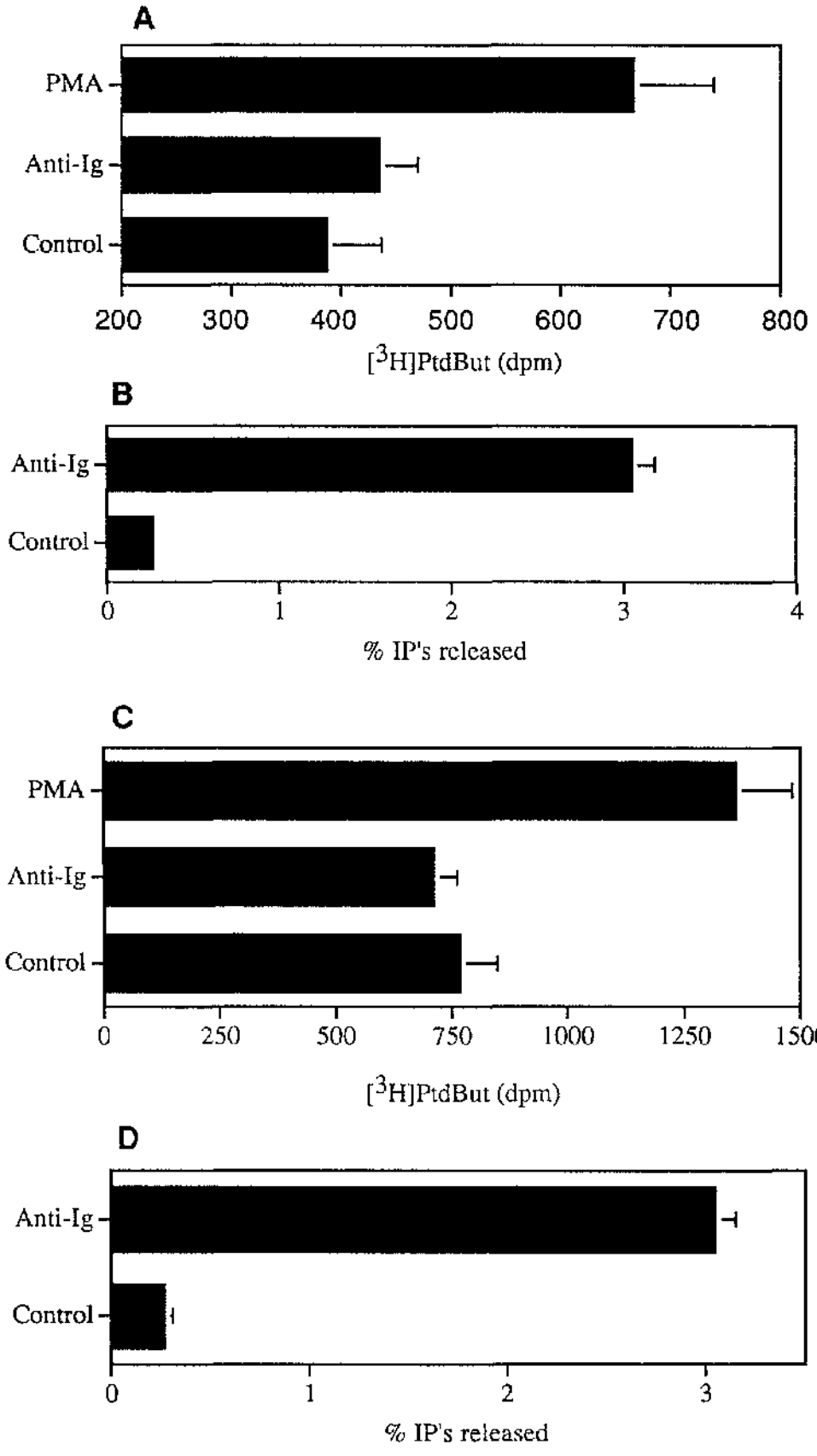

A
PMA
Anti-Ig
Control
200
300
400
500
600
700
800
[3H]PtdBut (dpm)
B
Anti-Ig
Control
0
1
2
3
4
% IP's released
C
PMA
Anti-Ig
Control
0
250
500
750
1000
1250
1500
[3H]PtdBut (dpm)
D
Anti-Ig
Control
0
1
2
3
% IP's released

Figure 15. Production of inositol phosphates, but not PtdCho-PLD activation, is induced by crosslinking of the antigen receptors on Ramos and Daudi human B cells. In *panels A* and *B* Ramos B cells were labelled with [^{3}H]palmitate (*panel A*) or [^{3}H]inositol (*panel B*), and stimulated (*panel A*) with anti-Ig (25 μg/ml) or PMA (100 ng/ml); or (*panel B*) with anti-Ig (25 μg/ml); for 30 min. In *panel C* and *D* Daudi B cells were labelled with [^{3}H]palmitate (*panel A*) or [^{3}H]inositol (*panel B*), and stimulated (*panel A*) with anti-Ig (25 μg/ml) or PMA (100 ng/ml); or (*panel B*) with anti-Ig (25 μg/ml); for 30 min. [^{3}H]PtdBut, or [^{3}H]inositol phosphates, were measured as described in "Materials and Methods". The results are expressed as means ± SD, n=3.

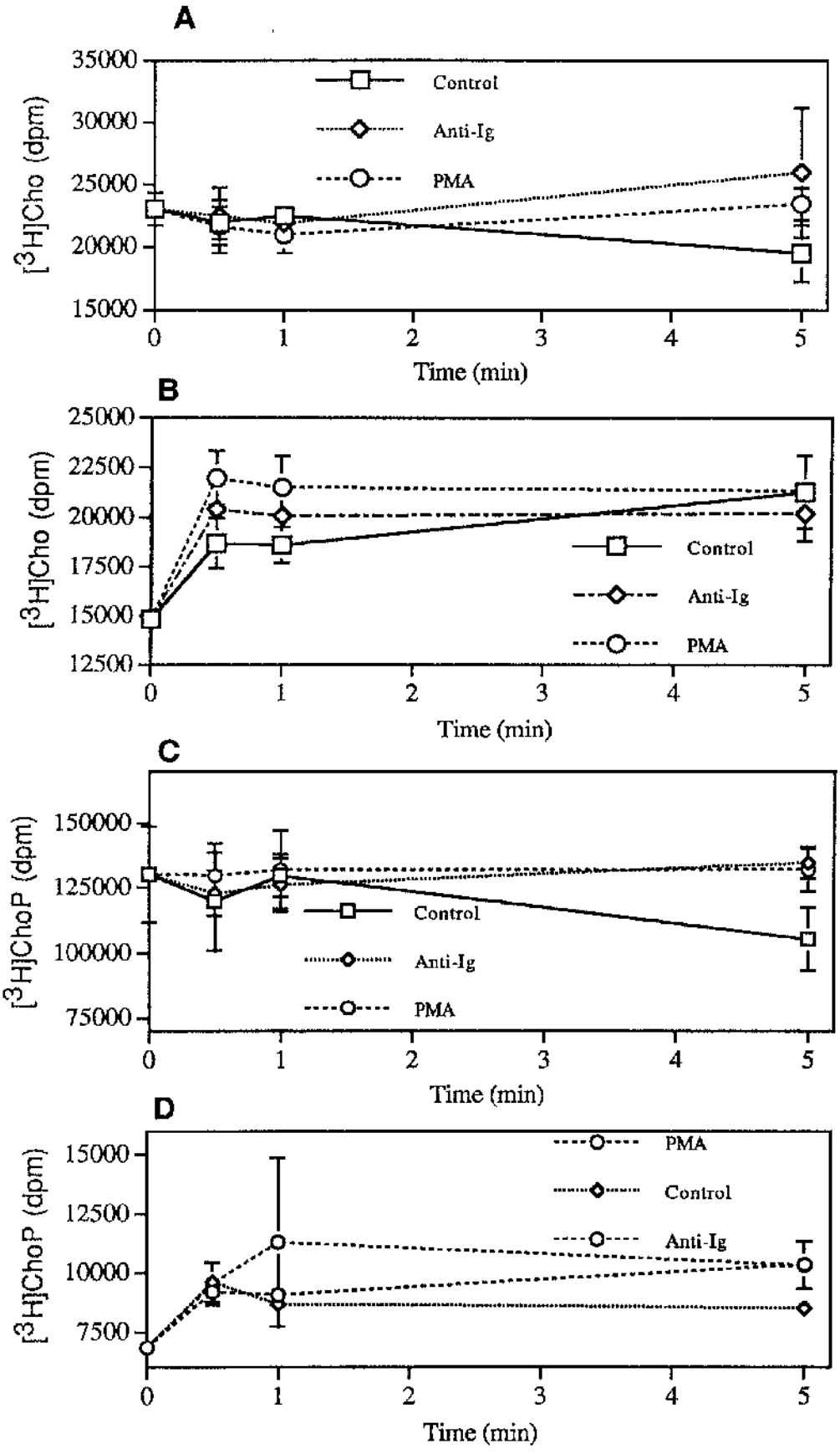
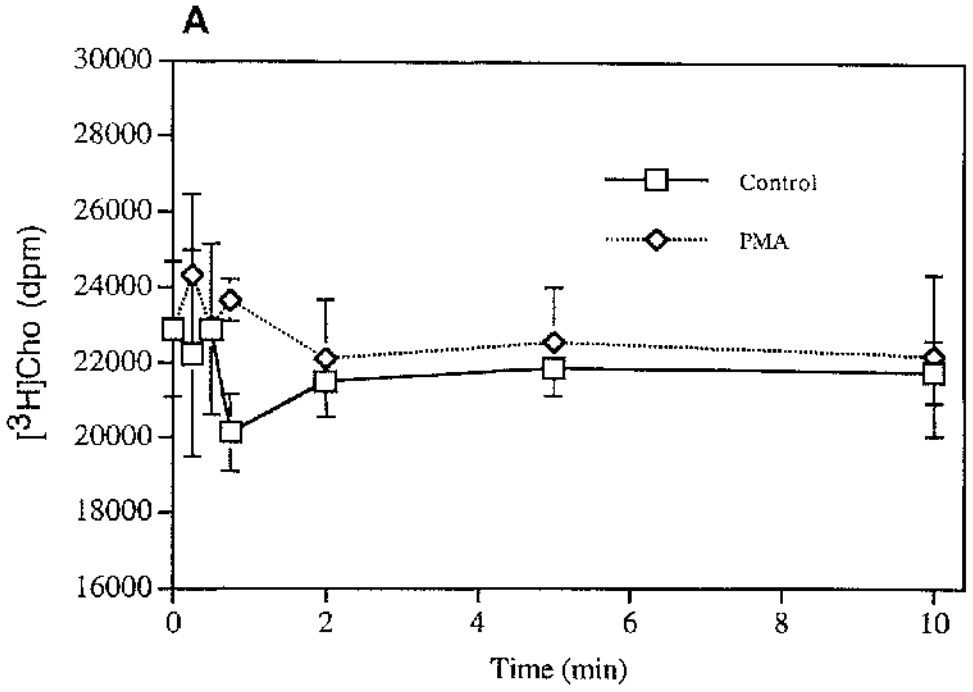

A
35000
30000
25000
20000
15000
[3H]Cho (dpm)
Control
Anti-Ig
PMA
0
1
2
3
4
5
Time (min)
B
25000
22500
20000
17500
15000
12500
[3H]Cho (dpm)
Control
Anti-Ig
PMA
0
1
2
3
4
5
Time (min)
C
150000
125000
100000
75000
[3H]ChoP (dpm)
Control
Anti-Ig
PMA
0
1
2
3
4
5
Time (min)
D
15000
12500
10000
7500
[3H]ChoP (dpm)
PMA
Control
Anti-Ig
0
1
2
3
4
5
Time (min)

Figure 16. Intracellular and extracellular levels of choline and choline phosphate in murine B cells. Resting murine splenic B cells were labelled with [^{3}H]methylcholine for 4 h and stimulated with anti-Ig (50 μg/ml) or PMA (100 ng/ml) for up to 5 min. Reactions were terminated and samples separated into intracellular and extracellular fractions as described in Materials and Methods. [^{3}H]Cho and [^{3}H]ChoP were then separated and measured to give intracellular and extracellular [^{3}H]Cho *(panels A and B, respectively)*, and intracellular and extracellular [^{3}H]ChoP *(panels C and D, respectively)*. The results are expressed as means ± SD, n=3.


A
30000
28000
26000
24000
22000
20000
18000
16000
[3H]Cho (dpm)
Control
PMA
0
2
4
6
8
10
Time (min)

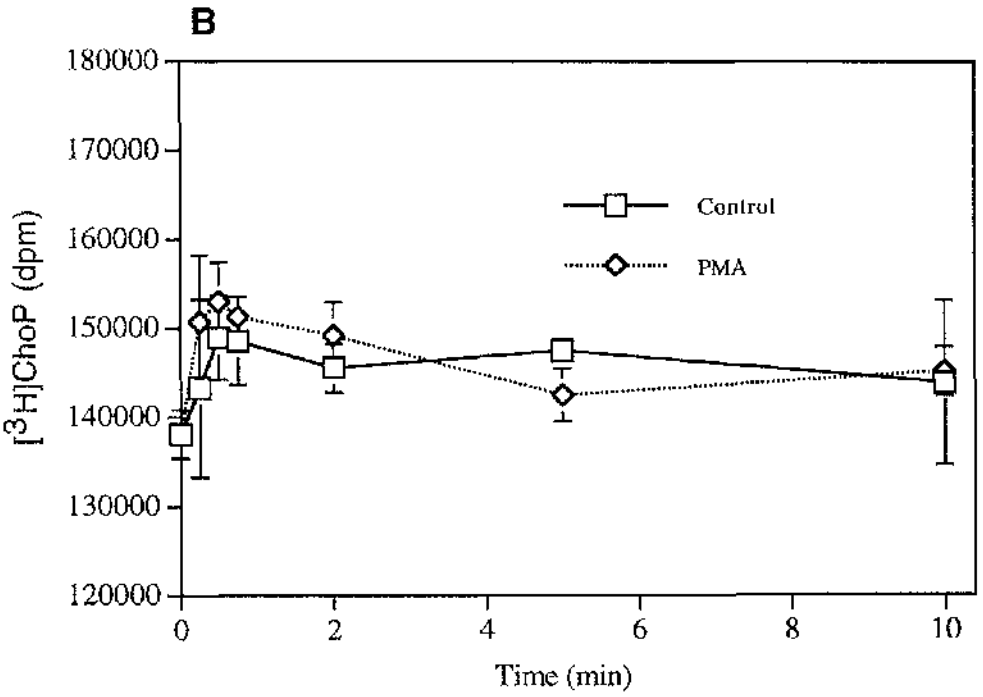

B
180000
170000
160000
150000
140000
130000
120000
[3H]ChoP (dpm)
Control
PMA
0
2
4
6
8
10
Time (min)

Figure 17. Total choline and choline phosphate levels in Jurkat human T cells. Jurkat cells were pre-labelled with [^{3}H]methylcholine overnight and stimulated with PMA (100ng/ml) for up to 20 min. [^{3}H]Cho *(panel A)* and [^{3}H]ChoP *(panel B)* were separated and measured as described in Materials and Methods. The results are expressed as means ± SD, n=3.

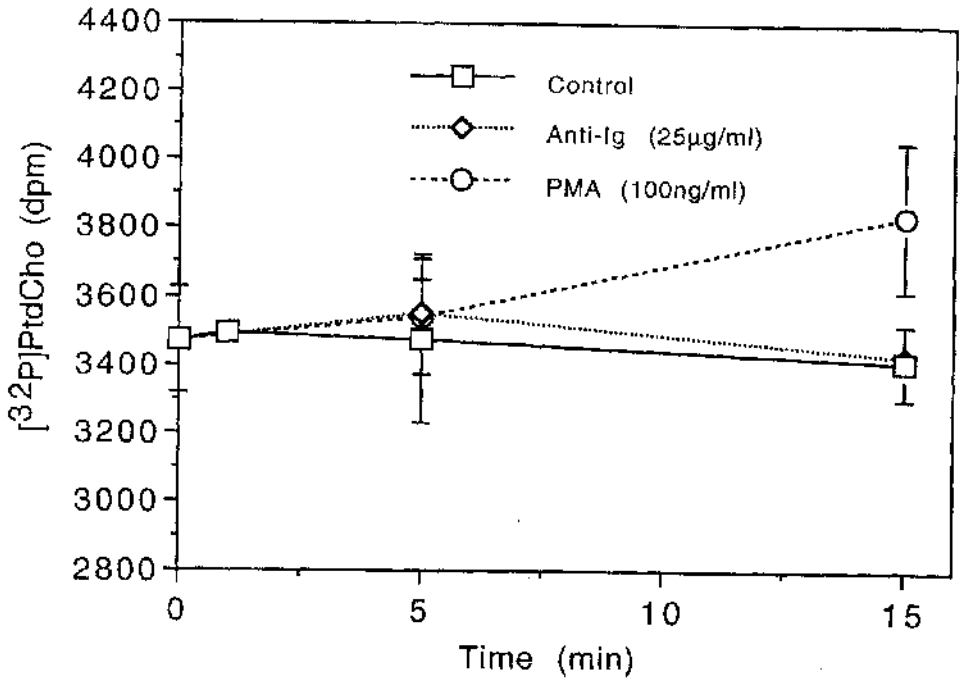

4400
4200
4000
3800
3600
3400
3200
3000
2800
[32P]PtdCho (dpm)
0
5
10
15
Time (min)
Control
Anti-Ig (25µg/ml)
PMA (100ng/ml)

Figure 18. Measurement of PtdCho levels in stimulated EDR human B cells. EDR human B cells were pre-labelled with [^{32}P]orthophosphate overnight and stimulated with anti-Ig (50 μg/ml) or PMA (100 ng/ml) for 1, 5, or 15 min. Phospholipids were then separated by tlc and [^{32}P]PtdCho levels determined as described under "Materials and Methods". The data are expressed as means ± SD, n=3.

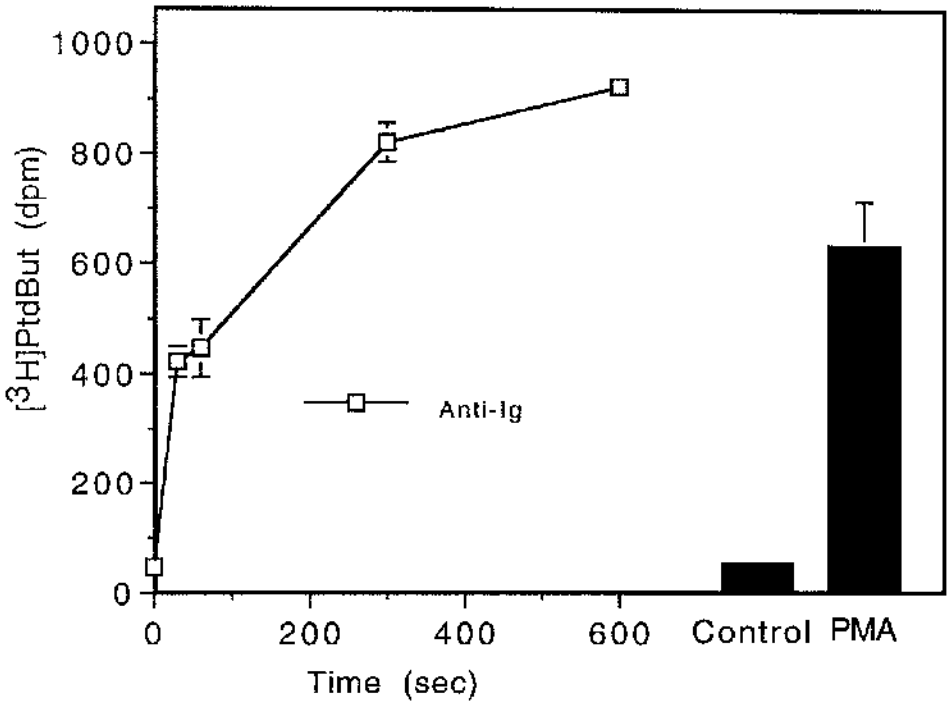

1000
800
600
400
200
0
[3H]PtdBut (dpm)
Anti-Ig
0
200
400
600
Time (sec)
Control
PMA

Figure 19. The antigen receptors on murine B cells are coupled to PLD activation. Murine splenic B cells were incubated with [^{3}H]butan-1-ol (20 μCi/ml) and stimulated with anti-Ig (50 μg/ml) for the indicated time, or with PMA (100 ng/ml) for 10 min. [^{3}H]PtdBut measured using tlc analysis as described under "Materials and Methods". The data are expressed as means ± SD, n=3.

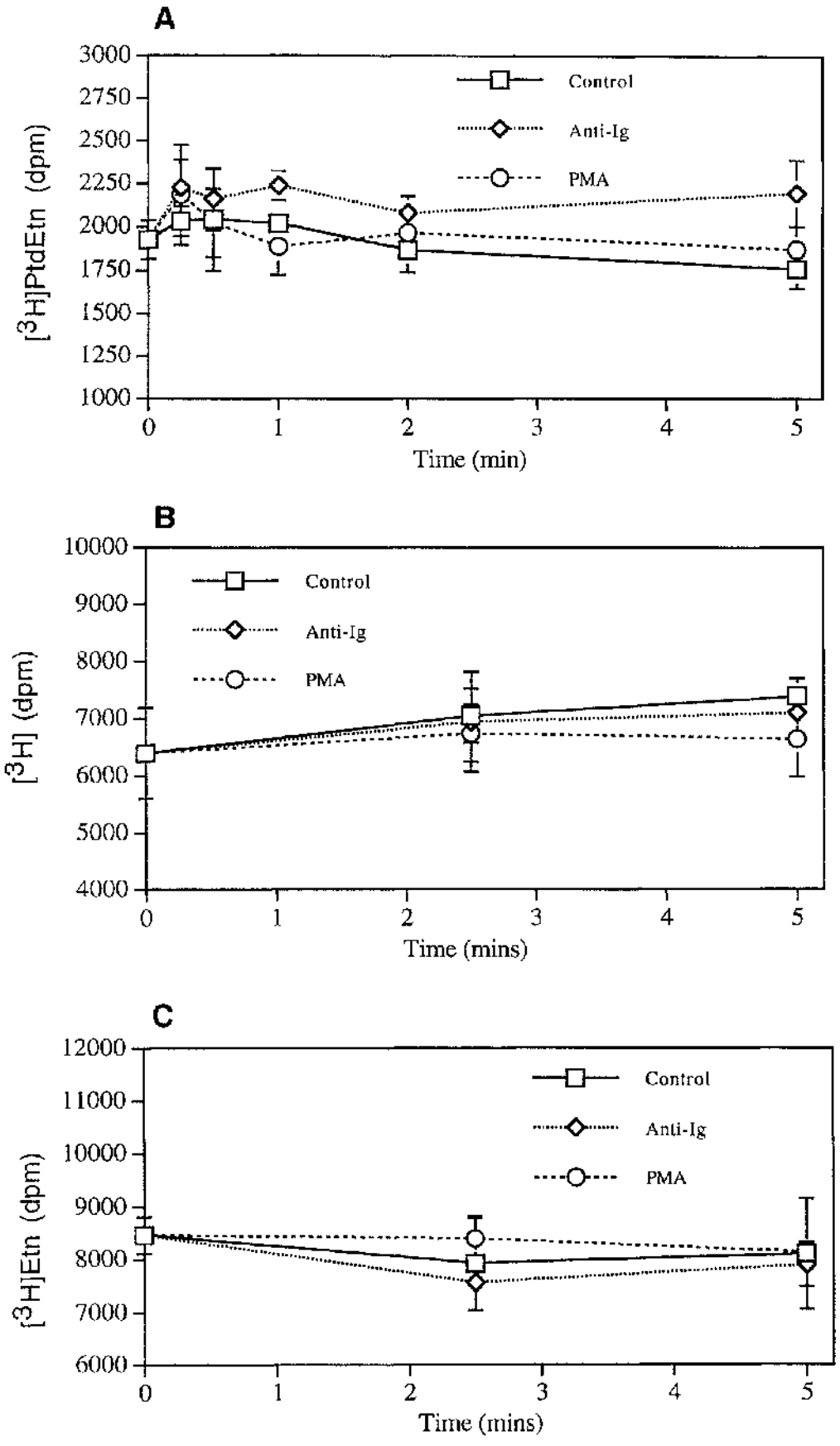

A
3000
2750
2500
2250
2000
1750
1500
1250
1000
[3H]PtdEtn (dpm)
Control
Anti-Ig
PMA
0
1
2
3
4
5
Time (min)
B
10000
9000
8000
7000
6000
5000
4000
[3H] (dpm)
Control
Anti-Ig
PMA
0
1
2
3
4
5
Time (mins)
C
12000
11000
10000
9000
8000
7000
6000
[3H]Etn (dpm)
Control
Anti-Ig
PMA
0
1
2
3
4
5
Time (mins)

Figure 20. PMA stimulation or sIg crosslinking on murine B cells does not induce hydrolysis of PtdEtn. Resting murine splenic B cells were pre-labelled with [^{3}H]ethanolamine for 4 h and stimulated with anti-Ig (50 μg/ml) or PMA (100 ng/ml) for the indicated time. [^{3}H]PtdEtn levels *(panel A)*, or [^{3}H]Etn and [^{3}H]PEtn levels *(panels B and C, respectively)* were then determined as described in Materials and Methods. The results are expressed as means ± SD, n=3.

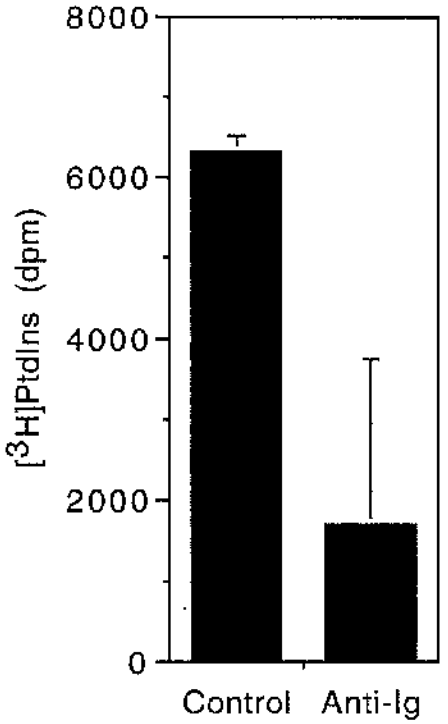

8000
6000
4000
2000
0
[3H]PtdIns (dpm)
Control
Anti-Ig

Figure 21. The antigen receptors on murine B cells are coupled to phosphatidylinositol breakdown. B cells were radiolabelled with [^{3}H]inositol and stimulated with anti-Ig (50 μg/ml) for 10 min. [^{3}H]PtdIns hydrolysis was measured as described in the methods section. The data are expressed as means ± SD, n=3.

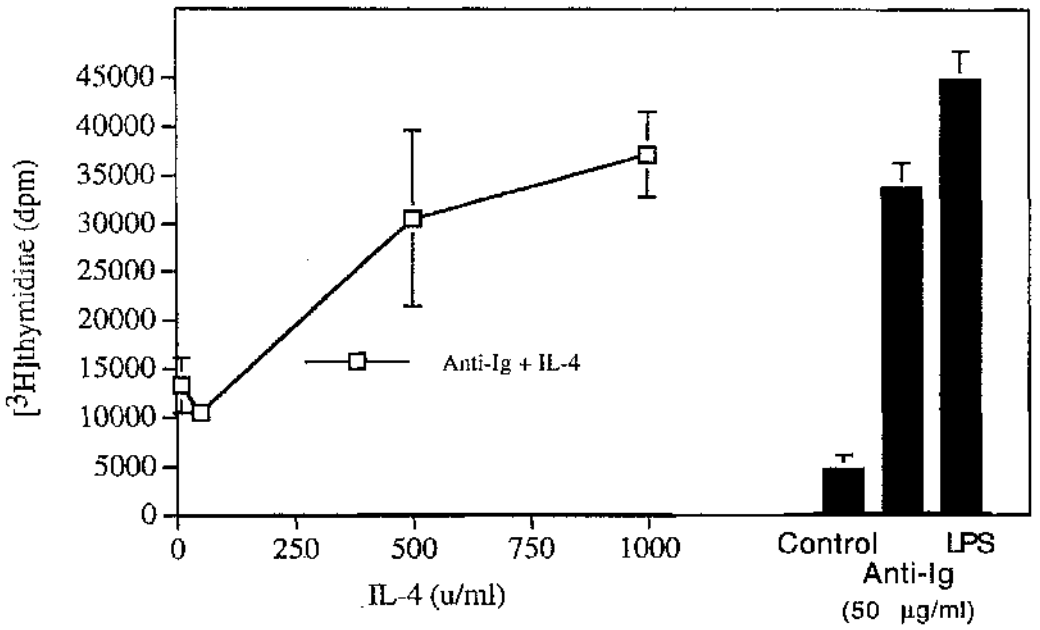
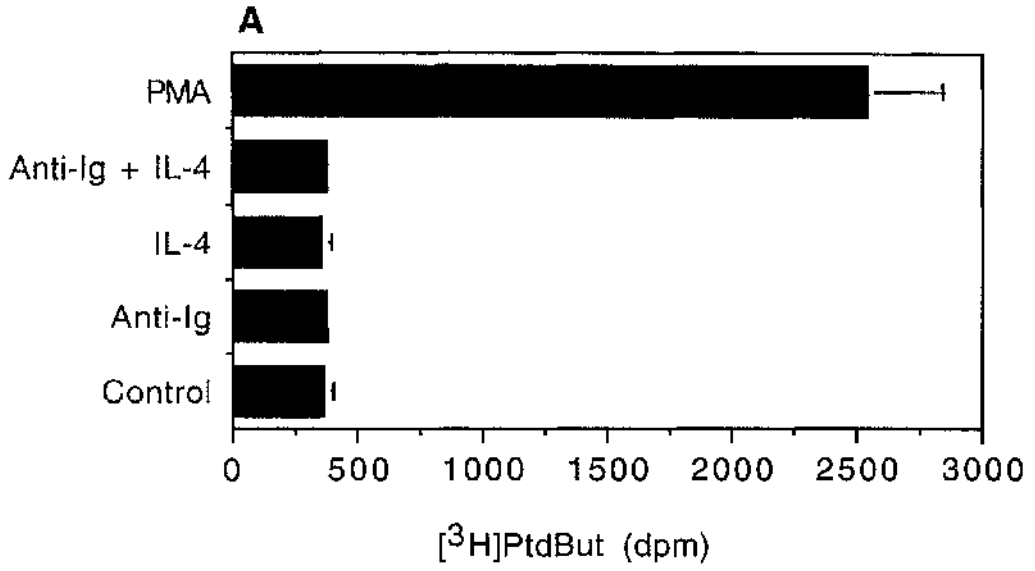

45000
40000
35000
30000
25000
20000
15000
10000
5000
0
[3H]thymidine (dpm)
Anti-Ig + IL-4
0
250
500
750
1000
IL-4 (u/ml)
Control
LPS
Anti-Ig
(50 µg/ml)

Figure 22. Anti-Ig, LPS or anti-Ig plus IL-4 induce DNA synthesis in murine B cells. Murine B cells (10^6/ml) were cultured in the presence of anti-Ig (50 μg/ml), LPS (50 μg/ml), or anti-Ig (5 μg/ml) plus IL-4 (10-1000 U/ml) and [^{3}H]thymidine uptake determined after 72 h as described in the methods section: data are presented as means ± SD, n=3.


A
PMA
Anti-Ig + IL-4
IL-4
Anti-Ig
Control
0
500
1000
1500
2000
2500
3000
[3H]PtdBut (dpm)

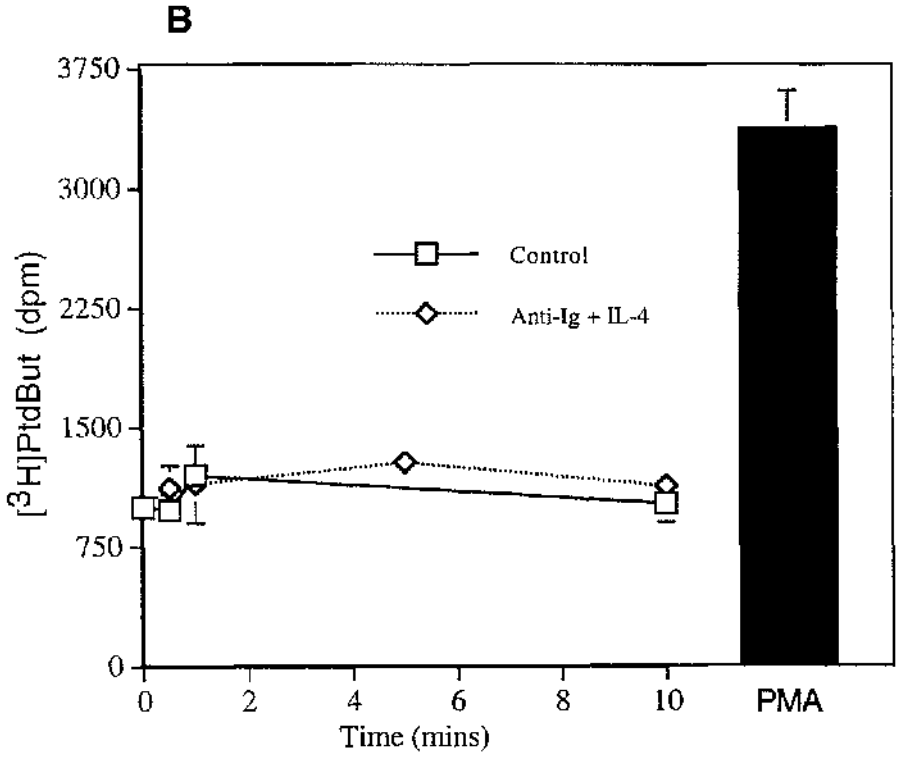

B
3750
3000
2250
1500
750
0
[3H]PtdBut (dpm)
Control
Anti-Ig + IL-4
0
2
4
6
8
10
Time (mins)
PMA

Figure 23. The IL-4 receptors on murine B cells are not coupled to PtdCho-PLD when ligated with rIL-4 alone, or following crosstalk with sIg-derived signals. Murine splenic B cells were pre-labelled with [^{3}H]palmitate for 4 h and stimulated, in *panel A*, with anti-Ig (5 μg/ml), IL-4 (100 U/ml), anti-Ig (5 μg/ml) plus IL-4 (100 U/ml), or PMA (100 ng/ml) for 30 min; or in *panel B* with anti-Ig (5 μg/ml) plus IL-4 (100 U/ml) for 30 sec, 1, 5 or 10 min, or PMA (100 ng/ml) for 10 min. [^{3}H]PtdBut was measured as described in the "Materials and Methods" section. The results are expressed as means ± SD, n=3.

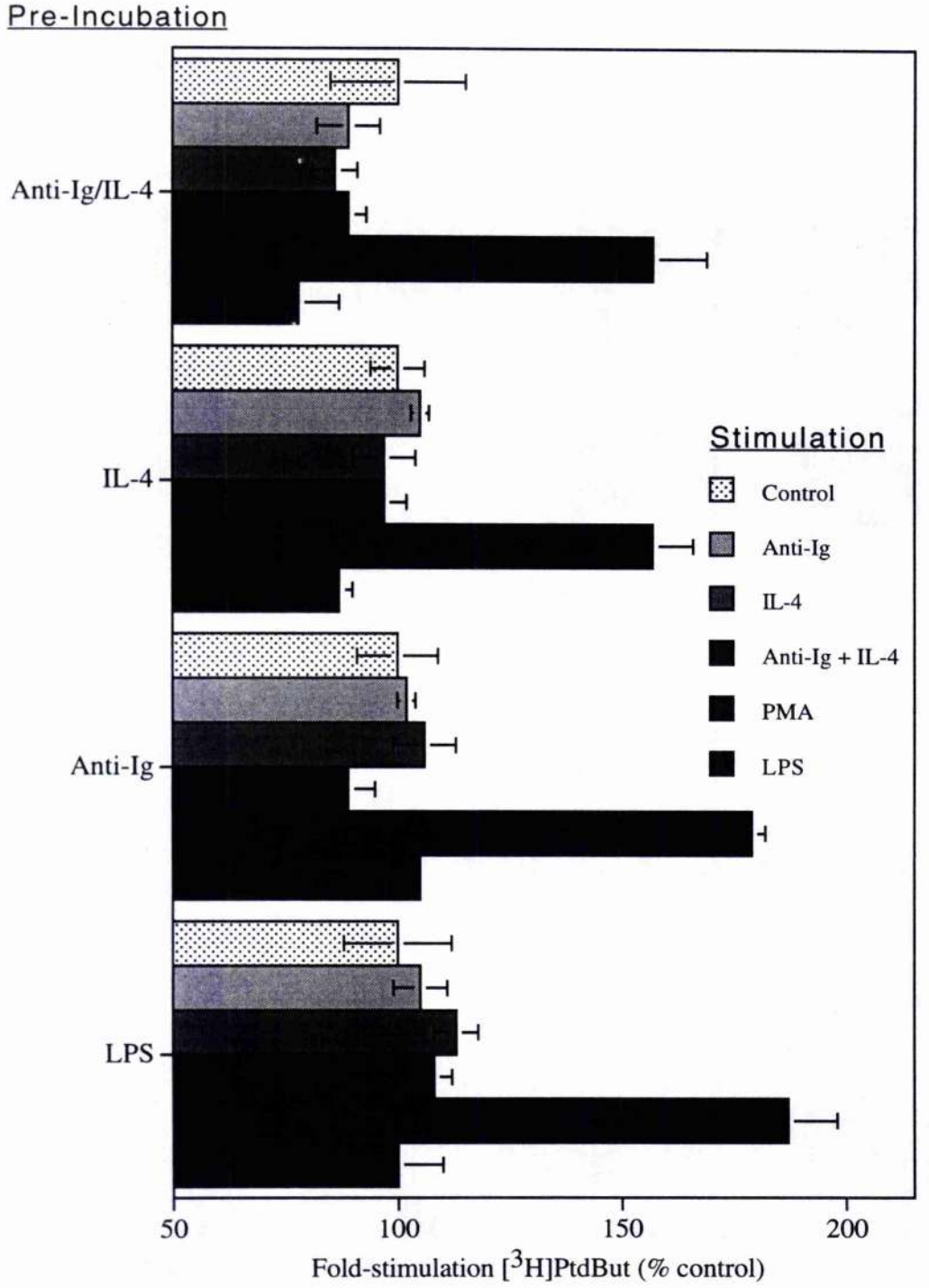
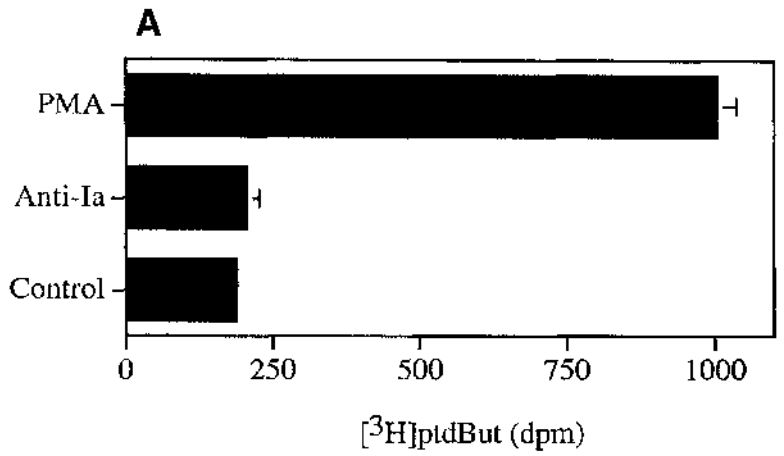
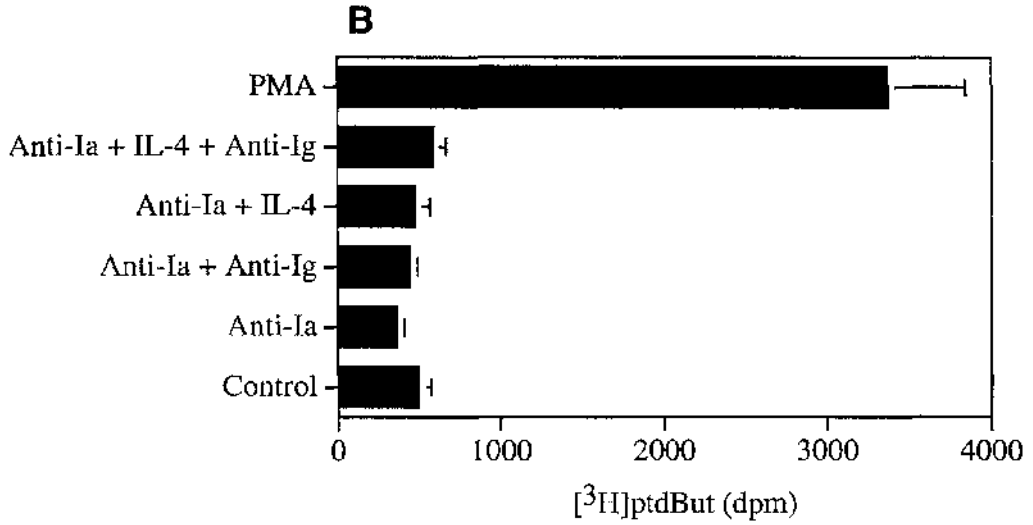

Pre-Incubation
Anti-Ig/IL-4
IL-4
Anti-Ig
LPS
Stimulation
Control
Anti-Ig
IL-4
Anti-Ig + IL-4
PMA
LPS
50
100
150
200
Fold-stimulation [3H]PtdBut (% control)

Figure 24. "Priming" of B cells with a variety of stimuli does not appear to alter coupling of PtdCho-PLD. Resting murine B cells were pre-labelled with [^{3}H]palmitate and concurrently pre-incubated with anti-Ig (50 μg/ml), IL-4 (100 U/ml), anti-Ig (5 μg/ml) plus IL-4 (100 U/ml), or LPS (50 μg/ml) overnight. Primed cells were then stimulated with anti-Ig (50 μg/ml), IL-4 (100 U/ml), anti-Ig (5 μg/ml) plus IL-4 (100 U/ml), LPS (50 μg/ml), or PMA (100 ng/ml), for 30 min. [^{3}H]PtdBut was measured as described in the "Materials and Methods" section. The results are expressed as means ± SD, n=3.


A
PMA
Anti-Ia
Control
0
250
500
750
1000
[3H]ptdBut (dpm)


B
PMA
Anti-Ia + IL-4 + Anti-Ig
Anti-Ia + IL-4
Anti-Ia + Anti-Ig
Anti-Ia
Control
0
1000
2000
3000
4000
[3H]ptdBut (dpm)

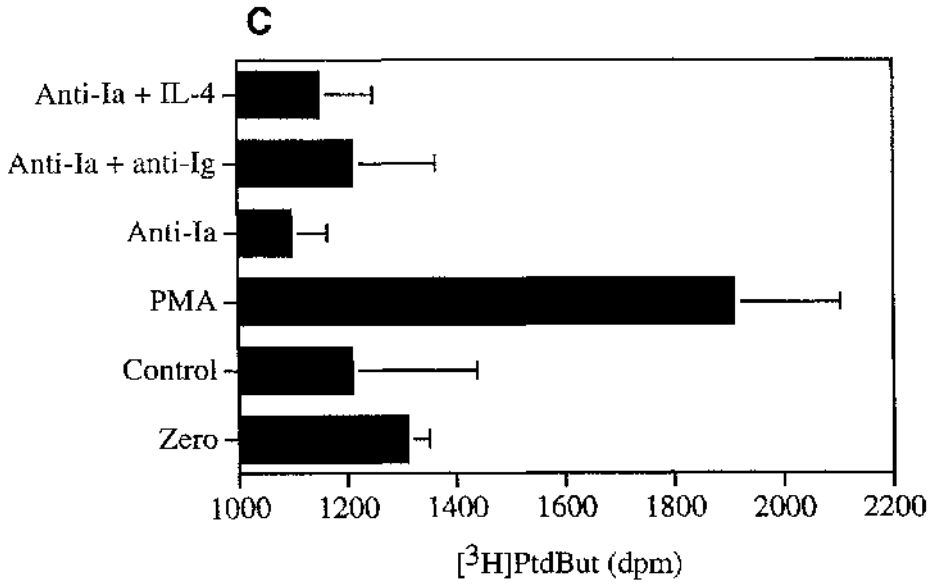

C
Anti-Ia + IL-4
Anti-Ia + anti-Ig
Anti-Ia
PMA
Control
Zero
1000
1200
1400
1600
1800
2000
2200
[3H]PtdBut (dpm)

Figure 25. Crosslinking of MHC class II molecules does not activate PtdCho-PLD in B cells. In *panel A:* resting murine splenic B cells were pre-labelled with [^{3}H]palmitate for 4 hours and stimulated with biotinylated anti-Ia (100 μg/ml) (before crosslinking with avidin) or PMA (100 ng/ml), for 30 min. In *panel B: in vivo* activated murine splenic B cells were pre-labelled with [^{3}H]palmitate for 4 hours and stimulated with biotinylated anti-Ia (100 μg/ml) (before crosslinking with avidin) in the presence or absence of anti-Ig (50 μg/ml), IL-4 (100 U/ml) or anti-Ig (5 μg/ml) plus IL-4 (100 U/ml), or with PMA (100 ng/ml), for 30 min. In *panel C:* resting murine B cells were pre-labelled with [^{3}H]palmitate and concurrently incubated with anti-Ig (5 μg/ml) plus IL-4 (100 U/ml) overnight. They were then stimulated with biotinylated anti-Ia (100 μg/ml) (before reaction initiated by crosslinking with avidin) in the presence or absence of anti-Ig (50 μg/ml) or IL-4 (100 U/ml), or with PMA (100 ng/ml), for 30 min. [^{3}H]PtdBut was measured as described in the methods section. The results are expressed as means ± SD, n=3.

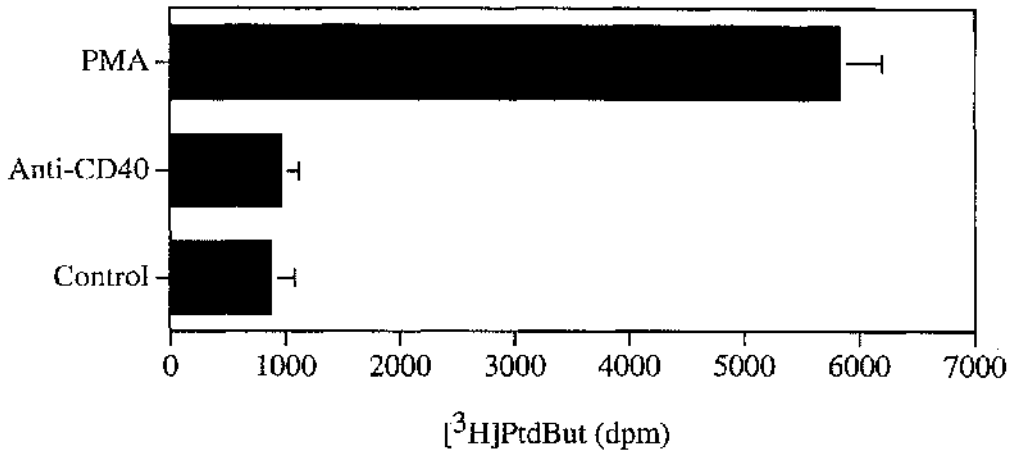

PMA
Anti-CD40
Control
0
1000
2000
3000
4000
5000
6000
7000
[3H]PtdBut (dpm)

Figure 26. Crosslinking of CD40 does not activate PtdCho-PLD in murine B cells. Resting murine B cells were pre-labelled with [^{3}H]palmitate for 4 h and stimulated with anti-CD40 antibody (FGK-45) (10 μg/ml) or PMA for 30 min. [^{3}H]PtdBut was measured as described in the methods section. The results are expressed as means ± SD, n=3.

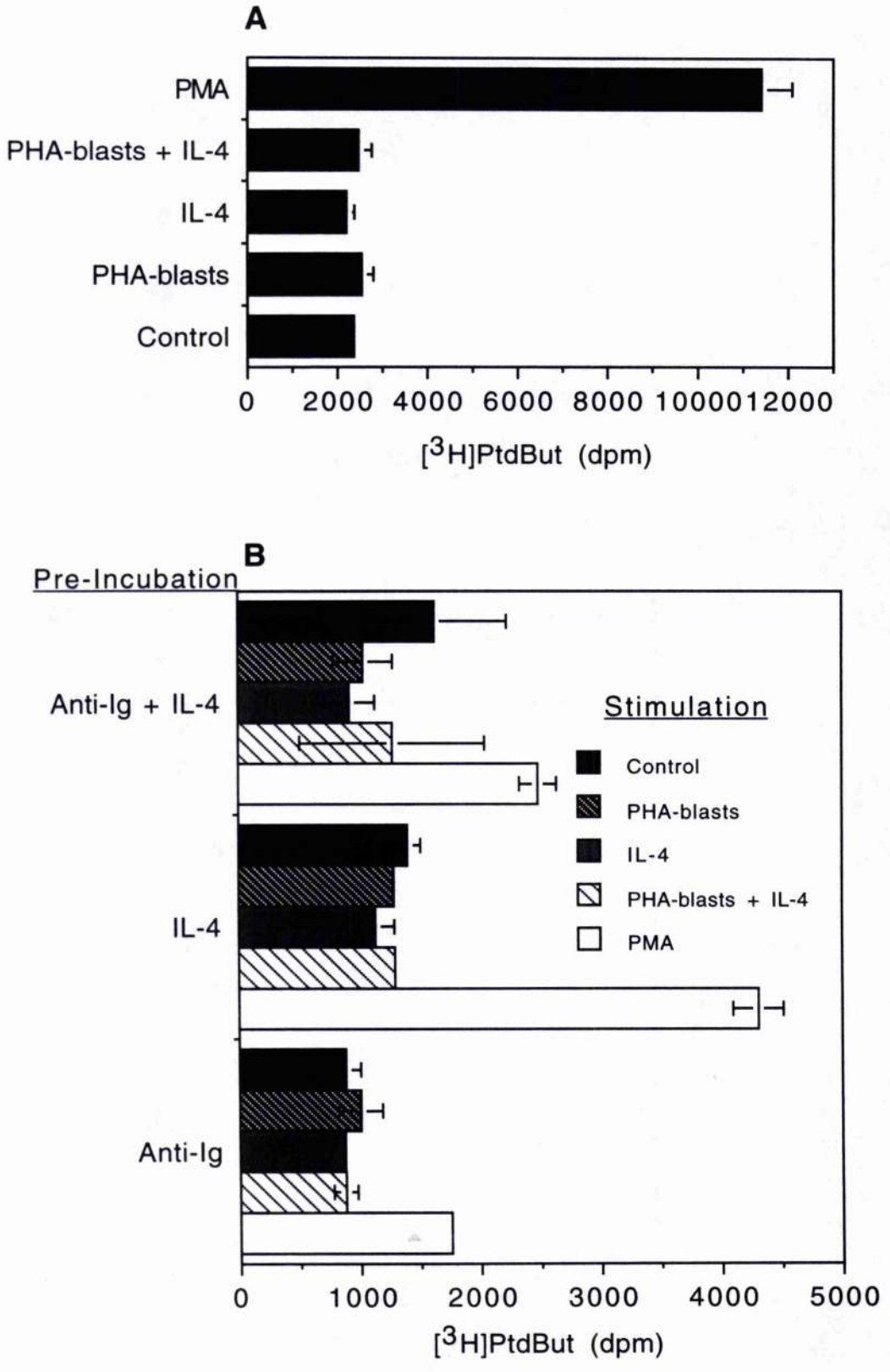

A
PMA
PHA-blasts + IL-4
IL-4
PHA-blasts
Control
0
2000
4000
6000
8000
10000
12000
[3H]PtdBut (dpm)
B
Pre-Incubation
Anti-Ig + IL-4
IL-4
Anti-Ig
Stimulation
Control
PHA-blasts
IL-4
PHA-blasts + IL-4
PMA
0
1000
2000
3000
4000
5000
[3H]PtdBut (dpm)

Figure 27. Contact with activated T cells does not activate PtdCho-PLD in B cells. In *panel A:* resting murine B cells were prelabelled with [^{3}H]palmitate for 4 h; in *panel B:* resting murine B cells were pre-labelled with [^{3}H]palmitate and concurrently incubated with anti-Ig (50 μg/ml), IL-4 (100 U/ml), or anti-Ig (5 μg/ml) plus IL-4 (100 U/ml), overnight. B cells were then stimulated with PHA-blasts (10^7 cells/ml) in the presence or absence of IL-4 (100 U/ml), or PMA (100 ng/ml), for 30 min. [^{3}H]PtdBut was measured as described in the methods section. The results are expressed as means ± SD, n=3.

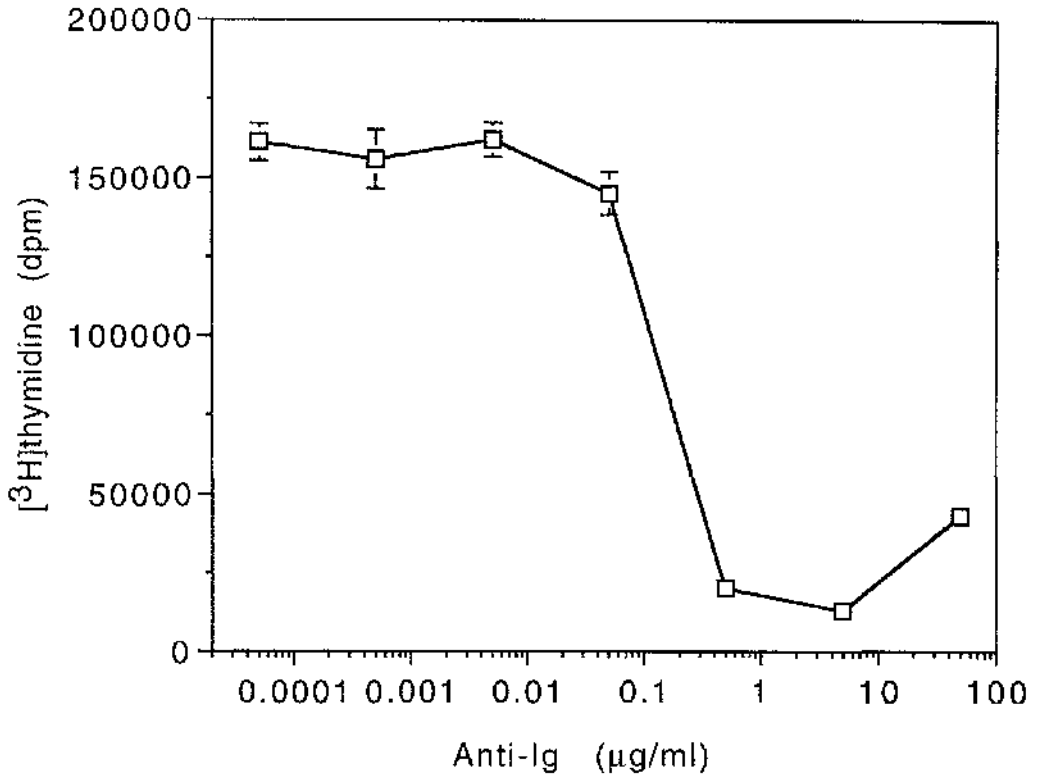

200000
150000
100000
50000
0
[3H]thymidine (dpm)
0.0001
0.001
0.01
0.1
1
10
100
Anti-Ig (µg/ml)

Figure 28. Crosslinking of sIg on WEHI 231 immature B cells induces growth arrest. WEHI 231 cells (2.5×10^5/ml) were cultured in the presence of anti-Ig (50 pg/ml - 50 μg/ml). [^{3}H]thymidine uptake was determined after 72 h as described in the methods section. Data are presented as means ± SD, n=3.

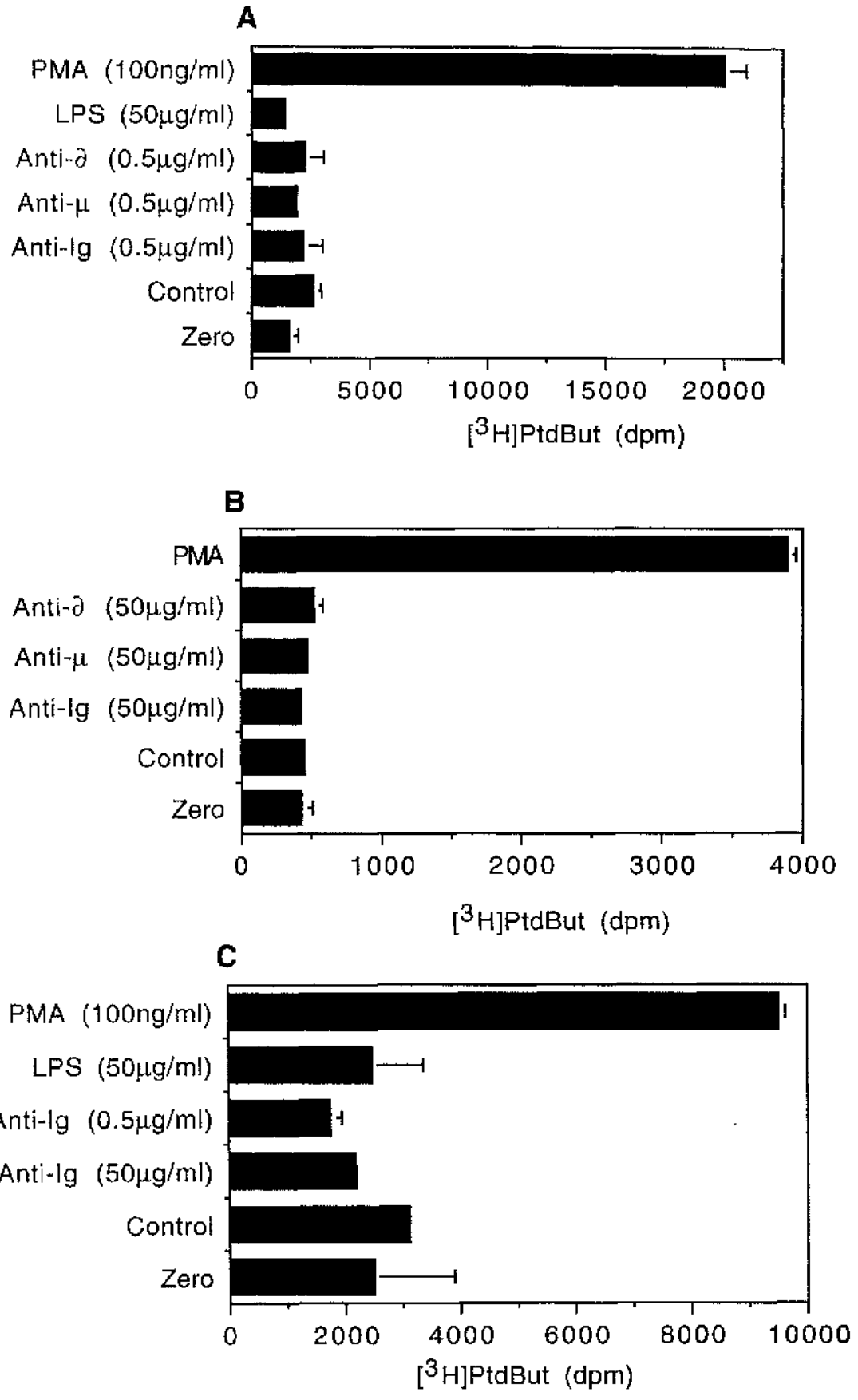
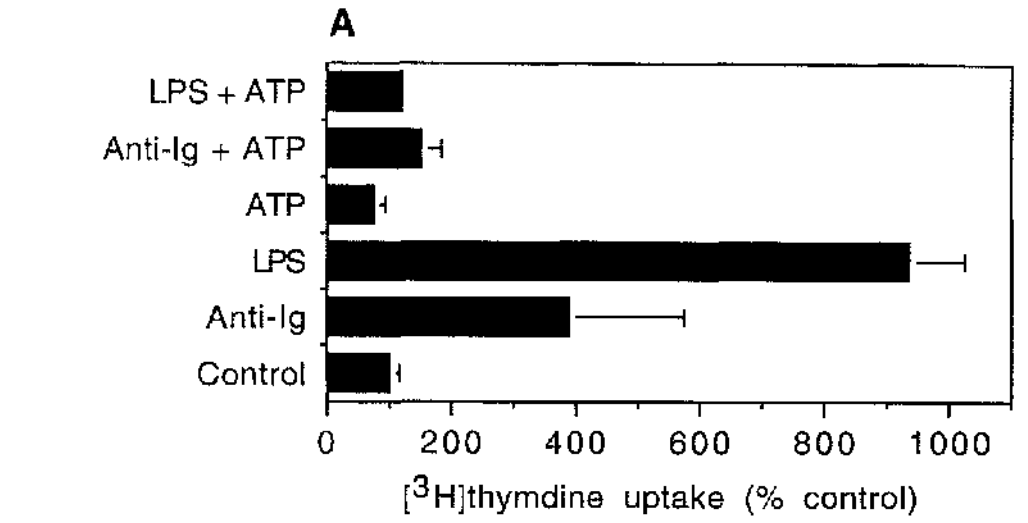
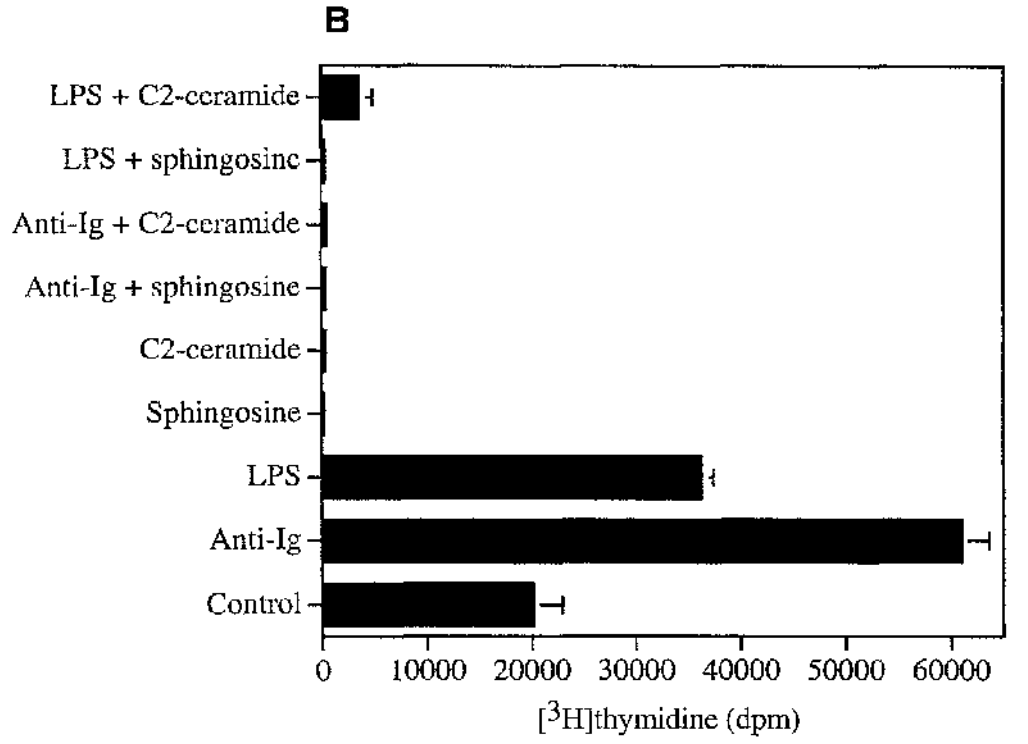

A
PMA (100ng/ml)
LPS (50μg/ml)
Anti-∂ (0.5μg/ml)
Anti-μ (0.5μg/ml)
Anti-Ig (0.5μg/ml)
Control
Zero
0
5000
10000
15000
20000
[3H]PtdBut (dpm)
B
PMA
Anti-∂ (50μg/ml)
Anti-μ (50μg/ml)
Anti-Ig (50μg/ml)
Control
Zero
0
1000
2000
3000
4000
[3H]PtdBut (dpm)
C
PMA (100ng/ml)
LPS (50μg/ml)
Anti-Ig (0.5μg/ml)
Anti-Ig (50μg/ml)
Control
Zero
0
2000
4000
6000
8000
10000
[3H]PtdBut (dpm)

Figure 29. The antigen receptors on immature B cells and the WEHI 231 immature B cell lymphoma cell line are not coupled to PtdCho-PLD. In *panels A and B:* murine splenic B cells from 4 week old mice were pre-labelled with [^{3}H]palmitate for 4 h and stimulated *(in panel A)* with anti-Ig (0.5 μg/ml), anti-IgM (0.5 μg/ml), anti-IgD (0.5 μg/ml), LPS (50 μg/ml) or PMA (100 ng/ml); or *(in panel B)* with anti-Ig (50 μg/ml), anti-IgM (50 μg/ml), anti-IgD (50 μg/ml), or PMA (100 ng/ml) for 30 min. In *panel C:* WEHI 231 cells were pre-labelled with [^{3}H]palmitate for 16 h and then stimulated with anti-Ig (0.5 or 50 μg/ml), LPS (50 μg/ml), or PMA (100 ng/ml) for 30 min. [^{3}H]PtdBut was measured as described under "Materials and Methods". The results are expressed as means ± SEM.


A
LPS + ATP
Anti-Ig + ATP
ATP
LPS
Anti-Ig
Control
0
200
400
600
800
1000
[3H]thymdine uptake (% control)


B
LPS + C2-ceramide
LPS + sphingosine
Anti-Ig + C2-ceramide
Anti-Ig + sphingosine
C2-ceramide
Sphingosine
LPS
Anti-Ig
Control
0
10000
20000
30000
40000
50000
60000
[3H]thymidine (dpm)

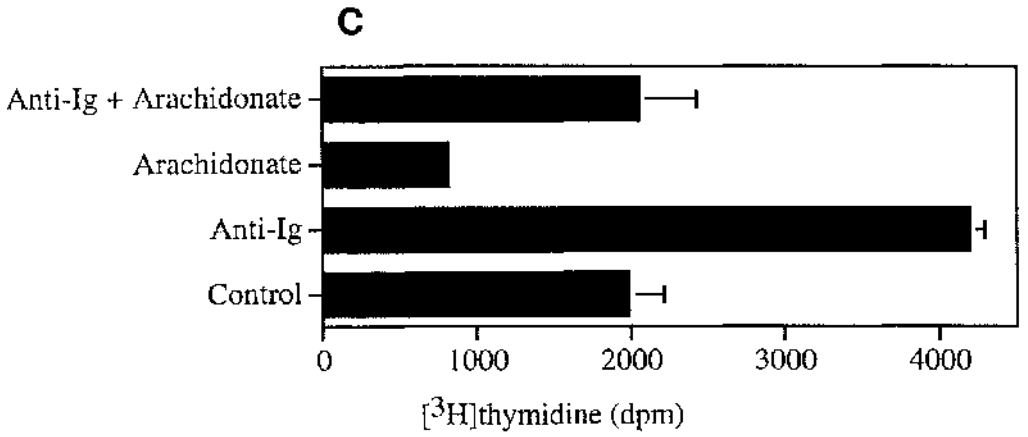
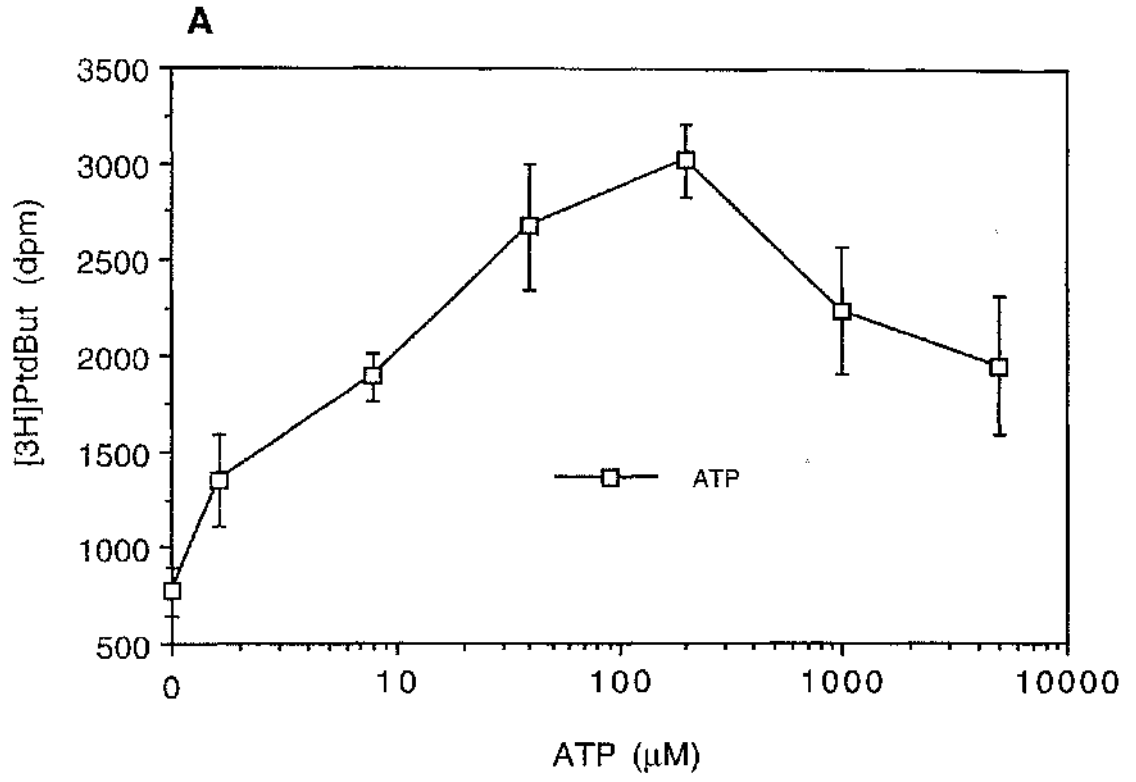

C
Anti-Ig + Arachidonate
Arachidonate
Anti-Ig
Control
0
1000
2000
3000
4000
[3H]thymidine (dpm)

Figure 30. ATP, sphingosine, ceramide and arachidonate have inhibitory effects on sIg-mediated DNA synthesis in B cells. Murine B cells (5 x 10^6/ml) were cultured in the presence of: *panel A:* anti-Ig (50 μg/ml), LPS (50 μg/ml), ATP (0.2 mM), anti-Ig (50 μg/ml) plus ATP (0.2 mM), or LPS (50 μg/ml) plus ATP (0.2 mM); *panel B:* anti-Ig (50 μg/ml), LPS (50 μg/ml), sphingosine (20 μM) ± anti-Ig (50 μg/ml) or LPS (50 μg/ml), or ceramide (20 μM) ± anti-Ig (50 μg/ml) or LPS (50 μg/ml); *panel C:* murine B cells (10^6/ml) were cultured in the presence of arachidonate (20 μM), anti-Ig (50 μg/ml), or arachidonate (20 μM) plus anti-Ig (50 μg/ml). [^{3}H]thymidine uptake was determined after 72 h as described in the methods section. Data are presented as means ± SD, n=3.


A
3500
3000
2500
2000
1500
1000
500
[3H]PtdBut (dpm)
ATP
0
10
100
1000
10000
ATP (μM)

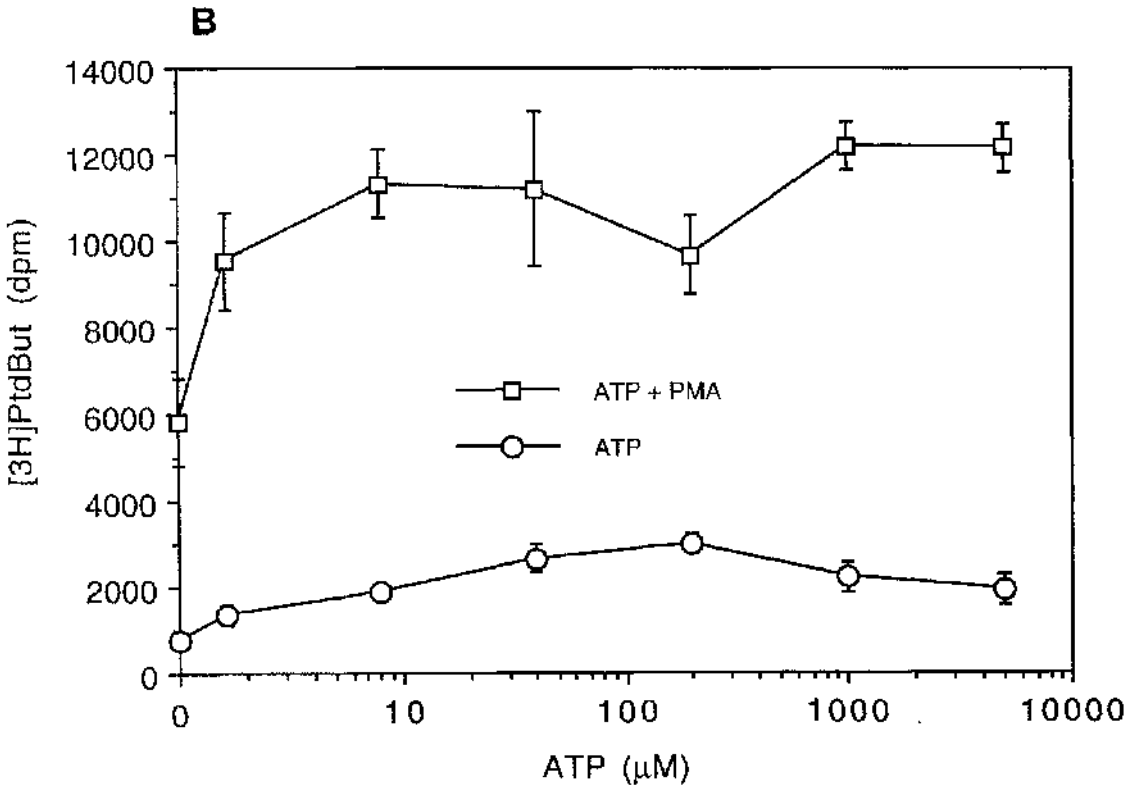

B
14000
12000
10000
8000
6000
4000
2000
0
[3H]PtdBut (dpm)
ATP + PMA
ATP
0
10
100
1000
10000
ATP (μM)

Figure 31. ATP induces PtdCho-PLD activation in murine B cells. *In panel A:* murine B cells were prelabelled with [^{3}H]palmitate and stimulated with ATP (2 μM-5 μM) +/- PMA (100 ng/ml) for 30 min. *In panel B:* stimulation of B cells with ATP (2 μM-5 μM) is represented on a more appropriate scale. [^{3}H]PtdBut was measured as described in "Materials and Methods". The data are expressed as means ± SD, n=3.

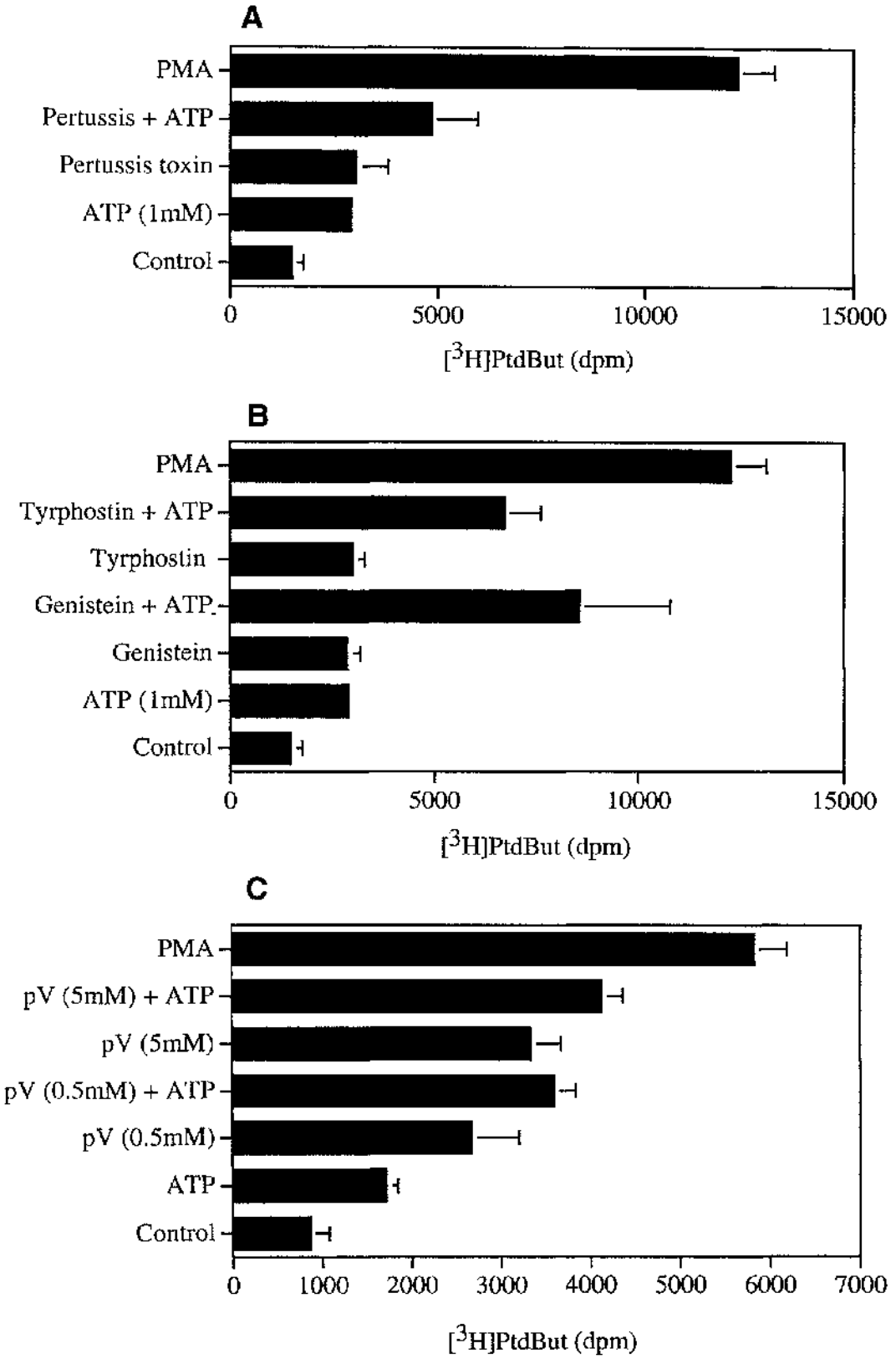

A
PMA
Pertussis + ATP
Pertussis toxin
ATP (1mM)
Control
0
5000
10000
15000
[3H]PtdBut (dpm)
B
PMA
Tyrphostin + ATP
Tyrphostin
Genistein + ATP
Genistein
ATP (1mM)
Control
0
5000
10000
15000
[3H]PtdBut (dpm)
C
PMA
pV (5mM) + ATP
pV (5mM)
pV (0.5mM) + ATP
pV (0.5mM)
ATP
Control
0
1000
2000
3000
4000
5000
6000
7000
[3H]PtdBut (dpm)

Figure 32. Analysis of coupling of P2-purinoceptors to PtdCho-PLD in murine B cells. In *panel A:* B cells were prelabelled with [^{3}H]palmitate in the presence or absence of pertussis toxin (1 μg/ml) for 4 h and stimulated with ATP (1 μM) for 30 min; control cells were also treated with PMA (100 ng/ml). In *panel B:* B cells were pretreated with medium, genistein (0.5 μM) or tyrphostin (0.1 μM) for the final hour of [^{3}H]palmitate-labelling (4 h), and stimulated with ATP (1 μM) for 30 min; control cells were also treated with PMA (100 ng/ml). In *panel C:* B cells were pre-labelled with [^{3}H]palmitate for 4 h and stimulated with pV (0.5 or 5 μM) ± ATP (1 μM) for 30 min. [^{3}H]PtdBut was measured as described in "Materials and Methods". The results are expressed as means ± SD, n=3.

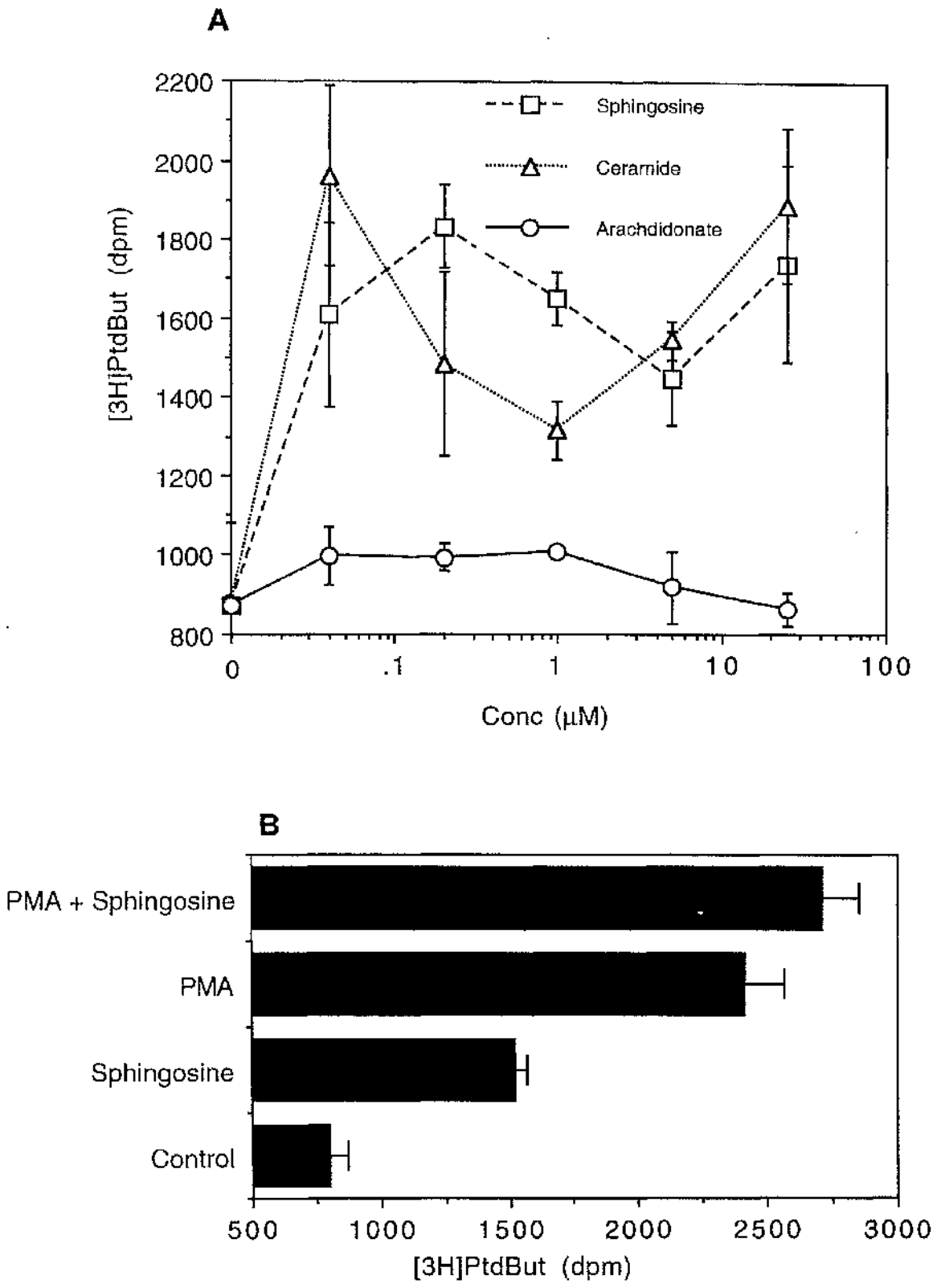

A
2200
2000
1800
1600
1400
1200
1000
800
[3H]PtdBut (dpm)
Sphingosine
Ceramide
Arachdidonate
0
.1
1
10
100
Conc (μM)
B
PMA + Sphingosine
PMA
Sphingosine
Control
500
1000
1500
2000
2500
3000
[3H]PtdBut (dpm)

Figure 33. Sphingosine and Ceramide induce activation of PtdCho-PLD in murine B cells. Resting murine B cells were pre-labelled with [^{3}H]palmitate and stimulated with: *panel A:* sphingosine (40 nM-25 μM), C2-ceramide (40 nM-25 μM) or arachidonate (40 nM-25 μM) for 30 min; *panel B:* sphingosine (25 μM), PMA (100 ng/ml) or sphingosine (25 μM) plus PMA (100 ng/ml) for 30 min. [^{3}H]PtdBut was measured as described in "Materials and Methods". The results are expressed as means ± SD, n=3.

4 Investigation of the role of cytosolic phospholipase A_2 in lymphoycte signalling.

Phosphatidylcholine (PtdCho) is hydrolysed by PLA_2 in response to a wide range of growth factors and mitogens (143, 232, 233). Agonist-induced PtdCho hydrolysis by phospholipase A_2 generates lyso-PtdCho (LPC) and a fatty acid (usually arachidonate). Although little is known about the functions of LPC, it may augment the activation of PKC by DAG, whereas arachidonate has a well-established role as a precursor for the intercellular inflammatory mediators, eicosanoids. In addition, arachidonate (as well as other fatty acids) has been increasingly recognised as a potential lipid second messenger. Indeed, arachidonate has been shown to be a co-mitogen for Swiss 3T3 cells (143) and has been implicated in the regulation of a number of signal transducing elements, including PLC-γ, sphingomyelinase, p21ras GAP and certain PKC isoforms such as PKC-α, -β and -γ (143, 232, 234). These findings suggested that the generation of arachidonate by phospholipase A_2 could play a role in antigen receptor-mediated signals in lymphocyte proliferation and differentiation.

4.1 The properties of PLA_2 enzymes.

Phospholipase A_2-mediated hydrolysis of PtdCho and release of arachidonate appears to be important both for the transduction of intracellular signals and the production of intercellular inflammatory mediators (127, 128). In addition, PLA_2 enzymes may also play a role in phospholipid digestion and metabolism (235). Two main classes of PLA_2 have been identified. These have no apparent sequence homology and differ significantly in other properties:

i) a family of "secretory" PLA_2s ($sPLA_2$s) of Mr approximately 14 kDa (235). These enzymes were originally identified as extracellular enzymes, but have increasingly been found in non-secretory locations, such as rat liver

mitochondria. $sPLA_2$s require millimolar concentrations of Ca^{2+} for full activity, a finding consistent with the proposal that $sPLA_2$ enzymes function mainly in the extracellular space.

ii) cytoplasmic PLA_2s ($cPLA_2$s) are predicted to have a molecular weight of 85 kDa (233, 235-237). These enzymes, however, migrate with an apparent Mr of 100-110 kDa on SDS-PAGE: this anomalous migration has been proposed to be due to proline-rich regions in the protein. $cPLA_2$s are found to be active at micromolar Ca^{2+} concentrations and are resistant to disulphide reducing agents, such as dithiothreitol, properties consistent with activity in the low-[Ca^{2+}], reducing environment of the cytosol (128, 235).

Another key difference between the two classes of PLA_2 activity is that $sPLA_2$s are not selective for any particular fatty acid species at the sn-2 position of the phospholipid substrate, whereas $cPLA_2$ enzymes exhibit a high degree of selectivity for specific acyl chains at the sn-2 position (235). Until recently $cPLA_2$ has always been considered to be specific for arachidonate: however, a recent report has suggested there may be $cPLA_2$ activities selective for other fatty acids, as an oleate-selective $cPLA_2$ which is regulated by GTP and protein tyrosine phosphorylation has been identifiedin HL60 and U937 myeloid cells (233).

4.2 Roles for $cPLA_2$ and $sPLA_2$ in intracellular signalling and eicosanoid production.

Although both classes of PLA_2 may be involved in the production of arachidonate for eicosanoid generation, it is generally accepted that it is the cytosolic PLA_2s which transduce receptor-coupled arachidonate generation leading to activation of signalling cascades. For example, overexpression of the two forms of PLA_2 in chinese hamster ovary (CHO) cells, has demonstrated that $cPLA_2$, but not $sPLA_2$, mediates the second messenger-like release of arachidonate in response to agonists such as ATP, thrombin,

calcium ionophores and phorbol esters (128). This investigation has therefore focused on the role of $cPLA_2$ activity in lymphocyte cellular signalling.

4.2.1 Arachidonate as a signalling precursor.

The supply of arachidonate is thought to be the rate limiting step in the agonist-stimulated production of eicosanoids (Figure 34) (238, 239), and there is evidence to suggest that either of the cytoplasmic and secretory forms of PLA_2 may be responsible for this in different receptor systems. For example, the stimulation of arachidonate release and thromboxane B_2 production in platelets by thrombin has been found to be inhibited by the arachidonate analogue, $AACOCF_3$, which is a tight-binding and selective inhibitor of $cPLA_2$, but not $sPLA_2$ (240, 241). Furthermore, platelets which had been induced to secrete 60% of their total $sPLA_2$ were found to produce normal amounts of thromboxane B_2 when stimulated with thrombin. These results therefore suggest that $cPLA_2$ is likely to be responsible for the thrombin-stimulated release of arachidonate required as a precursor substrate for the production of thromboxane B_2 by platelets. In contrast, $sPLA_2$ is stongly implicated in the generation of arachidonate for the agonist-induced production of prostaglandin E_2 in macrophages (233). Thus, transfection of the macrophage-like P388D1 cells with antisense cDNA to $sPLA_2$ was found to markedly decrease PLA_2 activity in cell homogenates and almost abolished the stimulation of arachidonate release and prostaglandin E_2 production by platelet activating factor (PAF) (242). Thus, both cytoplasmic and secretory forms of PLA_2 appear to be involved in the release of arachidonate for the agonist-stimulated generation of eicosanoids.

4.2.2 Functions of lysophosphatidic acid.

Lysophosphatidic acid (LPA) is formed from PtdOH by the action of an as yet undefined PLA_2, and is a putative lipid second messenger which can exert pleiotropic effects on cells and tissues when added exogenously (147, 148, 243), including smooth muscle contraction, the stimulation of DNA synthesis in Rat-1 fibroblasts, formation of actin stress fibres in Swiss 3T3 cells, and changes in cellular morphology. LPA is

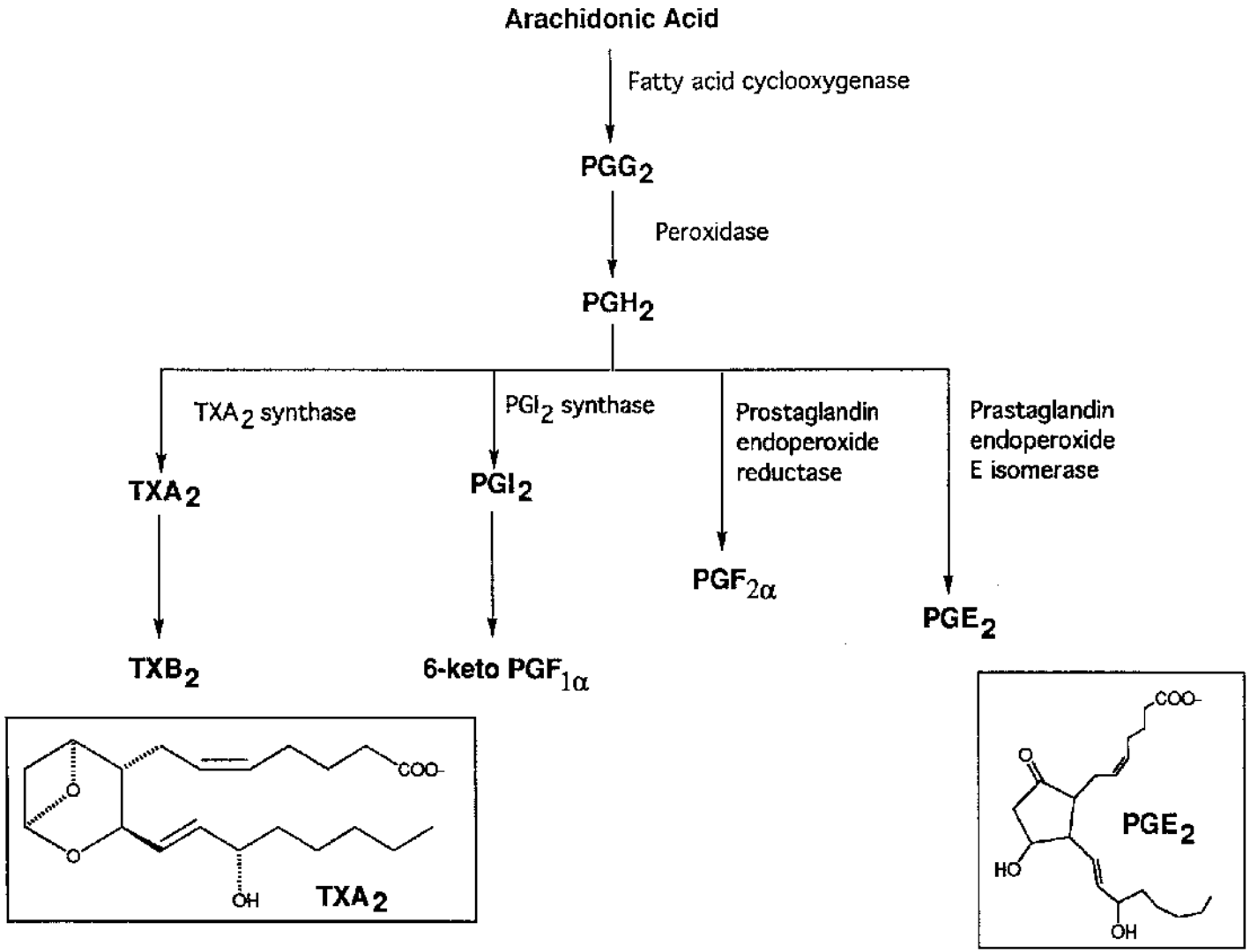

Arachidonic Acid
Fatty acid cyclooxygenase
PGG2
Peroxidase
PGH2
TXA2 synthase
PGI2 synthase
Prostaglandin endoperoxide reductase
Prastaglandin endoperoxide E isomerase
TXA2
PGI2
PGF2α
PGE2
TXB2
6-keto PGF1α
COO-
O
O
OH
TXA2
COO-
O
PGE2
HO
OH

Figure 34. The production of eicosanoids from arachidonic acid. The enzymatic pathways for production of various eicosanoids are depicted. The structures of two eicosanoids, thromboxane A_2 and prostaglandin E_2, are also shown.
PG, prostaglandin; TX, thromboxane.

believed to mediate its effects on cells through G protein-coupled receptors (GRE) (244), and its binding to receptors has been shown to initiate a number of intracellular signalling events, including $PtdInsP_2$ hydrolysis, the mobilisation of intracellular Ca^{2+} and the activation of Ras-like small G proteins (147, 148).

It would appear that the principal source of LPA is likely to be via the action of secretory PLA_2 rather than $cPLA_2$. Studies on a nonpancreatic $sPLA_2$ have indicated that this enzyme is responsible for the hydrolysis of phospholipids and the production of LPA in membrane microvesicles, platelets and whole cells challenged with inflammatory stimuli such as α-Toxin or LPS (245).

4.3 Regulation of $cPLA_2$ activity.

4.3.1 Receptor-mediated activation of $cPLA_2$.

Various studies have demonstrated the activation of cytosolic PLA_2 via both G protein-coupled receptors (GREs) and receptor tyrosine kinases (RTKs) (128). Further investigation, involving the use of pharmacological activators and inhibitors, reconstitution studies and the cloning of a gene encoding $cPLA_2$ have aided in the delineation of the pathways by which these receptors couple to $cPLA_2$, and considerable progress has recently been made in determining the mechanisms by which $cPLA_2$ activity is regulated.

A number of studies have reported the regulation of $cPLA_2$ activity by receptor protein tyrosine kinases (RTKs) (246-249). For example, epidermal growth factor (EGF), which mediates its effects on cells through a RTK, has been found to induce $cPLA_2$ activation in glomerular mesangial cells (246, 247). When $cPLA_2$ was purified from EGF-stimulated glomerular mesangial cells, its activation was found to be stable to solubilisation with detergent, indicating that the EGF-induced activation of $cPLA_2$ may be mediated by a mechanism involving direct modification of the enzyme, such as phosphorylation. EGF stimulation of $cPLA_2$ was also insensitive to PKC down-regulation, suggesting that such phosphorylation is likely to involve a PKC-independent

mechanism. Moreover, activation of $cPLA_2$ did not occur upon EGF stimulation of cells transfected with a kinase inactive mutant of the EGF receptor (EGF-R), suggesting the involvement of this activity in EGF-mediated $cPLA_2$ activation. Furthermore, TGF-α (250) which can bind to the EGF-R and activate its tyrosine kinase activity, was found to activate $cPLA_2$ in a mouse keratinocyte line. This activation was shown to be blocked by antibodies to the EGF-R, or by tyrphostin, a PTK inhibitor. These findings indicate a role for tyrosine kinase activities, and in particular the intrinsic activity of RTKs, in the growth factor-mediated activation of $cPLA_2$. It has been proposed that RTKs may activate $cPLA_2$ via MAP-kinase, which is activated by a kinase cascade initiated by agonist binding to many RTKs.

There is also evidence for G protein-dependent coupling of several receptors to $cPLA_2$ activation in several systems (128). Firstly, the non-hyrolysable guanine nucleotide analogue, GTPγS was found to stimulate basal and agonist-induced $cPLA_2$ activation in a number of permeabilised cells including neutrophils, platelets, and Swiss 3T3 fibroblasts (143). This effect was also inhibited by the non-hydrolysable GDP analogue, GDPβS, which locks G proteins in their inactive conformation. Secondly, ADP-ribosylation of specific G proteins, by pertussis toxin (G_i-like proteins) or cholera toxin (G-like proteins), has been shown to block agonist-mediated $cPLA_2$ activation in different receptor systems: pertussis toxin blocked stimulation of $cPLA_2$ activity in human neutrophils via fMetLeuPhe or ATP (251, 252); and cholera toxin was found to block the activation of $cPLA_2$ which is observed upon stimulation of outer rod segments with light or GTPγS (128). Finally, expression studies have supported the proposed role for G proteins in the regulation of agonist-mediated $cPLA_2$ in certain receptor systems (253-256). In one study, expression of an α_{s-i} chimera with the C-terminal 38 residues of α_s replaced by those of α_{i2}, which acts as a dominant negative form of α_{i2} in CHO cells, was found to inhibit α-thrombin and ATP stimulation of $cPLA_2$ activity (253-255). Another study involved the transfection of NIH 3T3 fibroblasts with wild type or mutant G_{i2} (with reduced GTPase activity, to prolong activation) (256). Enhanced activation of

$cPLA_2$ was observed in cells transfected with the mutant, as opposed to the wild type G protein, when the cells were stimulated with serum. These studies imply the involvement of α_{i2} in the regulation of $cPLA_2$ by these agonists.

The above reports clearly establish the coupling of receptors to $cPLA_2$ via G proteins: but, they do not determine whether this coupling is direct or mediated by other signalling elements. There are several additional lines of evidence indicating that the activation of $cPLA_2$ can be mediated through the direct coupling of G proteins to $cPLA_2$, rather than indirectly via other signalling elements.

i) Bombesin stimulation of Swiss 3T3 fibroblasts has been found to induce the activation of $cPLA_2$ in a biphasic manner (143). The first phase of this activation is very rapid: arachidonate release was detected following stimulation of the cells with bombesin for 2 seconds, reaching a peak at 10 sec and declining to basal levels within 1 min. This phase was found to be potentiated by GTPγS, which also stimulated basal arachidonate release, and to be inhibited by GDPβS. Furthermore, activation of $cPLA_2$ by bombesin in this phase was insensitive to a) thapsigargin (which induces calcium release), b) PKC downregulation by phorbol ester pretreatment, c) staurosporine, a selective PKC inhibitor in these cells, and d) the chelation of extracellular Ca^{2+}. These results suggest that $cPLA_2$ activation is not downstream of $PtdInsP_2$-PLC-mediated release of Ca^{2+} and PKC activation. Moreover, the rapidity of activation tends to indicate direct coupling of G proteins to $cPLA_2$. In contrast, the second phase of $cPLA_2$ activation, in which arachidonate release was detected within 1-2 min of stimulation and sustained for up to 60 min, was blocked by the chelation of extracellular Ca^{2+} and was shown to involve phosphorylation of $cPLA_2$. These findings therefore suggest that $cPLA_2$ can be activated by at least two distinct intracellular signalling pathways.

ii) The stimulation of $cPLA_2$ activity in outer rod segments by light or GTPγS was found to require the G protein, transducin (128). Importantly, the addition of transducin βγ subunits was found to activate $cPLA_2$ *in vitro*, indicating that these may be the primary effector molecules in the G protein-mediated activation of this enzyme. This possibility was supported by further *in vitro* studies which showed that, although transducin α subunits could induce slight stimulation of $cPLA_2$ activity by themselves, they inhibited βγ-induced $cPLA_2$ activation.

iii) Antibodies to G protein βγ subunits were found to differentially inhibit histamine and thrombin-stimulated $cPLA_2$ activation in permeabilised platelets: histamine stimulation of arachidonate release was inhibited, whereas thrombin-induced $cPLA_2$ activation was unaffected (257). Antibodies to G_i or G_0 were observed to inhibit both responses (258).

The studies described above support the G protein-dependent regulation of $cPLA_2$ reported in various receptor systems. They also suggest that G protein βγ subunits may be responsible for mediating this activation, at least in some systems. Furthermore, these findings have clearly suggested a direct role for G proteins in receptor coupling to $cPLA_2$ activation in some systems.

4.3.2 The regulation of $cPLA_2$ activity by protein kinases and calcium.

The precise details of receptor coupling to $cPLA_2$ activity independently of direct G protein coupling are not known, however, a number of studies have demonstrated roles for calcium mobilisation and protein kinase-mediated phosphorylation of $cPLA_2$ in the agonist-induced activation of $cPLA_2$ (128, 259, 260).

Cytosolic PLA_2 is phosphorylated by a number of protein kinases which are involved in the regulation of cell proliferation including PKC (261). Stimulation of cells with PMA has been shown to induce the phosphorylation and activation of $cPLA_2$ *in*

vivo, and *in vitro* studies have demonstrated the direct phosphorylation of $cPLA_2$ by PKC (262). Moreover, another investigation found that the down regulation of PKC-α, but not PKC-β (263), in MDCK cells led to the suppression of PMA-induced arachidonate release, indicating a specific role for this PKC isozyme in the regulation of $cPLA_2$. These findings therefore suggested that the PMA-induced activation of $cPLA_2$ might be mediated by direct PKC-mediated phosphorylation of $cPLA_2$.

Another protein kinase, which has also been shown to be involved in the regulation of $cPLA_2$ activity is mitogen activated protein (MAP)-kinase (260, 264). More specifically, this enzyme is the $p42^{erk\ 2}$ isoform of the MAP-kinase family, which includes three other members: $p40^{hera}$, $p44^{erk\ 1}$, and $p46^{erk\ 4}$; ($p42^{erk\ 2}$ will be referred to as MAP-kinase from this point). MAP-kinase is activated, via phosphorylation of both tyrosyl- and threonyl-regulatory sites in the enzyme, by a signalling cascade initiated by the stimulation of cells with mitogenic stimuli such as PDGF, EGF, and thrombin, agonists which are also found to induce activation of $cPLA_2$. Furthermore, MAP-kinase has been observed to phosphorylate and activate $cPLA_2$ *in vitro*, indicating that the MAP-kinase-mediated direct phosphorylation of this enzyme may be involved in agonist-induced stimulation of $cPLA_2$ activity and arachidonate release.

The cloning of a gene for $cPLA_2$, (236, 237) and structure-function studies on the enzyme, have rationalised the pharmacological evidence concerning the protein kinase-mediated phosphorylation and resultant activation of $cPLA_2$. MAP-kinase was observed to phosphorylate $cPLA_2$ at a specific serine residue (Ser-505) *in vitro* and *in vivo*, inducing activation of the enzyme and the resultant production of arachidonate. Mutation of this residue to alanine was found to abolish MAP-kinase-mediated phosphorylation of $cPLA_2$ and to greatly decrease the activation of $cPLA_2$ observed upon stimulation of transfected cells with mitogenic agonists such as thrombin (260). In contrast, PKC was observed to phosphorylate $cPLA_2$ *in vitro* on sites which are distinct from the MAP-kinase phosphorylation site. Treatment of cells with PMA, however, was found to induce phosphorylation of $cPLA_2$ at Ser-505, the MAP-kinase phosphorylation site. This finding suggests that, *in vivo*, protein kinase C mediates the phosphorylation

and activation of $cPLA_2$ indirectly, possibly via the activation of MAP-kinase. Activation of cAMP-dependent protein kinase (PKA) has also been reported to activate PLA_2 *in vivo*. However, although PKA was found to phosphorylate $cPLA_2$ *in vitro*, this did not result in any change in its activity. Any role for PKA-catalysed phosphorylation in the regulation of $cPLA_2$ therefore remains unclear.

In addition to regulation by protein kinases, $cPLA_2$ is rapidly activated by increases in the concentration of cytosolic Ca^{2+} (236, 265). This has now been found to involve the Ca^{2+}-induced translocation of $cPLA_2$ to the plasma membrane, a process which is thought to be mediated by the Ca^{2+}-dependent lipid binding (CalB) domain located at the amino terminus of $cPLA_2$. This domain has also been found in other signal transducing elements such as PKC, Ras GAP, and $PtdInsP_2$-PLC, and has been implicated in their recruitment to the plasma membrane and subsequent activation. As a result of these findings, a complex model of $cPLA_2$ activation has been proposed by Lin et al. (1993) (260), involving the synergistic actions of Ca^{2+} and MAP-kinase signals, both of which appear to be required for full activation (Figure 35).

4.4 Physiological functions of agonist-stimulated PtdCho hydrolysis by $cPLA_2$.

In contrast to other unsaturated free fatty acids (FFAs), arachidonate is present at very low basal levels in cells and is subject to very rapid turnover (133). This may be related to its function as an intracellular messenger (ie. its cellular levels may be tightly regulated in a manner analogous to other second messengers such as IP_3, DAG or cAMP), or it may reflect the fact that arachidonate can be metabolised very rapidly to generate eicosanoids (238, 239). Other unsaturated FFAs, and in particular oleate (see section 4.4.1), have also been implicated in intracellular signalling: oleate, since it is not metabolised as rapidly as arachidonate, has often been used in experiments to examine the effects of FFAs on biological responses (133).

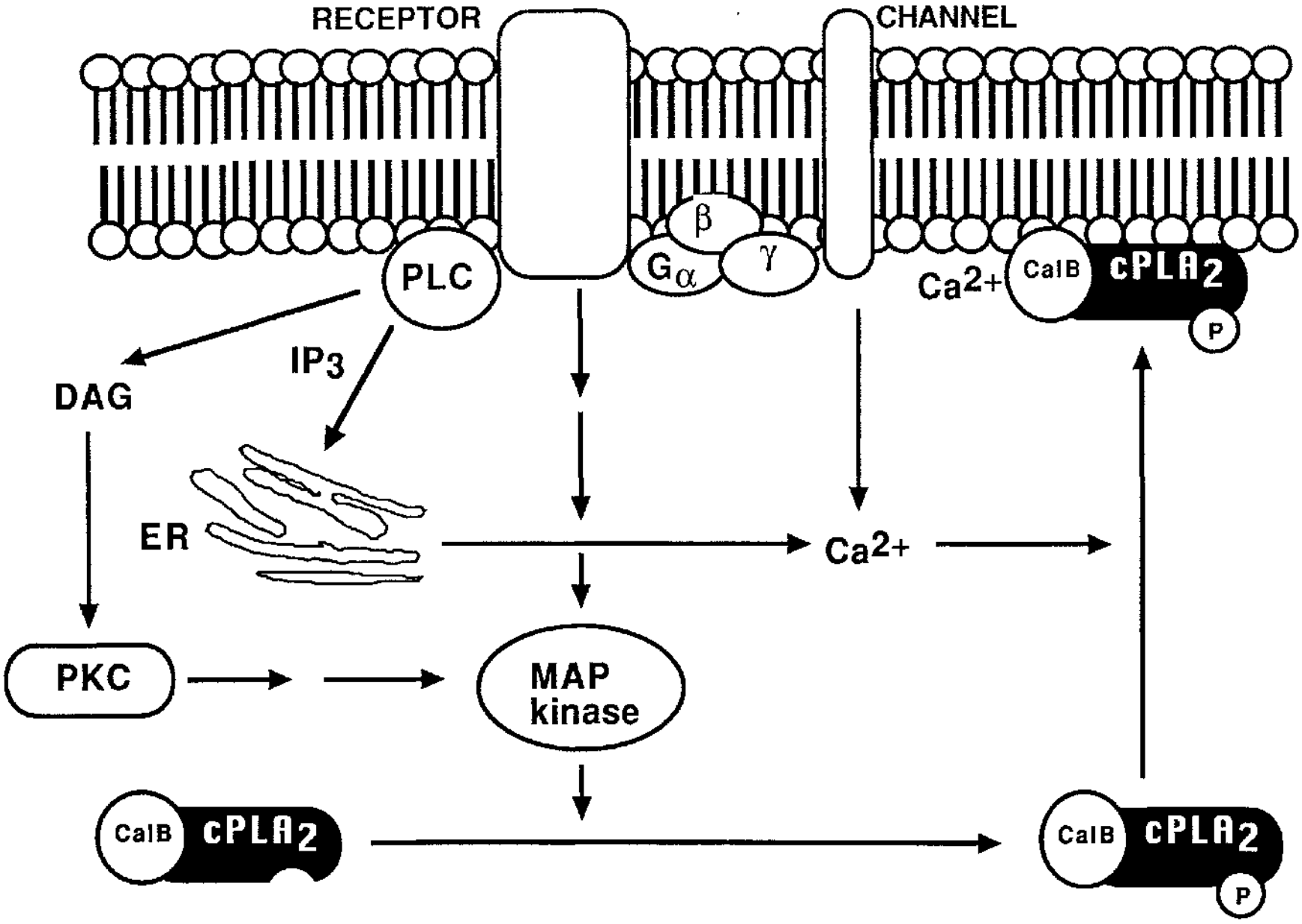

RECEPTOR
CHANNEL
β
Gα
γ
PLC
Ca2+
CalB
cPLA2
P
IP3
DAG
ER
Ca2+
PKC
MAP
kinase
CalB
cPLA2
CalB
cPLA2
P

Figure 35. Model for the activation of $cPLA_2$. In this model, proposed by Lin and coworkers (260), rapid activation of $cPLA_2$ is mediated by the phosphorylation of $cPLA_2$ by MAP-kinase and the Ca^{2+}-dependent recruitment of $cPLA_2$ to the plasma membrane. Receptor stimulation results in the activation of phospholipase C and the formation of $InsP_3$ and DAG. Mobilisation of Ca^{2+} then occurs as a result of the action of $InsP_3$ or the activation of receptor-coupled calcium channels. This induces the recruitment of $cPLA_2$ to the plasma membrane, which is mediated by the Ca^{2+}-dependent lipid-binding domain (CalB) located at the amino terminus of $cPLA_2$. MAP-kinase activation also occurs as a result of receptor stimulation, via PKC-dependent or -independent pathways. The activated MAP-kinase can then phosphorylate $cPLA_2$ at Ser-505, inducing its activation.
ER - endoplasmic reticulum.

4.4.1 Potential role of FFAs in mitogenic signalling.

FFAs have been proposed to regulate the activation of protein kinase C via a mechanism which is distinct from that requiring Ca^{2+} and DAG, or phorbol esters (133). Twelve isoforms of PKC have been identified to date and these have been classified into three main categories: Ca^{2+}-dependent PKCs (cPKCs) (α, βI, βII, γ); Ca^{2+}-independent novel PKCs (nPKCs) (δ, ε, θ, η, μ); and atypical PKCs (aPKCs) (ι, λ, ζ) (266, 267). Studies on the effect of FFAs on different PKC isoforms *in vivo* have demonstrated that nPKCs are activated by exogneously added FFAs in the range 5-10 μM, whereas cPKCs are activated by FFAs in the range 50-100 μM (268). This finding suggests that FFAs are most likely to play a physiological role in the activation and regulation of the Ca^{2+}-independent isoforms of protein kinase C (nPKCs). The regulation of the atypical PKC isoforms by FFAs has not yet been investigated. The activation of PKCs by FFAs is reported to be independent of PtdSer, calcium, and DAG, *in vitro* (133, 269, 270) Further studies have shown that oleate is unable to inhibit the binding of phorbol esters to PKC, indicating that that FFAs bind PKCs at a site which is distinct from the phorbol ester/DAG binding site. Moreover, in contrast to such classical PKC activators, FFAs preferentially activate soluble rather than membrane-bound PKC, and this activation appears to be mediated by free, and not membrane-partitioned, FFAs (270). Free fatty acids such as arachidonate may therefore regulate the activation of cytosolic, rather than membrane-bound, protein kinase C, resulting in the phosphorylation of target proteins without the requirement for their recruitment to the plasma membrane. The existence of such a mechanism, as well as the DAG/PtdSer/Ca^{2+}-dependent mechanism, would enable the differential regulation of the ser/thr phosphorylation of cytosolic and plasma membrane proteins by PKC.

Arachidonate has also been reported to activate other cellular signalling transducers which have been implicated in mitogenic signalling, such as phospholipases: arachidonate and oleate have been reported to activate a PtdInsP_2-PLC in microsomes prepared from the MCF-7 cell line (133, 239). Moreover, FFAs, particularly arachidonate, have been reported to induce activation of a novel protein which can

activate PLC-γ and PLC-δ isoforms, and which may be involved in the EGF-mediated activation of PLC-γ in hepatocytes (271). Furthermore, FFAs have also been shown to activate both PtdCho-PLD (272) and PPH *in vitro*. Other proposed roles for FFAs in the regulation of cellular activation include: i) the regulation of ion channels (133); ii) the inhibition of Ras GAP (ie. prolonging the activation of Ras) (273) and iii) the inhibition of cyclic AMP-dependent protein kinase (PKA) (133). Thus, arachidonate and other FFAs may play a role in the regulation of a wide range of intracellular signals, although the precise mechanistic details of these regulatory functions are, at present unknown.

4.4.2 Functions of lysophosphatidylcholine.

The other product of the PLA_2-catalysed hydrolysis of PtdCho is lysoPtdCho (LPC) (128). LPC is a putative lipid second messenger which has also been proposed to potentiate the activation of PKC isoforms by DAG (274-277). Thus, LPC has been reported to enhance the activation of the PKC isoforms α, β, γ, and induced by DAG, *in vitro* (132). Consistent with this finding, exogenously added LPC has been reported to enhance the activation of T cells (274), and the differentiation of HL60 cells to macrophages, resulting from the addition of permeant species of DAG (131). The mechanism (s) underlying LPC activation of PKC is, as yet, poorly defined, but LPC may also have non specific regulatory effects on proteins, due to its detergent-like properties. It is therefore unclear whether the observed effects of LPC *in vitro* indicate a physiological signalling role for this putative lipid second messenger.

4.4.3 Arachidonate as a transducer of differentiation or apoptotic signals.

A recent study by Jayadev et al. (1994) (61) has suggested a key role for arachidonate generation in the Tumor Necrosis Factor-α (TNF-α)-mediated activation of sphingomyelinase (SMase) in HL60 monocytic cells. SMase hydrolyses sphingomyelin (SM) to generate the lipid second messenger, ceramide, which has been widely proposed to play a role in the regulation of proliferation and apoptosis (61, 180-182, 222). Stimulation of HL60 cells with TNF-α (30 nM) was found to induce the hydrolysis of

approximately 30% of cellular SM within 45- 60 min. Such SM hydrolysis was preceded by the generation of arachidonate, suggesting a possible role for released arachidonate in the activation of SMase. Furthermore, exogenously added arachidonate was found to stimulate SM hydrolysis and the generation of ceramide within 20 min, ie. more rapidly than TNF-α, providing further circumstantial evidence that arachidonate-mediated activation of SMase is downstream of TNF-α receptor ligation. Other FFAs, particularly oleate, were found to have a similar effect on SMase activity when added exogenously, indicating that the effects of arachidonate on SMase activity were not due to its metabolism to eicosanoids. These findings were further supported by the finding that eicosatriynoic acid (ETI), a non-hydolysable analogue of arachidonate, also induced SM hydrolysis in these cells. Furthermore, the methyl ester and alkyl analogues of fatty acids did not activate SMase, indicating that the activation of this enzyme by fatty acids is likely to be due to a specific effect of these lipid second messengers, rather than nonspecific effects membrane-directed of fatty acids, on the plasma membrane. Furthermore, melittin, a potent PLA_2 activator, stimulated SM hydrolysis *in vivo*, but failed to induce direct activation of SMase *in vitro*, indicating that its effects are likely to be mediated indirectly, via PLA_2. Moreover, *in vitro* studies showed that a neutral cytosolic SMase activity prepared from HL60 cells could be activated by arachidonate (10-100 μM) in a dose-dependent manner, indicating that the activation of SMase is directly coupled to arachidonate generation. Finally, arachidonate was found to exert anti-proliferative effects on HL60 cells, as were ceramide and TNF-α, suggesting that the TNF-α-induced suppression of proliferation and induction of apoptosis in HL60 cells may be mediated via the activation of PLA_2 and subsequent arachidonate-induced activation of SMase.

4.5 Aims and objectives.

The reports described above indicate that $cPLA_2$ could be involved in the transduction of mitogenic, differentiation or apoptotic signals. $cPLA_2$ has been shown to be regulated by at least two distinct mechanisms, i) by direct interaction with G proteins, or ii) downstream of MAP-kinase and calcium mobilisation. Thus, since many of the

cellular signals implicated in the regulation of $cPLA_2$ are initiated by the ligation of antigen receptors on B and T cells, cytosolic PLA_2 represents a good potential candidate for the transduction of intracellular signals via the antigen receptors on lymphocytes.

The objectives of this investigation were as follows:

i) to identify and characterise $cPLA_2$ activity in B and T cells;

ii) to determine whether the antigen receptors, or other surface receptors, on B and T cells are coupled to $cPLA_2$ activation;

iii) to characterise the mechanisms of antigen receptor coupling to $cPLA_2$ activation, and the regulation of $cPLA_2$ activity;

iv) to examine the expression of $cPLA_2$ throughout lymphocyte maturation to determine whether $cPLA_2$ is expressed and activated in a maturation stage-specific manner;

iv) to investigate the role(s) of $cPLA_2$ in mitogenic, differentiation or anti-proliferative/apoptotic signalling in lymphocytes.

RESULTS

4.6 $cPLA_2$ expression and activation in mature lymphocytes.

The initial aim was to investigate the role of $cPLA_2$ in mature B and T cell activation. The work described in this section was carried out in collaboration with Stewart and Wakelam (now at Birmingham University), and with Courtney, Fleming, Reid, and Harnett (this laboratory). Thus, only the key results of the work on mature lymphocytes are presented here. The full results are currently submitted for publication.

4.6.1 $cPLA_2$ activity in mature B and T cells.

Crosslinking of the antigen receptors on murine splenic B cells (sIg), using a mitogenic concentration (50 μg/ml) of anti-Ig antibodies, did not stimulate the release of [^{3}H]arachidonate from prelabelled cells (Figure 36) over a short (15-60 sec) (data not shown) or a longer (10 min) (Figure 36) stimulation. Under comparable conditions, anti-Ig induced release of [^{3}H]InsPs from [^{3}H]inositol-labelled cells, indicating that these cells were still responsive to stimulation via sIg (results not shown). Furthermore, PMA, which can activate $cPLA_2$ via MAP-kinase in most cell types, also failed to induce the release of [^{3}H]arachidonate from these cells (Figure 36A). Similar results were obtained following crosslinking of the antigen receptors on the human B lymphoblastoid cell lines, Daudi and Ramos (results not shown), even when the incubation period was extended to 30 min. These findings indicate that the antigen receptors on mature B cells are not coupled to $cPLA_2$ activation.

In order to determine whether there was a differential coupling of the antigen receptors to PLA_2 activation in B and T cells, arachidonate release following T cell activation was investigated (Figure 36B): activation of the antigen receptors (TCR) on the human leukaemic T cell line, Jurkat, with optimally stimulatory concentrations of anti-CD3 antibodies did not induce [^{3}H]arachidonate release. Moreover, PMA also failed to stimulate arachidonate generation over a 30 min time period (results not shown). In contrast, anti-CD3 and PHA did induce the release of [^{3}H]InsPs from [^{3}H]inositol-

labelled cells (results not shown) indicating that these cells were responsive to stimuation via the TCR and associated molecules.

The recent identification of an oleate-selective PLA_2 activity in myeloid cells, which is subject to regulation by GTP and tyrosine phosphorylation (233), raised the possibility that the antigen receptors on B and T cells could be coupled to a non-arachidonate-selective $cPLA_2$ activity. However, studies in [^{3}H]oleate-labelled Daudi B cells (Figure 37A) and Jurkat T cells (Figure 37B) showed that the antigen receptors on B and T cells are also not coupled to an oleate-selective PLA_2.

4.6.2 $cPLA_2$ expression in mature lymphocytes.

These findings raised the possibility that mature B and T cells might not express $cPLA_2$. Western blot analysis of a range of lymphocyte cell lysates (100 μg/lane), prepared from murine splenic B and T cell lines (Figure 38A), human tonsillar B and T cells (Figure 38B) and human B and T cell lines (Figure 38A and B) demonstrated that $cPLA_2$ expression could not be detected in these cells. In contrast, $cPLA_2$ expression in a partially purified preparation of the enzyme (5 μg/lane) and HL60 lysates (40 μg/lane) was strongly recognised by this anti-$cPLA_2$ reagent.

Further studies investigated the possibility that certain cytokines known to upregulate $cPLA_2$ expression in fibroblasts (278) and/or to induce lymphocyte proliferation/differentiation (279), might induce expression of $cPLA_2$ in mature lymphocytes. However, incubation of Jurkat T cells or Daudi B cells with the cytokines IL-1, IL-2, IL-4, IL-10, TNF- or IL-13 for up to 3 days did not induce $cPLA_2$ expression in these cells *in vitro* (results not shown).

These results therefore indicate that $cPLA_2$ is not expressed by mature lymphocyte cells or cell lines, and suggest that the upregulation of $cPLA_2$ expression does not play a role in T or B cell activation during an immune response.

4.7 Arachidonate has an anti-proliferative effect on B cells.

Taken together, the above data strongly suggested that $cPLA_2$ activation, and resultant arachidonate generation, does not play a role in the transduction of proliferative signals in B or T cells. Indeed, addition of exogenous arachidonate (20 μM) was found to inhibit basal and anti-Ig-stimulated DNA synthesis of murine splenic B cells by 40-50% (Figure 39). As discussed above, arachidonate has been reported to play a central role in the TNF-α-mediated sphingomyelinase activity leading to induction of anti-proliferative and/or differentiation signals in monocytes (61). This finding could therefore suggest a role for the production of arachidonate in anti-proliferative and/or apoptotic signalling in lymphocytes.

As already discussed, ligation of the antigen receptors on the majority of immature B and T (thymocytes) cells, *in vivo*, induces apoptosis in order to delete autoreactive clones and prevent inappropriate activation by autoantigens. Thus, in order to investigate whether $cPLA_2$ may play a role in the processes underlying B or T cell maturation and/or apoptosis, it was decided to investigate $cPLA_2$ expression and activation at earlier stages of lymphocyte ontogeny.

4.8 $cPLA_2$ in immature lymphocytes.

The initial aims of this investigation were to i) determine whether $cPLA_2$ is expressed at the early stages of lympocyte maturation, ie. in pre-B cells or in immature B and T cells, and ii) determine whether $cPLA_2$, if expressed, can be activated by antigen receptor ligation, or by pharmacological stimuli known to activate $cPLA_2$ in other cells.

4.8.1 $cPLA_2$ is expressed by immature lymphocytes.

Cytosolic PLA_2 expression was examined in a range of human pre-B cell lines: 697 cells, 207 cells and REH cells; and in immature murine splenic lymphocytes (B and T cells) and murine thymocytes. Western blot analysis, using specific anti-$cPLA_2$ C-terminal peptide antibodies, was used to assess the relative levels of $cPLA_2$ in the appropriate cell lysates (100 μg/lane). Cytoplasmic PLA_2 was not expressed by any of

the pre-B cell lines, but was strongly expressed in both murine thymocytes and immature murine splenic lymphocytes (Figure 40). This was a particularly interesting finding becaused it demonstrated the expression of $cPLA_2$ in lymphocytes at a maturation stage in which they may undergo apoptosis in response to antigen receptor crosslinking.

4.8.2 $cPLA_2$ activity in thymocytes and immature splenic B cells.

Crosslinking of the antigen receptors on thymocytes has been found to induce apoptosis, and cytosolic PLA_2 has been proposed to play a role in the transduction of apoptotic signals in some receptor systems, eg. TNF-α stimulation of HL60 monocytic cells. Therefore, in view of the finding that thymocytes express $cPLA_2$, it was decided to determine whether the antigen receptors on thymocytes are coupled to $cPLA_2$ activation. Ligation of the antigen receptors on prelabelled thymocytes with anti-CD3 or Con A stimulated substantial generation of [^{3}H]arachidonate release within 30 min (Figure 41), strongly indicating the coupling of the antigen receptors on these cells to $cPLA_2$ activation. In an attempt to determine whether arachidonate is being released as an intracellular messenger in thymocytes, some further experiments (carried out in collaboration with A. Wise in this laboratory) involved the separation of intracellular and extracellular pools of [^{3}H]arachidonate prior to analysis. This was achieved by the pelleting of thymocytes to separate them from the extracellular medium, following termination of reactions by the addition of ice-cold PBS. Kinetic analysis showed that, after an initial lag phase of at least 1 min, ligation of the antigen receptors with Con A or anti-CD3 stimulated substantial intracellular generation of [^{3}H]arachidonate within 10 min (Figure 42A and B). In addition, the calcium ionophore, ionomycin (1 μM), which can also induce apoptosis in thymocytes, similarly stimulated intracellular [^{3}H]arachidonate release in murine thymocytes, following a lag phase of at least 1 min (Figure 42C). Moreover, ionomycin and Con A were found to stimulate rises in extracellular [^{3}H]arachidonate within 10 min (Figure 43). The kinetics of [^{3}H]arachidonate generation following ligation of the antigen receptor, and the effects of pharmacological agents on $cPLA_2$ activity, were consistent with $cPLA_2$ activation being

downstream of MAP-kinase activation rather than by direct TCR/CD3-$cPLA_2$ coupling. These data therefore stongly indicate that the antigen receptors are coupled to $cPLA_2$ activation in murine thymocytes.

Western blots had also shown the expression of $cPLA_2$ by immature murine splenic lymphocytes (B and T cells) (Figure 40), suggesting that $cPLA_2$ could be expressed by immature B lymphocytes. The possibility that the antigen receptors on immature splenic (IS) murine B cells are coupled to $cPLA_2$ activation was therefore investigated. However, stimulation of [^{3}H]arachidonate-prelabelled IS B cells with anti-Ig (0.5-50 μg/ml) for 30 min did not stimulate the release of [^{3}H]arachidonate (Figure 44). Moreover, stimulation of IS B cells with PMA, or LPS which has been implicated in priming and/or activation of $cPLA_2$ in other cells, did not induce the release of [^{3}H]arachidonate at 5, 15 or 30 min (Figure 44, and results not shown). However, the difference between the zero time and unstimulated levels of [^{3}H]arachidonate generation observed at 30 min (Figure 44) did indicate a high basal level of arachidonate release by IS B cells, suggesting that $cPLA_2$ is constitutively and fully activated in these IS B cells.

Interpretation of these results has been complicated by the fact that IS B cells are a heterogenous population comprising a number of maturation states. Thus, antigen receptor crosslinking may result in proliferation or apoptosis, depending on the precise maturation state of any given cell. Indeed, it has been demonstrated that the crosslinking of sIg on B cells from 4 week old mice stimulates significant DNA synthesis, indicating that at this age a large proportion of B lymphocytes are mature and can be activated by antigen receptor crosslinking (280). Moreover, approximately 84% of B cells from the spleens of 4 week old mice were found to be $sIgM^+sIgD^+$, indicating that they had a mature phenotype (280). It was therefore decided to investigate $cPLA_2$ expression and antigen receptor coupling to $cPLA_2$ in homogenous immature T and B cell populations by using immature thymoma and B lymphoma cell lines.

4.9 $cPLA_2$ expression and activation in T3 thymoma cells.

T3 thymoma cells, which are derived from a double-positive (CD4+CD8+) thymocyte, have been demonstrated to undergo growth arrest upon crosslinking of the antigen receptors with Con A (50 μg/ml) or PHA (50 μg/ml) (Figure 45A), or following incubation with ionomycin (0.1 μg/ml) (Figure 45B). These agents have also been shown to induce apoptosis in these cells (R. Zamoyska, NIMR, London; personal communication). However, growth arrest was not observed in response to crosslinking of the antigen receptors with soluble anti-CD3 antibodies (.001-1 μg/ml) or stimulation with PMA (Figure 45B). (Results from Figure 45: personal communication A. Michie). The failure of anti-CD3 to induce growth arrest in T3 cells, however, was probably due to insufficient crosslinking of the antigen receptors (R. Zamoyska, personal communication). These findings confirmed that the antigen receptors on these proliferating thymoma cells were coupled to intact signalling pathways, and that T3 thymoma cells would be suitable for investigating the transduction of apoptotic signals via the TCR in immature T cells.

Western blotting of anti-$cPLA_2$ immunoprecipitates from T3 cell lysates demonstrated that these cells express cytoplasmic PLA_2 (Figure 46), and it was decided to investigate the coupling of the antigen receptors on T3 cells to $cPLA_2$ activation. Stimulation of prelabelled T3 cells with Con A or ionomycin induced a rapid (within 1 min) release of [^{3}H]arachidonate which was sustained for at least 30 min (Figure 47). In contrast, PMA stimulation failed to induce the production of [^{3}H]arachidonate within 30 min (Figure 47). These initial results suggested that the activation of $cPLA_2$ might be associated with stimulation of T3 cells by agents which induce apoptosis. Indeed, further studies examining intracellular and extracellular release of [^{3}H]arachidonate, demonstrated that anti-CD3 antibodies, which did not induce growth arrest in the studies detailed above but which have been found to induce growth arrest and apoptosis when used in immobilised forms (R. Zamoyska, personal communication), stimulated the rapid and sustained release of intracellular [^{3}H]arachidonate, in a manner similar to Con A (Figure 48A and B). Ionomycin also stimulated sustained release of intracellular

[^{3}H]arachidonate in a linear manner for at least 30 min (Figure 48C), whereas PMA again failed to induce [^{3}H]arachidonate production (Figure 48A, B and C). This could indicate that although the influx of extracellular calcium is sufficient for the activation of $cPLA_2$ in these cells, the phorbol-ester mediated activation of PKC is not sufficient for $cPLA_2$ activation in the absence of calcium-induced translocation of $cPLA_2$ to the plasma membrane.

These findings clearly support the coupling of the antigen receptors on immature T lymphocytes to the activation of $cPLA_2$, and suggest that the release of arachidonate may be involved, at least in part, in the transduction of growth arrest and/or apoptotic signals in these cells.

4.10 $cPLA_2$ expression and activation in WEHI 231 immature murine B cells.

The possibility that immature B cells express $cPLA_2$, and that B cell antigen receptors are coupled to this activity in such cells, was investigated in the immature B lymphoma, WEHI 231. Many investigators have reported that WEHI 231 cells undergo growth arrest and apoptosis in response to crosslinking of *sIg* with anti-Ig (87, 88), and this was reconfirmed by a DNA synthesis assay showing that stimulation of WEHI 231 cells with anti-Ig antibodies (>0.1 μg/ml) inhibited DNA synthesis in a dose-dependent manner (Figure 49A). In contrast, stimulation of WEHI 231 cells with LPS (50 μg/ml), a polyclonal B cell activator, was found to stimulate an increase in the rate of DNA synthesis (Figure 49B).

Western blot analysis demonstrated the expression of $cPLA_2$ by WEHI 231 cells (Figure 50), and in view of this, the coupling of the antigen receptors on these cells to $cPLA_2$ activation was investigated. WEHI 231 cells, prelabelled with [^{3}H]arachidonate, were stimulated with anti-Ig at concentrations that i) did not significantly affect DNA synthesis (0.0125 μg/ml); ii) induced about 50% inhibition of DNA synthesis (0.125 μg/ml); and iii) essentially induced complete abrogation of DNA synthesis in these cells (1.25 μg/ml) (Figure 51A). Interestingly, WEHI 231 cells exhibited a time-dependent

basal release of arachidonate, indicating a measure of constitutive $cPLA_2$ activation, at least in some of these cells. No stimulated [^{3}H]arachidonate generation could be detected following ligation of the antigen receptors with anti-Ig at concentrations (0.0125 μg/ml) which did not induce growth arrest (Figure 51A). However, under conditions in which anti-Ig (0.125-1.25 μg/ml) abrogated DNA synthesis in WEHI 231 cells, crosslinking of the antigen receptors induced a substantial, and sustained (for up to at least 60 min) generation of [^{3}H]arachidonate (Figure 51A). This finding suggested the association of [^{3}H]arachidonate release with the induction of growth arrest and/or apoptosis by antigen receptor crosslinking on WEHI 231 cells. However, a preliminary characterisation of the concentration-dependence of anti-Ig-stimulated $cPLA_2$ activity demonstrated that, at higher concentrations of anti-Ig (> 5 μg/ml), stimulation of [^{3}H]arachidonate release became desensitised (Figure 51B). This finding could possibly contradict a role for $cPLA_2$ in the transduction of growth arrest (and apoptotic) signals as these higher concentrations of anti-Ig also induce apoptosis in WEHI 231 cells. However, this desensitisation of anti-Ig-induced $cPLA_2$ activation was observed in an experiment only measuring a single time point of 30 min, and a simpler explanation is that higher concentrations of anti-Ig will induce a greater, but more rapid and transient release of arachidonate, leading to activation of downstream signals and more rapid desensitisation of receptor-coupling, possibly as the result of a negative feedback mechanism. Thus, arachidonate levels could peak and decline before 30 min, resulting in no apparent release of [^{3}H]arachidonate. It would therefore be necessary to investigate the time- and concentration-dependence of anti-Ig-mediated $cPLA_2$ activation to determine whether this is the case.

These investigations also showed that stimulation of WEHI 231 cells with PMA (100 ng/ml) or ionomycin (1 μM), did not induce the release of [^{3}H]arachidonate, under conditions in which anti-Ig (0.5 μg/ml) did stimulate $cPLA_2$ (Figure 52A). This indicated that neither calcium influx nor PKC activation were sufficient on their own to induce $cPLA_2$ activation in WEHI 231 cells. Further investigation could involve the co-

stimulation of WEHI 231 cells with PMA and ionomycin to determine whether $cPLA_2$ activation requires both Ca^{2+} influx and phosphorylation in WEHI 231 cells.

4.10.1 The effects of a $cPLA_2$ inhibitor on DNA synthesis and arachidonate release in WEHI 231 cells.

As already discussed in section 4.2.1, studies on the role and activation of PLA_2 in platelets have involved the use of the arachidonate analogue, $AACOCF_3$, which is a tight-binding inhibitor of cytosolic PLA_2, but not secretory PLA_2 (240, 241). This inhibitor was found to block agonist-induced arachidonate and eicosanoid production in platelets, suggesting that $cPLA_2$ is responsible for the production of arachidonate as a substrate for the eicosanoid cascade in these cells. It was thus decided to investigate the effects of arachidonate and this $cPLA_2$ inhibitor on anti-Ig-induced growth arrest in WEHI 231 cells. Interestingly, $AACOCF_3$ (0-50 μM) did not inhibit the growth arrest induced by anti-Ig (0.5 or 5 μg/ml), but induced growth arrest itself in a dose-dependent manner (Figure 53B). WEHI 231 cells were also incubated with arachidonate (50 nM-50 μM) for 72 h before DNA synthesis was determined. Arachidonate inhibited DNA synthesis in WEHI 231 cells, but only at concentrations >16 μM (Figure 54), which was consistent with the finding that arachidonate (20 μM) induced growth arrest in mature B cells. In contrast, at lower concentrations arachidonate (0.16-16 μM) stimulated a small, but significant increase in DNA synthesis (Figure 54).

Since $AACOCF_3$ did not block anti-Ig-mediated growth arrest it was decided to investigate the effects of $AACOCF_3$ on anti-Ig-mediated activation of $cPLA_2$ in immature B cells. WEHI 231 cells, prelabelled with [^{3}H]arachidonate, were incubated with $AACOCF_3$ (0-20 μM) in the presence or absence of anti-Ig (0.25 μg/ml) (Figure 53A). Interestingly, $AACOCF_3$ (0.1-20 μM) appeared to inhibit basal archidonate release but had no effect upon anti-Ig-stimulated arachidonate release. This finding may indicate that $AACOCF_3$ will only inhibit $cPLA_2$ activity if it binds the enzyme prior to activation, perhaps explaining why $AACOCF_3$ did not inhibit anti-Ig-mediated growth arrest in WEHI 231 B cells (although any effect might be masked by its cytotoxic properties),

because anti-Ig and $AACOCF_3$ were added simultaneously, and the arachidonate analogue probably did not induce inhibition of $cPLA_2$. Further experiments would be needed to examine these possibilities. In addition, it is possible the observed effects of $AACOCF_3$ were due to its mimicking the effects of arachidonate.

4.10.2 The effects of stimuli which protect WEHI 231 cells from anti-Ig-induced apoptosis on $cPLA_2$ activity.

A number of stimuli have been reported to abrogate the growth arrest and apoptosis induced in WEHI 231 cells by antigen receptor crosslinking (3, 115, 120, 281). These include i) the T cell-derived cytokine, IL-4 (120); ii) crosslinking of CD40 molecules on B cells (116-119) (mediated *in vivo* by CD40 ligand on activated T cells); iii) the polyclonal B cell activator, LPS (120); and iv) phorbol esters, such as PMA (116). PMA stimulation of WEHI 231 cells had already been investigated, and no activation of $cPLA_2$ was observed. The effects of the other stimuli on $cPLA_2$ activity in WEHI 231 cells were examined: WEHI 231 cells, prelabelled with [^{3}H]arachidonate, were stimulated with IL-4 (10-200 U/ml), anti-CD40 (0.1-10 μg/ml), or LPS (50 μg/ml) for 30 min. However, none of these stimuli induced the release of [^{3}H]arachidonate under conditions in which anti-Ig (0.5 μg/ml) did, suggesting that arachidonate release is unlikely to play a role in the transduction of protective and/or mitogenic signals in WEHI 231 cells (results not shown). The effect of IL-4 on the activation of $cPLA_2$ by anti-Ig was also examined in order to determine whether stimuli which inhibit negative growth responses and apoptosis in immature B cells might also inhibit the activation of $cPLA_2$. WEHI 231 cells, prelabelled with [^{3}H]arachidonate, were stimulated with IL-4 (0-200 U/ml) in the presence or absence of anti-Ig (0.3 μg/ml) for 30 min. The basal level of arachidonate release was unaffected by IL-4, but IL-4 was found to inhibit anti-Ig-mediated [^{3}H]arachidonate release in a dose-dependent manner (Figure 55A and B). The inhibition of the anti-Ig-mediated activation of $cPLA_2$ in WEHI 231 cells by IL-4 could therefore be important in the inhibition of anti-Ig-induced growth arrest and programmed cell death in these cells. These findings support the hypothesis that the production of

arachidonate by $cPLA_2$ is involved in the transduction of anti-proliferative signals induced by the crosslinking of antigen receptors on immature lymphocytes. It would therefore be useful to examine the effects of the other protective stimuli, CD40 crosslinking, LPS and PMA, on anti-Ig-mediated arachidonate release.

4.11 Anti-Ig stimulates ceramide generation in WEHI 231 cells.

Recently, reports have demonstrated a role for the agonist-induced release of arachidonate in the activation of sphingomyelinase and the resultant hydrolysis of sphingomyelin and release of ceramide (61). This pathway is strongly implicated in the transduction of apoptotic signals in a number of cell types (61, 180-182, 222), and it was therefore decided to investigate the possibility that the antigen receptors on WEHI 231 cells are coupled to the activation of sphingomyelinase (this work was carried out in collaboration with S. Gardner, this laboratory). WEHI 231 cells were stimulated with anti-Ig for 15 or 30 min, and ceramide mass levels were determined using an *in vitro* DAG-kinase assay which catalyses the transfer of the γ-phosphate of [γ-^{32}P]ATP to ceramide to generate [^{32}P]ceramide phosphate, which was then determined by thin layer chromatography analysis. Anti-Ig stimulation of WEHI 231 cells was found to induce the production of ceramide within 30 min (Figure 56), but not 15 min (data not shown) in a dose-dependent manner (0.001-100 μg/ml). These findings suggest that SMase activation and ceramide production could be involved in the transduction of anti-proliferative signals upon ligation of the antigen receptors on immature B cells. The finding that anti-Ig-mediated ceramide release is temporally downstream of $cPLA_2$ activation also suggests that the stimulated release of arachidonate could play a role in the activation of SMase in these cells, in a manner analogous to that observed for TNF-α-signalling in HL60 monocytic cells.

DISCUSSION

4.12 Cytosolic PLA_2 in mature lymphocytes.

The results presented here demonstrate that the antigen receptors on mature B and T cells are not coupled to either an arachidonate-selective $cPLA_2$ activity or to an oleate-selective PLA_2 activity following mitogenic stimulation (Figures 36 and 37). Moreover, $cPLA_2$ does not appear to be expressed in mature tonsillar splenic B and T cells or in a number of B and T cell lines representing mature lymphocytes (Figure 38). These data suggest that arachidonate, or other free fatty acids, are unlikely to play a role as lipid second messengers in transducing proliferative signals in mature, immunocompetent B or T cells.

These results were initially rather surprising as arachidonate generation has been implicated in the regulation of signal transduction events leading to proliferation in a number of cell types (143, 232, 234) and because many of the receptors known to couple to $cPLA_2$ are, like the antigen receptors on B and T cells, calcium-mobilising receptors (278). In addition, whilst several groups had reported an inability to detect eicosanoid formation by human lymphocyte preparations and cell lines (282-284), others (285-287) had previously reported arachidonate release and PLA_2 activation from B and T cell lines stimulated with receptor ligands or pharmacological activators, such PMA and/or calcium ionophores. However, the apparent discrepancies with these earlier published studies may be easily resolved since these authors have described arachidonate release from either an antigen-specific $CD4^+$ T cell clone (286) or human EBV-transformed human B cell lines, including the SKW6.4 B cell line (285, 287). Whilst the results of this investigation indicate that simply culturing Jurkat T cells, *in vitro*, with IL-2 for up to 3 days does not induce $cPLA_2$ expression (data not shown), it is possible that induction of antigen-specific T cell clones followed by their repeated stimulation and culture with antigen and IL-2 may induce $cPLA_2$ expression in these T cells. Moreover, examination of $cPLA_2$ expression in B cell lines, has shown that certain EBV-transformed cells, including SKW and EDR cells, do indeed express $cPLA_2$ (data not shown), suggesting that EBV-mediated deregulation of cellular proliferation may overcome, or lie distal, to

any potentially deleterious effects of $cPLA_2$ activation on B cell proliferation. However, expression of $cPLA_2$ does not appear to be a ubiquitous feature of EBV-transformed B cells as we have found that other EBV-transformed B cell lines (including Daudi B cells) do not appear to express $cPLA_2$. Moreover, investigation of $cPLA_2$ activity in EDR cells has demonstrated that arachidonate generation is not induced following antigen receptor crosslinking, or stimulation with PMA, IL-4 (alone or in combination with anti-Ig), LPS or ATP (results not shown), suggesting that transformation may have uncoupled $cPLA_2$ from the antigen receptors in these cells.

Recent findings that inflammatory mediators such as IL-1 and TNF-α, which can promote lymphocyte proliferation and differentiation, dramatically upregulate $cPLA_2$ expression and activity in fibroblasts (278, 279), raised the possibility that, although $cPLA_2$ does not appear to be expressed in resting B and T cells or proliferating lymphocyte cell lines (Figure 38), it may play a role in lymphocyte activation during an immune response. However, culture of B and T cell lines with these cytokines did not induce $cPLA_2$ expression, at least *in vitro* (results not shown). Although the precise profile of $cPLA_2$ expression has not been defined, it is clear that it is expressed by a wide variety of cell lineages including kidney mesangial cells, fibroblasts, epithelial cells, macrophages and platelets (278, 288-292). Thus, taken together with the increasing evidence that fatty acids, such as arachidonic acid, can mediate anti-proliferative or even cytolytic effects in a number of cell types (61, 233, 293, 294), including B cells (Figure 39), the lack of expression of $cPLA_2$ in B and T cells suggested that $cPLA_2$ activity could be (i) deleterious for lymphocyte survival and proliferation and (ii) involved in antiproliferative responses such lymphocyte differentiation and/or apoptosis.

4.13 $cPLA_2$ expression and activation in immature B and T cells.

As a result of the finding that $cPLA_2$ is not expressed by mature lymphocytes, and that arachidonate release may be involved in negative signalling in lymphocytes, it was decided to screen a range of pre-B cell lines, thymocytes, immature (4 week old) splenic lymphocytes, murine T3 thymoma cells, and the immature murine B lymphoma,

WEHI 231, for $cPLA_2$ expression. Although $cPLA_2$ was not expressed in pre-B cells, it was highly expressed in thymocytes, lymphocytes prepared from immature murine spleens (Figure 40), murine T3 thymoma cells, (Figure 46) and WEHI 231 cells (Figure 50), suggesting that $cPLA_2$ may transduce maturation stage-specific signals.

4.13.1 $cPLA_2$ activity in murine thymocytes and T3 thymoma cells.

Investigation initially focused on the use of primary murine thymocytes. Ligation of the antigen receptors on murine thymocytes with anti-CD3 or Con A stimulated the intracellular release of arachidonate (Figure 42), demonstrating that the antigen receptors on these cells are coupled to $cPLA_2$ activation. Incubation of these cells with ionomycin, a calcium ionophore, also stimulated the release of arachidonate (Figure 42). Taken together with the time course of $cPLA_2$ activation, the results suggest that the TCR may be coupled to $cPLA_2$, at least in part, through the mobilisation of calcium and the Ca^{2+}-dependent recruitment of $cPLA_2$ to the plasma membrane. Preliminary findings indicated that PMA does not induce $cPLA_2$ activation in these cells, suggesting that PKC activation is not sufficient for, or not involved in, $cPLA_2$ activation in these cells.

It was then decided to investigate antigen receptor-$cPLA_2$ coupling in T3 murine thymoma cells, which also been found to express $cPLA_2$, because these cells represent a more homogeneous immature T cell population. T3 cells have been found to undergo apoptosis in response to antigen receptor crosslinking by Con A and following stimulation with ionomycin or hydrocortisone, but not PMA (R. Zamoyska, personal communication). Proliferation assays had also shown that growth arrest is induced in these cells in response to Con A, PHA or ionomycin (Figure 45), but not anti-CD3 (0.001-1 μg/ml) (A. Michie, personal communication). However, the failure of anti-CD3 to induce growth arrest in T3 cells was apparently because insufficient TCR crosslinking is mediated by soluble anti-CD3 Ab (R. Zamoyska, personal communication). These cells were therefore appropriate for the study of apoptotic signals in immature T cells.

Following these findings and reports, it was decided to investigate TCR-$cPLA_2$ coupling in T3 cells. Interestingly, stimulation of T3 cells with Con A (50 μg/ml), anti-

CD3 (1 μg/ml) or ionomycin (1 μM) stimulated rapid and sustained release (for at least 30 min) of arachidonate, whereas PMA (100 ng/ml) induced no arachidonate generation. These findings strongly support the findings in thymocytes that the antigen receptors on immature T cells are coupled to $cPLA_2$ activation. The results obtained with stimulation of T3 cells with ionomycin and PMA were also very interesting because i) sustained calcium release has been implicated in the induction of apoptosis in a number of cell types, and ionomycin has been shown to induce apoptosis in T3 cells, and ii) phorbol esters have been shown to inhibit apoptosis in several cell types, including T3 thymoma cells. Furthermore, it has been found that PKC is not activated following crosslinking of the antigen receptors on immature lymphocytes to the same extent as that observed in mature lymphocytes (93), and it is possible that PKC activation is inhibitory to the induction of apoptosis. These results therefore support the proposal that arachidonate generation may be involved in the induction of apoptosis of immature lymphocytes, as only stimuli known to induce apoptosis were found to stimulate $cPLA_2$ activity.

The analysis of $cPLA_2$ activation in T3 cells also found that Con A or anti-CD3 induced a much more rapid release of arachidonate (within 1 min), than ionomycin (>1 min, <10 min). This could simply be because antigen receptor crosslinking induces a number of signals in addition to Ca^{2+} influx (such as MAP-kinase activation), and therefore induces a more rapid activation of $cPLA_2$ than the ionomycin-mediated influx of Ca^{2+} does on its own. It is also possible, however, that the antigen receptors on immature T cells are coupled to $cPLA_2$ activity via alternative pathways (eg. G proteins), which would elicit more rapid activation of $cPLA_2$. It would therefore be appropriate to investigate the coupling of the TCR to $cPLA_2$ via both Ca^{2+}/protein kinase-dependent and G protein-dependent pathways.

Overall, the findings demonstrate the expression of $cPLA_2$ and coupling of antigen receptors to PLA_2 activation, in murine thymocytes and T3 thymoma cells. These results therefore indicate that the generation of arachidonate may be involved in the transduction of apoptotic signals via the antigen receptors on immature T cells.

4.13.2 $cPLA_2$ activity in immature murine B cells.

Western blots also showed that $cPLA_2$ is also expressed in murine immature splenic lymphocytes from 4 week old mice (B and T cells), indicating that $cPLA_2$ could be expressed (and possibly activated via the antigen receptors) in immature B cells in a manner analogous to that observed for immature T cells. However, investigation of antigen receptor coupling in IS B cells showed that antigen receptor crosslinking, or stimulation with PMA or LPS, did not induce the release of arachidonate above that observed in unstimulated cells (Figure 44). However, comparison of the level of [^{3}H]arachidonate release in unstimulated cells at zero time and following culture for 30 min, indicated that the there was an extremely high basal level of arachidonate release, suggesting that, if arachidonate release constitutes a signal for the induction of anergy or apoptosis, then some of these B cells might be spontaneously undergoing this process, possibly as the result of prior encounter with self antigen or their removal from their physiological environment. Another problem associated with investigating the coupling of the antigen receptors to $cPLA_2$ activity in IS B cells is that the spleens of 4 week old mice contain a rather mixed population of B cells of varying maturation states. Indeed, other workers have found that splenic B cells from 4 week old mice proliferate in response to antigen receptor crosslinking, and a large proportion (approximately 84%) are IgM^+IgD^{++}, indicating that they are mature B cells (280). Interestingly, the results suggested that stimulation of IS B cells with anti-Ig or LPS might induce a slight suppression of arachidonate release, possibly indicating that mitogenic stimulation of IgD^+ B cells may suppress $cPLA_2$ activity.

As a result of these complications it was decided to study $cPLA_2$ activation in WEHI 231 B lymphoma cells, which had also been found to express $cPLA_2$ (Figure 50). These cells have been reported to undergo apoptosis in response to sIg crosslinking with anti-Ig antibodies, and proliferation assays demonstrated that anti-Ig (>50 ng/ml) inhibited DNA synthesis (Figure 49). Moreover, stimulation with LPS (50 μg/ml), which has been reported to promote proliferation and to abrogate anti-Ig-induced growth arrest

and apoptosis in WEHI 231 cells, was found to promote DNA synthesis (Figure 49), demonstrating that these cells are responsive to this polyclonal B cell activator.

The antigen receptor coupling and regulation of $cPLA_2$ activity was then investigated in WEHI 231 lymphoma cells. Stimulation of WEHI 231 cells with anti-Ig at concentrations below that which induce apoptosis (ie. <0.1 μg/ml) did not induce the release of arachidonate, whereas stimulation with anti-Ig concentrations which suppress DNA synthesis by 50% (0.125 μg/ml) or completely inhibit DNA synthesis (1.25 μg/ml) was found to induce significant release of arachidonate at 5 (results not shown), 10, 30 and 60 min (Figure 51), indicating that the antigen receptors on WEHI 231 B cells are coupled to $cPLA_2$ activation, and that arachidonate generation could be involved in the transduction of growth arrest and/or apoptotic signals in immature B cells. However, further investigation of the concentration-dependence of the anti-Ig-induced arachidonate generation demonstrated that stimulation of WEHI 231 cells with substantially higher concentrations of anti-Ig (5, 50 μg/ml) resulted in an apparent densensitisation of $cPLA_2$ activation (Figure 51), despite the fact that these concentrations of anti-Ig induce growth arrest and apoptosis in WEHI 231 cells. However, since the coupling of sIg to $cPLA_2$ under conditions of extensive crosslinking were only investigated at a single time point of 30 min, it is possible that $cPLA_2$ is deactivated by negative feedback once downstream signals have been fully activated by arachidonate. Such a situation might suggest that sustained arachidonate release is not required for the maintenance, but only for the initiation, of the cellular events leading to the induction of apoptosis in WEHI 231 cells.

Further investigation of the regulation of $cPLA_2$ activity in WEHI 231 cells found that arachidonate release was not stimulated by ionomycin (1 μM) or PMA (100 ng/ml). This could indicate that calcium influx and PKC activation are not sufficient on their own to induce $cPLA_2$ activation. Indeed, PMA has been found to rescue cells (including WEHI 231 cells) from growth arrest and apoptosis (94), and so activation of $cPLA_2$ by PMA would not be predicted if arachidonate release is a signal for the inhibition of growth or induction of apoptosis. Reports also suggest that the antigen receptors on immature lymphocytes may be uncoupled from the full activation of PKC, indicating that

sIg-cPLA$_2$ coupling is likely to be mediated by alternative pathways such as the ras-raf-MAP-kinase cascade. It is possible that sIg on immature B cells may couple to cPLA$_2$ activity via alternative mechanisms, such as the G protein-dependent activation of cPLA$_2$ induced by bombesin in Swiss 3T3 cells (143). However, it is also possible that activation of cPLA$_2$ in WEHI 231 cells simply requires both Ca^{2+} influx and PKC activation, signals resulting from sIg crosslinking.

Overall, the results therefore demonstrate the expression of cPLA$_2$ by immature B cells, and the coupling of the antigen receptors on these cells to arachidonate release in a manner analogous to that observed in immature T cells, suggesting that this signalling pathway may be important for the induction of anergy and/or apoptosis in these lymphocytes.

4.13.3 The effects of stimuli which protect WEHI 231 cells from apoptosis on the activation of cPLA$_2$.

Further studies carried out in WEHI 231 cells examined the effects on cPLA$_2$ activity of number of stimuli which have been shown to abrogate anti-Ig-mediated growth arrest (3, 115, 120, 281). Stimulation of WEHI 231 cells with IL-4, CD40 crosslinking or LPS failed to induce activation of cPLA$_2$. Furthermore, preliminary findings indicated that such stimuli might inhibit the anti-Ig-induced activation of cPLA$_2$ in WEHI 231 cells. IL-4 was found to inhibit anti-Ig-mediated release of arachidonate in a dose-dependent manner, although it had no effect on basal arachidonate release, suggesting that this cytokine probably has no direct inhibitory effect on cPLA$_2$ activity, but modulates an upstream signal initiated by the crosslinking of sIg. These preliminary results suggest that the inhibition of anti-Ig-mediated signals, such as activation of cPLA$_2$, by IL-4 (and possibly other stimuli), may be important for the protection of WEHI 231 cells from anti-Ig-induced growth arrest and apoptosis. The findings are therefore consistent with a role for arachidonate release in the transduction of negative signals in lymphocytes.

4.13.4 The effects of an arachidonate analogue on DNA synthesis and $cPLA_2$ activity in WEHI 231 cells.

Further studies investigated the effects of the direct inhibition of $cPLA_2$ activity on anti-Ig-mediated growth arrest and arachidonate release in WEHI 231 cells. These employed the use of an arachidonate analogue, $AACOCF_3$, which has been shown to inhibit $cPLA_2$ but not $sPLA_2$ activity (240, 241). Firstly, WEHI 231 cells were incubated with anti-Ig (0, 0.05, 0.5, or 5 μg/ml) in the presence of $AACOCF_3$ (0-50 μM) and the effects on DNA synthesis were determined. The effects of arachidonate (0-50 μM) on WEHI 231 DNA synthesis were also examined. The results were somewhat surprising: $AACOCF_3$ inhibited DNA synthesis in WEHI 231 cells in a dose-dependent manner, whereas arachidonate (0.16-16 μM) induced a small, but significant increase in DNA synthesis, only inhibiting DNA synthesis at higher concentrations (50 μM). Although these results could suggest that low levels of released arachidonate could be involved in mitogenic, rather than apoptotic, signalling, it is possible that exogenously added arachidonate will have different effects to stimulated release of arachidonate by cells. Moreover, the different effects of arachidonate and $AACOCF_3$ may be due to the fact that, unlike arachidonate, $AACOCF_3$ is probably not metabolised by cells (240, 241), resulting in the sustained presence of $AACOCF_3$, as compared to the removal of arachidonate through its metabolism by the cells, and/or conversion of arachidonate to other molecules such as eicosanoids. $AACOCF_3$ appeared to reduce the concentration dependence for anti-Ig in the induction of growth arrest in WEHI 231 cells. Thus, in WEHI 231 cells incubated with a concentration of anti-Ig (0.5 μg/ml) which markedly inhibited growth on its own, addition of $AACOCF_3$ induced total growth inhibition at a concentration of 5-16 μM; whereas WEHI 231 cells which were incubated with no anti-Ig or a "sub-apoptotic" concentration of anti-Ig (0.05 μg/ml), required $AACOCF_3$ at a concentration of 50 μM for the total inhibition of DNA synthesis. Furthermore, the results from the experiments examining the effects of $AACOCF_3$ on anti-Ig-mediated $cPLA_2$ activation suggest that it is unlikely that $AACOCF_3$ inhibited arachidonate release during the DNA synthesis assay. In any case, the cytotoxic effects would probably mask

any effects of $AACOCF_3$ on cell proliferation resulting from the inhibition of $cPLA_2$ activity. These results are inconclusive regarding the role of $cPLA_2$ activation and arachidonate release in the control of growth and apoptosis in WEHI 231 cells.

4.14 The coupling of the antigen receptors on WEHI 231 B cells to the sphingomyelinase pathway.

A preliminary study was carried out (in collaboration with S. Gardner in this laboratory), to determine whether crosslinking of the antigen receptors on immature B cells results in the activation of sphingomyelinase, which catalyses the hydrolysis of sphingomyelin and the release of ceramide, another lipid second messenger implicated in the control of apoptosis in several cell types (61, 180-182, 222). Stimulation of WEHI 231 cells with anti-Ig (0.001-100 μg/ml) induced the release of ceramide in dose-dependent manner within 30 min, although no ceramide production was observed by 15 min. Other workers have previously shown that WEHI 231 cells generate ceramide in response to antigen receptor crosslinking. However, this was only observed 8 h after the initiation of sIg crosslinking. These discrepancies may be resolved by the fact that the other study involved the determination of ceramide levels in [^{3}H]palmitate-labelled WEHI 231 cells. In contrast, the DAG-kinase assay is a highly sensitive mass assay and should detect the production of ceramide from all species of sphingomyelin. However, taken together these findings indicate that antigen receptor crosslinking on immature B cells stimulates ceramide production which is sustained for several hours. The results also demonstrate that, following sIg crosslinking, sphingomyelinase activation is temporally downstream of $cPLA_2$ activation, suggesting that, as with TNF-α stimulation of HL60 monocytes (61), the stimulated release of arachidonate may activate sphingomyelinase. Further studies to investigate this possibility could involve examination of the effects of exogenous arachidonate, and arachidonate analogues such as $AACOCF_3$ and ETI, on sphingomyelinase activity in WEHI 231 cells and other immature lymphocytes. Interestingly, in mutant WEHI 231 cells, which do not undergo growth arrest or apoptosis in response to sIgM crosslinking, crosslinking of the antigen receptors has

been found to be uncoupled from SMase activation and ceramide generation (295). Further investigation of the uncoupling of the antigen receptors on these cells from SMase activation and the induction of growth arrest/ apoptosis could involve studies on sIgM coupling to $cPLA_2$ activation.

4.15 Summary.

This investigation has generated a number of findings which indicate a key role for $cPLA_2$ in lymphocyte maturation:

i) $cPLA_2$ is expressed by B and T cells in a maturation state-specific manner. Western blotting studies demonstrated that mature lymphocytes, and a number of cell lines representing this maturation state, do not express $cPLA_2$. In contrast, $cPLA_2$ was found to be expressed in immature murine splenocytes, murine thymocytes, T3 murine thymoma cells and WEHI 231 murine immature B cells, although not in the pre-B cell lines 697, 207 and REH.

ii) the antigen receptors on immature B and T cells were found to be coupled to $cPLA_2$ activation, indicating that arachidonate release may be involved in the antigen receptor-mediated induction of growth arrest and/or apoptosis.

iii) Pharmacological studies demonstrated that certain agents which induce apoptosis (eg. ionomycin in T3 cells) activate $cPLA_2$, whereas those which do not induce apoptosis (eg. PMA in all the immature lymphocytes investigated) failed to induce arachidonate release.

iv) stimulation of WEHI 231 immature B cells with specific stimuli known to rescue these cells from sIgM-mediated apoptosis, including IL-4, CD40 crosslinking and LPS, did not stimulate $cPLA_2$ activity. Moreover, IL-4 was found to block anti-Ig-mediated release of arachidonate, suggesting that these

stimuli may effect B cell rescue from apoptosis through the inhibition of intracellular signals such as $cPLA_2$ activation.

v) the antigen receptors on WEHI 231 cells were found to be coupled to the downstream activation of sphingomyelinase and the resultant generation of ceramide.

The findings of this investigation have therefore demonstrated a role for $cPLA_2$ in maturation state-specific signalling in lymphocytes. The antigen receptor-mediated release of arachidonate has also been strongly implicated in the induction of growth arrest and/or apoptosis. Furthermore, sphingomyelinase, a signal transducer which has been shown to be activated by released arachidonate in other cell types, has been found to be temporally downstream of $cPLA_2$ activation following crosslinking of the antigen receptors on WEHI 231 immature B cells. Taken together, these results suggest that the sIg-coupled activation of $cPLA_2$ and SMase may be analogous to that observed in the TNF-α receptor system.

4.16 Future perspectives.

The investigation of $cPLA_2$ expression and activation in lymphocytes has clearly established a role for $cPLA_2$ in maturation state-dependent signalling via the antigen receptor in immature B and T cells. These findings have suggested a role for the stimulated release of arachidonate in the induction of growth arrest and/or apoptosis. However, there is as yet, no conclusive evidence for a role for $cPLA_2$ in negative selection of lymphocytes, and a number of questions remain unanswered as to the receptor coupling and regulation of $cPLA_2$ activity, as well as the downstream effects of stimulated arachidonate release.

i) Coupling mechanism.

The crosslinking of the antigen receptors on B or T cells leads to the rapid and sustained production of arachidonate. However, the mechanism(s) by which the antigen receptors on lymphocytes couple to $cPLA_2$ has not been determined. The most obvious mechanism, established in other cell types, involves the phosphorylation of $cPLA_2$ by MAP-kinase and its calcium-dependent recruitment to the plasma membrane (260). However, although ionomycin, a calcium ionophore, was found to activate $cPLA_2$ in immature T cells, there is no evidence for the calcium-dependent activation of $cPLA_2$ in immature B cells. Another possibility is that the lymphocyte antigen receptors may couple to $cPLA_2$ activation via a G-protein-dependent mechanism (possibly in addition to the Ca^{2+}/MAP-kinase-dependent pathway). There are a number of experiments which could be conducted to define the coupling mechanism(s) in B and T cells:

i) a more detailed time course (ie. at shorter time points) to determine the precise lag between TCR or sIg crosslinking, or ionomycin stimulation, and $cPLA_2$ activation in T3 and WEHI 231 cells.

ii) analysis of the effects of the guanine nucleotide analogues GTPγS and GDPβS on $cPLA_2$ activation following TCR or BCR crosslinking on permeabilised T3 or WEHI 231 cells, respectively. Determination of whether $cPLA_2$ is activated subsequent to PtdInsP2-PLC: dose response on intact cells or in the presence of neomycin in permeabilised cells.

iii) analysis of the effects of G protein ADP-ribosylation, by pertussis toxin (G_i-like proteins) or cholera toxin (G_α-like proteins), on the rapid-phase of $cPLA_2$ activation following TCR or BCR crosslinking crosslinking.

iv) analysis of the Ca^{2+}-dependence of of $cPLA_2$ activation in T3 and WEHI 231 cells, using chelation of extracellular calcium or intracellular calcium (in permeabilised cells).

v) analysis of the effects of protein kinase (PTK and PKC) inhibitors on antigen receptor-mediated $cPLA_2$ activation in immature B and T cells.

ii) Rescue from growth arrest.

The investigation also demonstrated that IL-4 inhibits sIg-$cPLA_2$ coupling in WEHI 231 cells. It is possible that other stimuli which rescue B cells from anti-Ig induced apoptosis could inhibit anti-Ig-mediated $cPLA_2$ activation. Thus, it would be interesting to examine the effects of CD40 crosslinking, LPS and PMA on anti-Ig-induced $cPLA_2$ activation. It would also be useful to determine whether these stimuli have any effect on $cPLA_2$ expression.

iii) The role of arachidonate and prostaglandins in lymphocyte proliferation and apoptosis.

DNA synthesis assays demonstrated differences between the effects of exogenous arachidonate and its analogue, $AACOCF_3$, on the proliferation of WEHI 231 cells. Indeed, at low concentrations arachidonate slightly stimulated DNA synthesis, and there are a number of possible reasons for this. Arachidonate can be converted to eicosanoids, whereas $AACOCF_3$ does not appear to be metabolised. The production of these intracellular inflammatory mediators by WEHI 231 cells could have an autocrine effect, possibly inducing the increase in proliferation observed upon incubation with arachidonate (0.16-16 μM). Indeed it has been reported that murine fetal thymic lobes will convert exogenous arachidonate to 6-keto prostaglandin F_1 (PGF_1), PGE_2, and PGF_2, which were found to promote proliferation of such cells, although their production has also been implicated in the negative modulation of growth responses to lymphokines such as IL-1 in thymocytes. [^{3}H]arachidonate could be added exogenously

to WEHI 231 cells and then the formation of [^{3}H]eicosanoids could be analysed using HPLC. These cells could also be stimulated with anti-Ig to determine whether antigen receptor crosslinking induces any changes in the formation of eicosanoids. In addition, the effects of eicosanoids such as PGE_2 on WEHI 231 cell proliferation could be determined.

iv) Signals downstream of arachidonate generation.

In addition to antigen receptor-mediated arachidonate release, sIg crosslinking was found to induce the production of ceramide in WEHI 231 cells. This pathway has been found to be activated following TNF-α-stimulated arachidonate release by $cPLA_2$ in HL60 monocytes. The role of arachidonate generation in coupling sIg on B cells to downstream signals, such as sphingomyelin hydrolysis, could be analysed. WEHI 231 cells could be stimulated with exogenous arachidonate, $AACOCF_3$ or ETI, and the generation of ceramide measured using the DAG-kinase assay. The involvement of arachidonate in the regulation of such pathways might indicate that the primary role of anti-Ig-induced arachidonate release is as an intracellular second messenger, rather than as a precursor for eicosanoid biosynthesis. The effect of specific eicosanoids (if they are produced by WEHI 231 cells from exogenous arachidonate) on this pathway could also be examined. The sustained production of ceramide following sIg crosslinking on WEHI 231 cells has also been reported by other researchers. The temporal relationship between arachidonate and ceramide generation following antigen receptor crosslinking suggests that released arachidonate may directly activate sphingomyelinase and indirectly to the induction of growth arrest and/or apoptosis. The above experiments, as well as further investigation of the anti-Ig-mediated production of ceramide would help to substantiate this possibility. This investigation has also demonstrated the activation of PtdCho-PLD activity in B cells following addition of exogenous ceramide. This raises the possibility that the anti-Ig-stimulated release of ceramide could activate PtdCho-PLD in immature B cells, but over a much longer time course than was previously examined. Such activation would be consistent with a role for PtdCho-PLD in negative signalling in B cells.

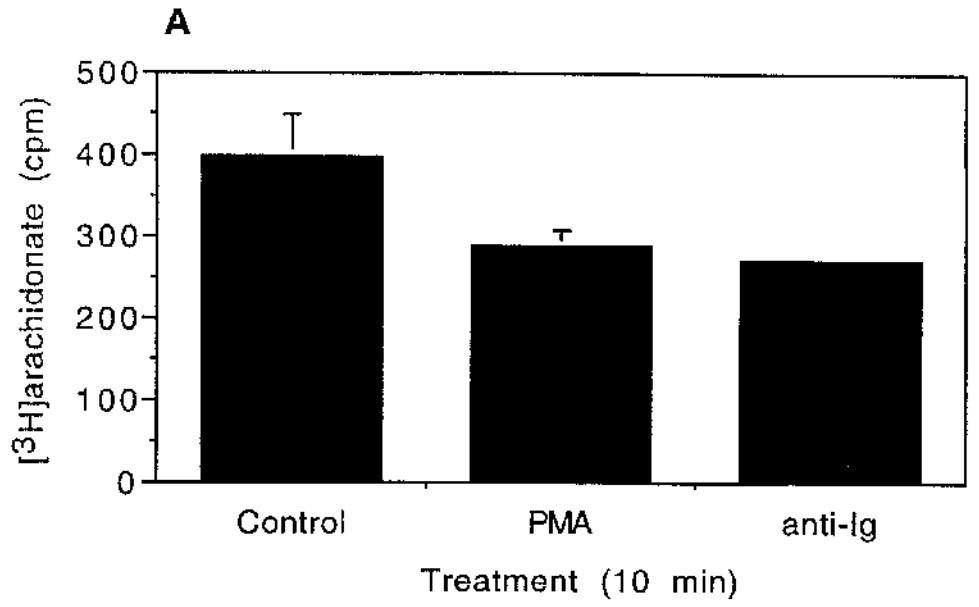
v) Demonstration of a causal link between $cPLA_2$ activation and growth arrest/apoptosis.

Some other reports have suggested another possible strategy for investigating the role of $cPLA_2$ in the transduction of negative signals from sIg on WEIII 231 cells. Growth arrest of WEHI 231 cells has been used to examine the cell cycle-dependence of anti-Ig-mediated growth inhibition. WEHI 231 cells were growth-arrested and synchronized in G_2M phase using nocodazole (2), which was then removed allowing them to progress and enabling the analysis of their sensitivity to anti-Ig at different points in the cell cycle. It was found that WEHI 231 cells are sensitive to anti-Ig growth inhibition immediately following cell division, as the cells enter G_1 phase, but not after that, suggesting that antigen receptor crosslinking modulates some key intracellular events at that point. Interestingly, a report on the cell cycle-dependence of $cPLA_2$ activation in HeLa cells (296), demonstrated that incubation of interphase-arrested HeLa cells with histamine, thapsigargin or ionomycin led to the release of arachidonate, whereas $cPLA_2$ activation was totally inhibited in metaphase-arrested cells. It might therefore be worthwhile to examine the cell cycle-dependence of $cPLA_2$ activation by anti-Ig in WEHI 231 cells. If anti-Ig-induced arachidonate release constitutes an apoptotic signal in these cells, then it is likely that periods where anti-Ig will induce arachidonate release and growth arrest would coincide. Such a finding would not prove the involvement of $cPLA_2$ in the transduction of apoptotic signals from antigen receptors on immature lymphocytes, but it would lend considerable weight to that hypothesis.

Further studies investigating the role of arachidonate release in the induction of apoptosis in immature T cells, will involve the characterisation of the regulation of $cPLA_2$ activity in a number of mutant T3 cell lines. These mutants, all of which have been found to express $cPLA_2$ (Figure 46), were derived from T3 murine thymoma cells on the basis of their resistance to apoptosis induced by ionomycin (I1-6A10, I3-2F7), concanavalin A (C3-6H12), or hydrocortisone (H1-5F12). The examination of whether these stimuli, or indeed antigen receptors, are uncoupled from specific intracellular signals, particularly

$cPLA_2$ activation, would give further insight into the signals involved in the induction of apoptosis in immature lymphocytes.

Finally, work in progress (in collaboration with M. Wakelam, Birmingham) will provide a much more definitive answer to the question of whether $cPLA_2$ activation is involved in the induction of growth arrest or apoptosis in immature lymphocytes. The cloning of the gene encoding $cPLA_2$ has enabled the synthesis of a dominant negative $cPLA_2$ gene which can be used to suppress $cPLA_2$ activity in cells. The transfection of cells with this gene will therefore permit analysis of the requirement of these cells for $cPLA_2$ and downstream signal events such as ceramide generation in the induction of specific cellular responses such as growth arrest and apoptosis. Both WEHI 231 murine B lymphoma cells and T3 murine thymoma cells constitute appropriate cell lines in which to conduct such a study. Moreover, it will be possible to prepare $cPLA_2$ deletion mutants of various lymphocyte cell lines which will allow further study of its function in these cells.


A
500
400
300
200
100
0
[3H]arachidonate (cpm)
Control
PMA
anti-Ig
Treatment (10 min)

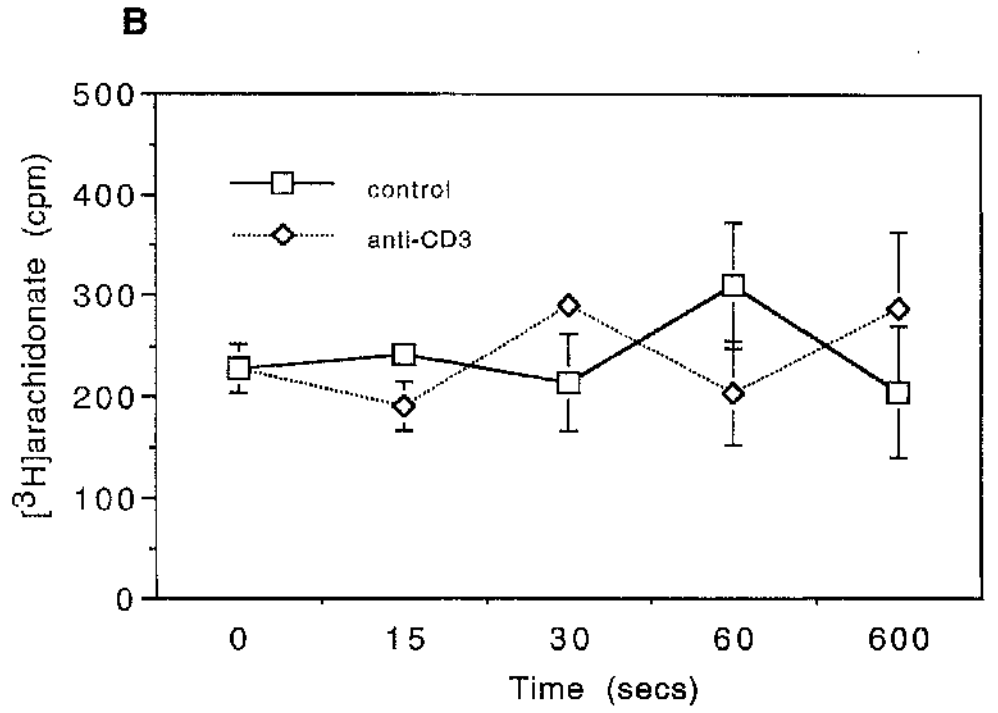
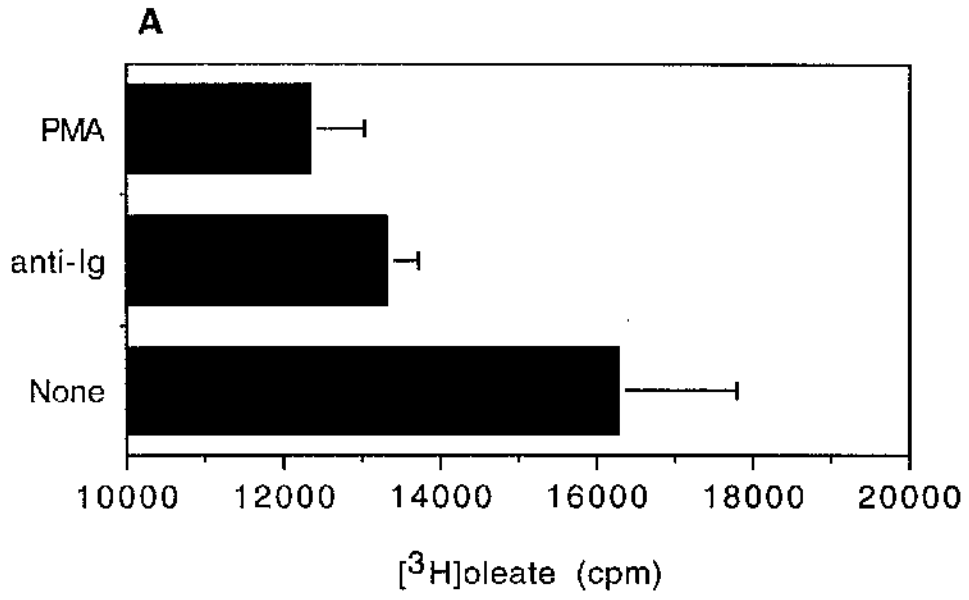

B
500
400
300
200
100
0
[3H]arachidonate (cpm)
control
anti-CD3
0
15
30
60
600
Time (secs)

Figure 36. The antigen receptors on B and T cells are not coupled to $cPLA_2$ activation. Murine splenic B cells, purified as described under "Materials and Methods" were radiolabelled and in *panel A*, stimulated with anti-Ig (50 μg/ml), PMA (1 μg/ml) or medium (control) for 10 min. In *panel B*, Jurkat T cells, treated as described under "Materials and Methods" were radiolabelled with [^{3}H]arachidonate and stimulated with anti-CD3 (1 μg/ml;) or medium for the indicated time. Arachidonic acid was measured by tlc analysis as described. The results are expressed as means ± SEM from single typical experiments where n=3.


A
PMA
anti-Ig
None
10000
12000
14000
16000
18000
20000
[3H]oleate (cpm)

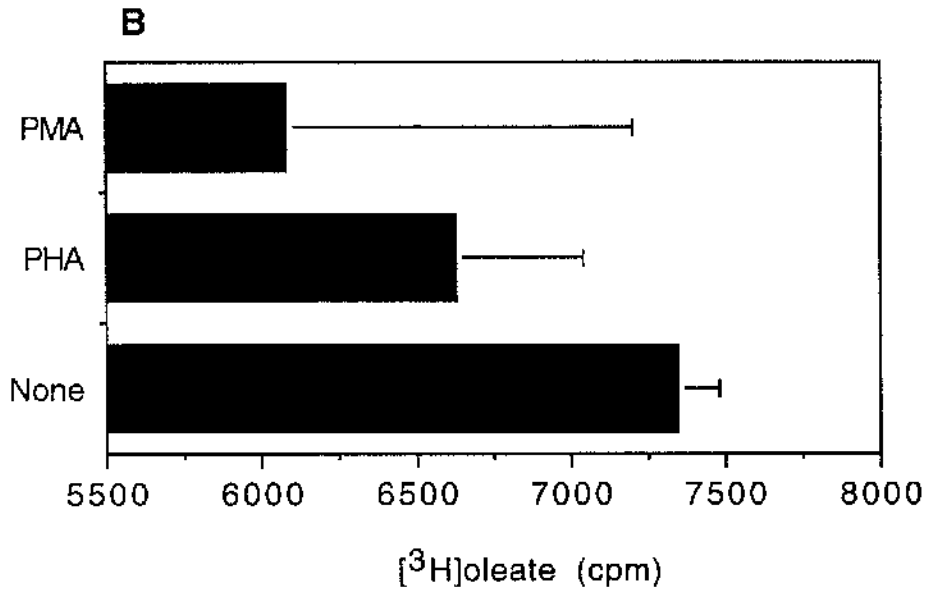

B
PMA
PHA
None
5500
6000
6500
7000
7500
8000
[3H]oleate (cpm)

Figure 37. The antigen receptors on B and T cells are not coupled to oleate-specific $cPLA_2$ activity. Human Daudi B and Jurkat T cell lines, treated as described under "Materials and Methods" were radiolabelled with [^{3}H]oleate. In *panel A* Daudi B cells were stimulated with anti-Ig (25 μg/ml), PMA (1 μg/ml) or medium; in *panel B*, Jurkat T cells were stimulated with PHA (50 μg/ml), PMA (1 μg/ml) or medium. Oleic acid was measured by tlc analysis as described. The results are expressed as means ± SEM from single typical experiments where n=3.

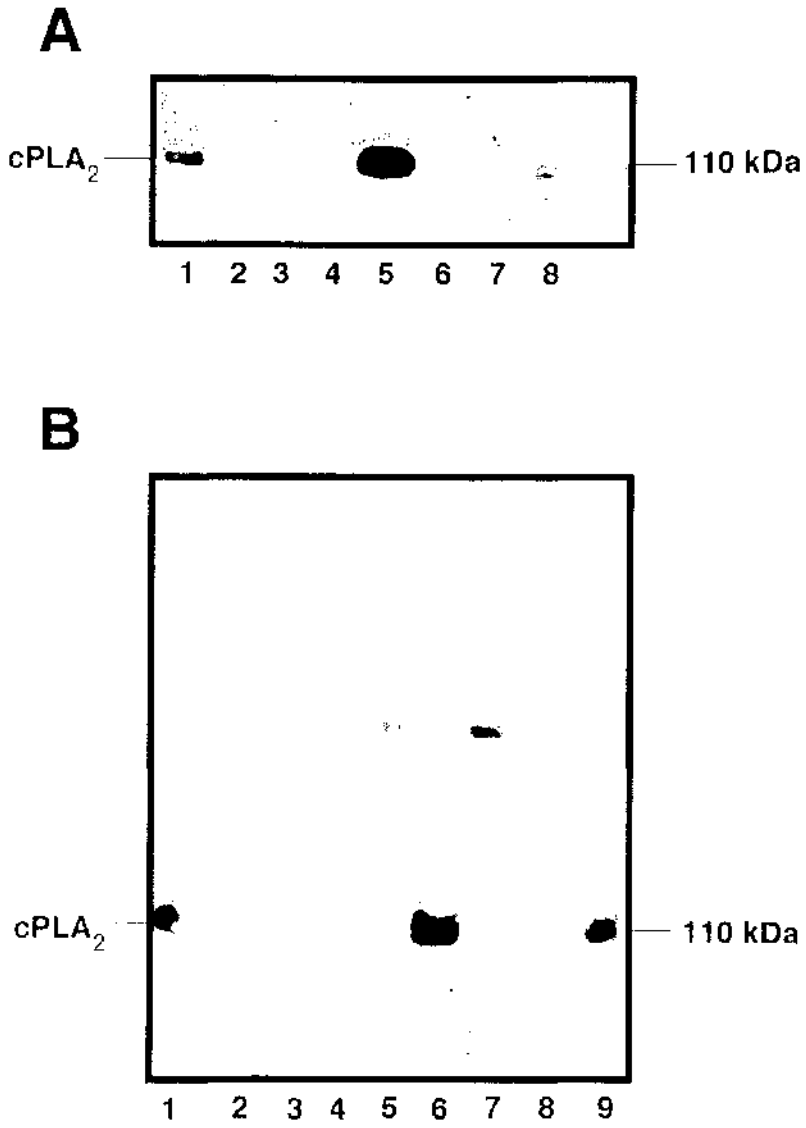

A
cPLA2
110 kDa
1 2 3 4 5 6 7 8
B
cPLA2
110 kDa
1 2 3 4 5 6 7 8 9

Figure 38. Cytosolic PLA_2 is not expressed by mature B or T lymphocytes. Cell lysates were prepared and immunoblotted as described in "Materials and Methods". In panel *A*, lane1: partially purified $cPLA_2$; lane 2: murine splenic resting B cells; lane 3: murine splenic resting T cells; lane 4: Daudi B cells; lane 5: HL60 cells; lane 6: Jurkat T cells; lane 7: Ramos B cells; lane 8: partially purified $cPLA_2$. In panel *B*, lane1: partially purified $cPLA_2$; lane 2: human tonsillar resting T cells; lane 3: human tonsillar *in vivo* activated T cells; lane 4: human tonsillar *in vivo* activated B cells; lane 5: Daudi B cells; lane 6: HL60 cells; lane 7: Jurkat T cells; lane 8: Ramos B cells; lane 9: partially purified $cPLA_2$. All lymphocyte cell lysates were loaded as 100 μg/lane whereas HL60 lysates were 40 μg/lane and partially purified $cPLA_2$ samples were 5 μg/lane.

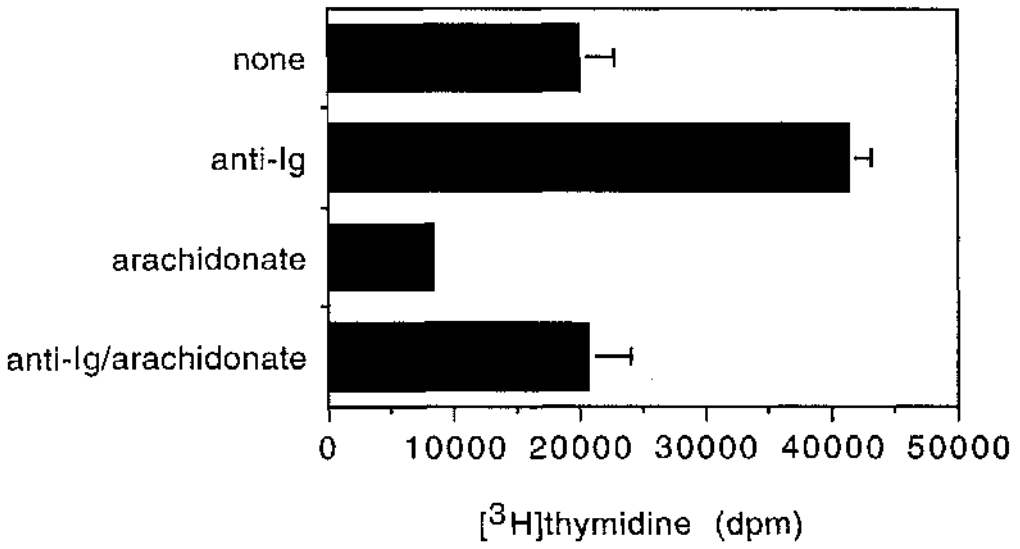

none
anti-Ig
arachidonate
anti-Ig/arachidonate
0
10000
20000
30000
40000
50000
[3H]thymidine (dpm)

Figure 39. Exogenous arachidonate inhibits basal and anti-Ig stimulated DNA synthesis in murine B cells. Murine splenic B cells, purified as described under "Materials and Methods" were cultured (5×10^6/ml) in the presence of anti-Ig (50 μg/ml), arachidonate (20 μM), or anti-Ig (50 μg/ml) plus arachidonate (20 μM). [^{3}H]thymidine uptake was determined after 72 h as described in the methods section. Data are presented as means ± SD where n=3.

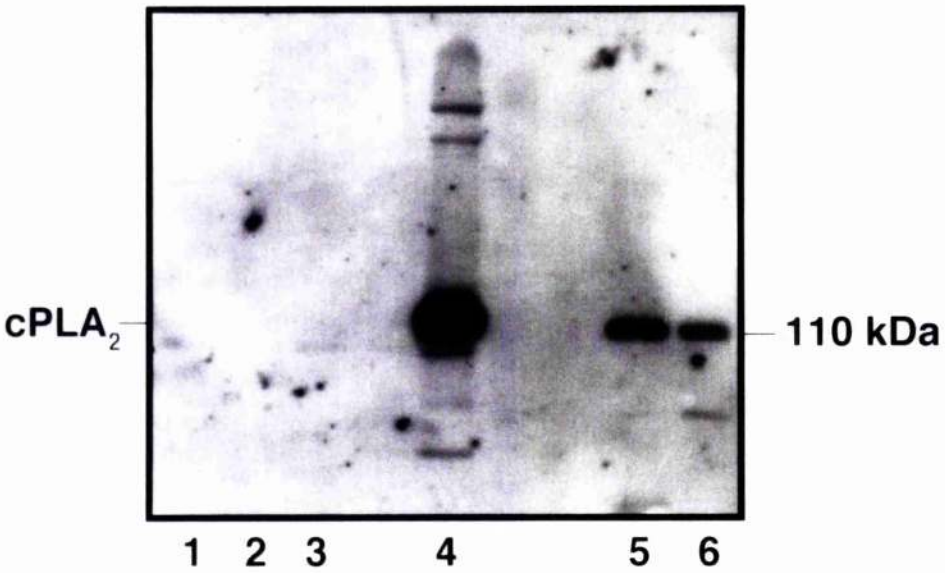

cPLA2
110 kDa
1
2
3
4
5
6

Figure 40. Cytosolic PLA_2 is expressed by murine thymocytes and immature splenocytes. Cell lysates were prepared and immunoblotted as described in "Materials and Methods". In lane 1: 697 pre-B cells; lane 2: 207 pre-B cells; lane 3: REH pre-B cells; lane 4: partially purified $cPLA_2$; lane 5: immature murine lymphocytes; lane 6: murine thymocytes. All lymphocyte cell lysates were loaded as 100 µg/lane whereas partially purified $cPLA_2$ samples were 5 µg/lane.

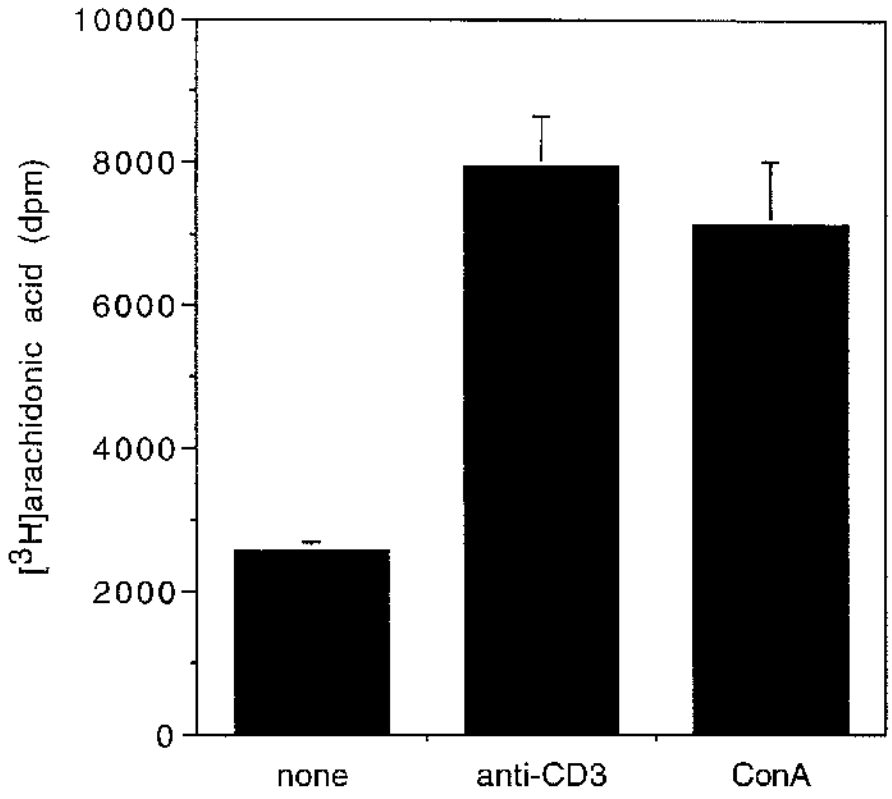

10000
8000
6000
4000
2000
0
[3H]arachidonic acid (dpm)
none
anti-CD3
ConA

Figure 41. cPLA2 is activated via the antigen receptors on murine thymocytes. Murine thymocytes, treated as described under "Materials and Methods" were radiolabelled with [^{3}H]arachidonic acid and stimulated with anti-CD3 (1 μg/ml) or ConA (50 μg/ml) for 30 min at 37°C. Arachidonic acid was measured by tlc analysis as described. The results are expressed as means ± SEM from single typical experiments where n=3.

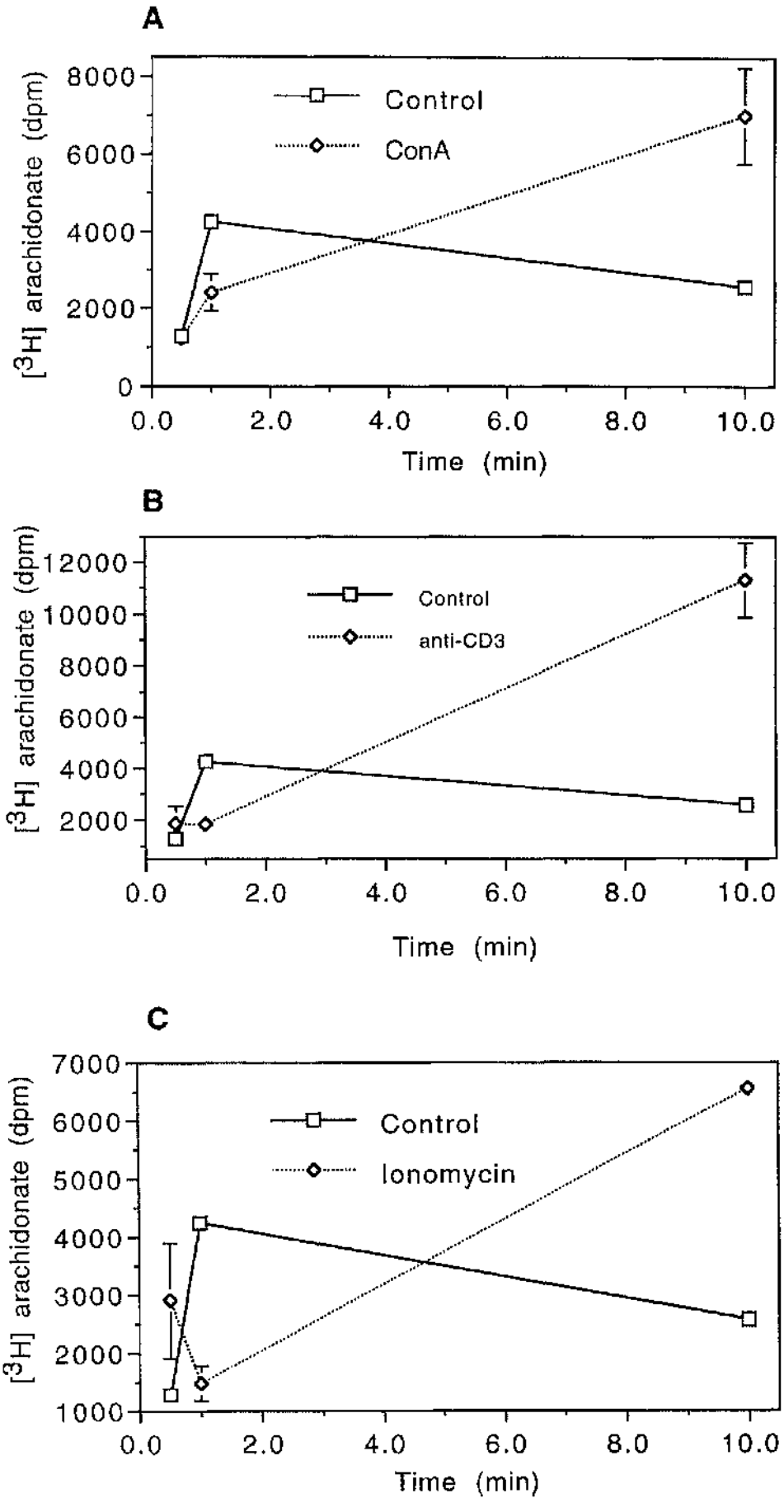

A
8000
6000
4000
2000
0
[3H] arachidonate (dpm)
Control
ConA
0.0
2.0
4.0
6.0
8.0
10.0
Time (min)
B
12000
10000
8000
6000
4000
2000
[3H] arachidonate (dpm)
Control
anti-CD3
0.0
2.0
4.0
6.0
8.0
10.0
Time (min)
C
7000
6000
5000
4000
3000
2000
1000
[3H] arachidonate (dpm)
Control
Ionomycin
0.0
2.0
4.0
6.0
8.0
10.0
Time (min)

Figure 42. Intracellular arachidonate release is stimulated in murine thymocytes by antigen receptor crosslinking or ionomycin. Murine thymocytes, treated as described under "Materials and Methods" were radiolabelled with [^{3}H]arachidonic acid. Intracellular levels of [^{3}H]arachidonic acid were measured in murine thymocytes following stimulation, in *panel A* with ConA (50 μg/ml); in *panel B* with anti-CD3 (1 μg/ml); or in *panel C* with ionomycin (1 μM); for 10 min. Arachidonic acid was measured by tlc analysis as described. The results are expressed as means ± SEM from single typical experiments where n=3.

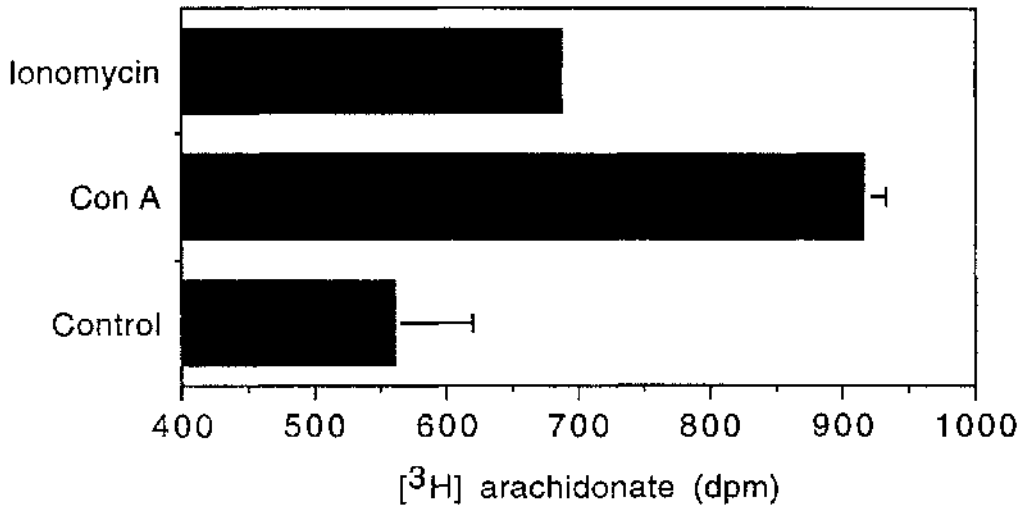

Ionomycin
Con A
Control
400
500
600
700
800
900
1000
[3H] arachidonate (dpm)

Figure 43. Con A and ionomycin induce the release of extracellular arachidonate by murine thymocytes. Murine thymocytes, treated as described under "Materials and Methods" were radiolabelled with [^{3}H]arachidonic acid, and extracellular levels of [^{3}H]arachidonic acid were measured following stimulation with ConA (50 μg/ml) or with ionomycin (1 μM) for 10 min. Arachidonic acid was measured by tlc analysis as described. The results are expressed as means ± SEM from single typical experiments where n=3.

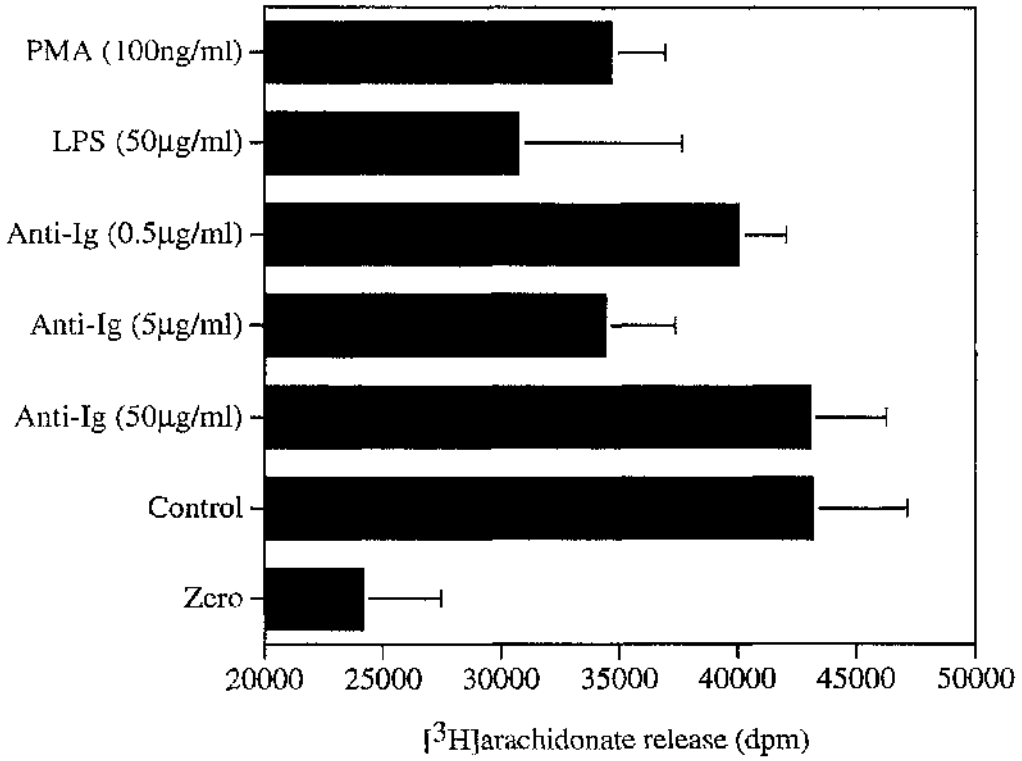
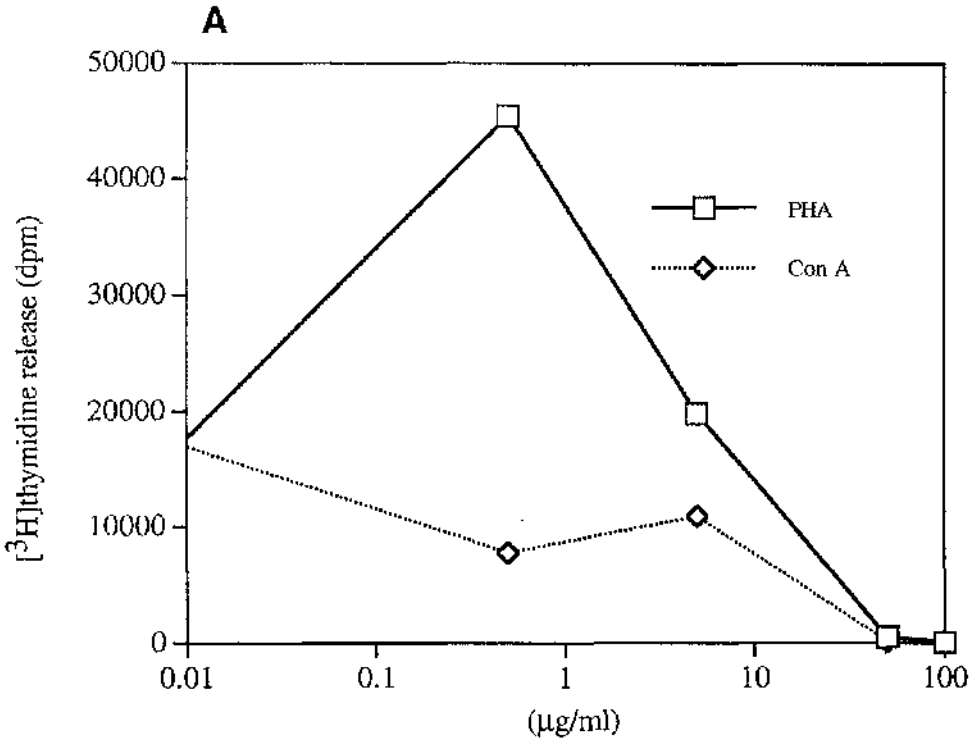

PMA (100ng/ml)
LPS (50µg/ml)
Anti-Ig (0.5µg/ml)
Anti-Ig (5µg/ml)
Anti-Ig (50µg/ml)
Control
Zero
20000
25000
30000
35000
40000
45000
50000
[3H]arachidonate release (dpm)

Figure 44. $cPLA_2$ is not activated in murine immature splenic B cells upon stimulation with anti-Ig, LPS or PMA. Immature murine splenic B cells, purified as described under "Materials and Methods", were radiolabelled with [^{3}H]arachidonic acid and stimulated with anti-Ig (0.5-50 μg/ml), LPS (50 μg/ml) or PMA (100 ng/ml) for 30 min. Arachidonic acid was measured by tlc analysis as described. The results are expressed as means ± SEM from single typical experiments where n=3.


A
50000
40000
30000
20000
10000
0
[3H]thymidine release (dpm)
PHA
Con A
0.01
0.1
1
10
100
(µg/ml)

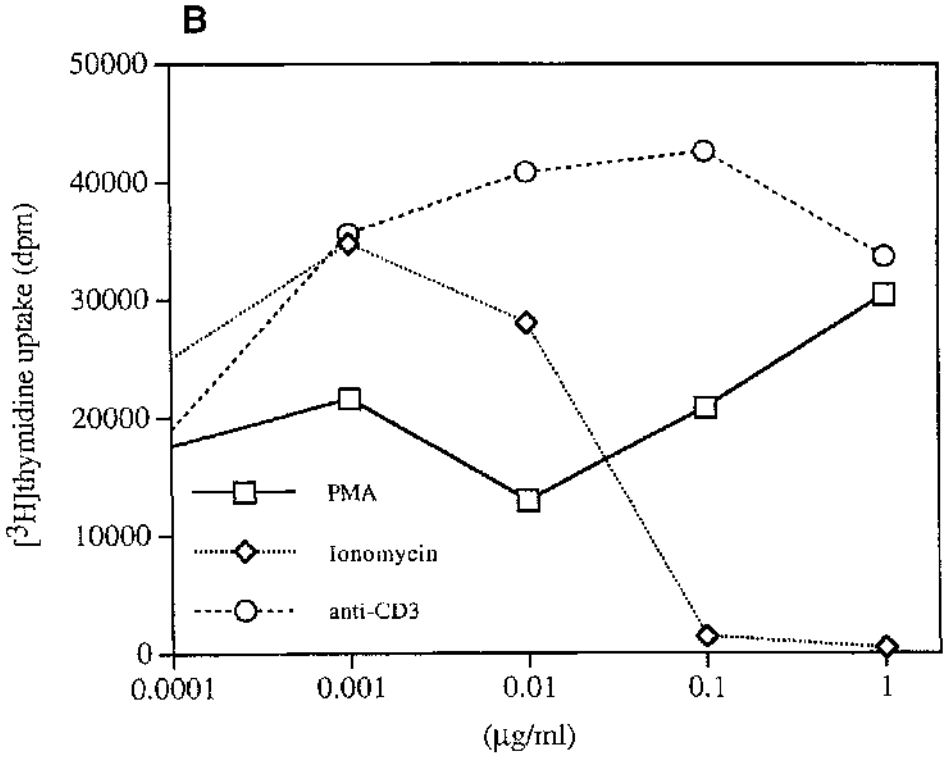
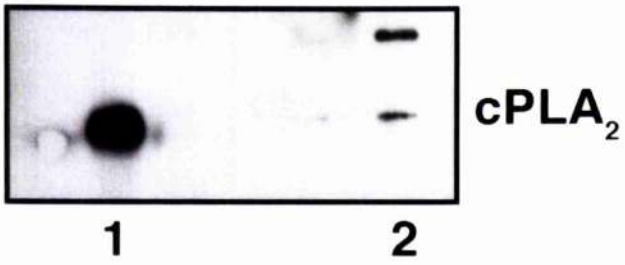

B
50000
40000
30000
20000
10000
0
[3H]thymidine uptake (dpm)
PMA
Ionomycin
anti-CD3
0.0001
0.001
0.01
0.1
1
(µg/ml)

Figure 45. Growth arrest is induced in T3 murine thymoma cells by antigen receptor crosslinking or stimulation with ionomycin. T3 murine thymoma cells, treated as described under "Materials and Methods", were cultured (2.5 x 10^5/ml), in *panel A* in the presence of PHA (0-100 μg/ml), or concanavalin A (0-100 μg/ml); in *panel B* with PMA (0.001-1 μg/ml), ionomycin (0.01-10 μg/ml), or anti-CD3 (0.01-10 μg/ml). [^{3}H]thymidine uptake was determined after 72 h as described in the methods section. Data are presented as means ± SD where n=3.



1) T3
2) $cPLA_2$

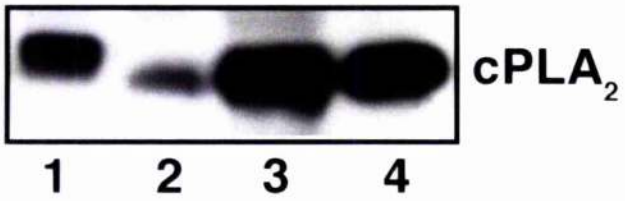


Figure 46. Cytosolic PLA_2 is expressed in T3 murine thymoma cells. Cell lysates were prepared, immunoprecipitated and then blotted as described in "Materials and Methods". In the top panel, lane 1: T3 murine thymoma cells (100 μg); lane 2: partially purified $cPLA_2$ (5 μg). In the bottom panel, lane 1: T3 murine thymoma cells (100 μg); lane 2: I3 cells (100μg); lane 3: H1 cells (100 μg); lane 4: C3 cells (100 μg),

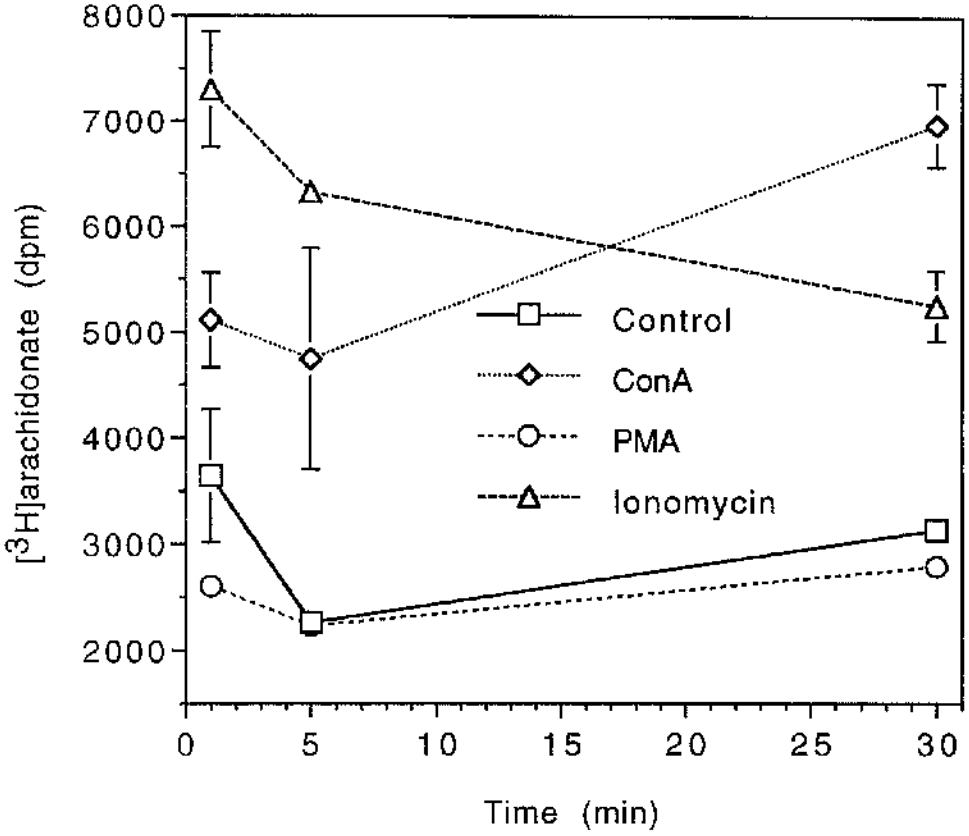

8000
7000
6000
5000
4000
3000
2000
[3H]arachidonate (dpm)
0
5
10
15
20
25
30
Time (min)
Control
ConA
PMA
Ionomycin

Figure 47. Stimulation of T3 thymoma cells with Con A or ionomycin induces release of arachidonate. Murine T3 thymoma cells, treated as described under "Materials and Methods", were radiolabelled with [^{3}H]arachidonic acid and stimulated with Con A (50 μg/ml), PMA (100 ng/ml) or ionomycin (1 μM) for the indicated time. Arachidonic acid was measured by tlc analysis as described. The results are expressed as means ± SEM from single typical experiments where n=3.

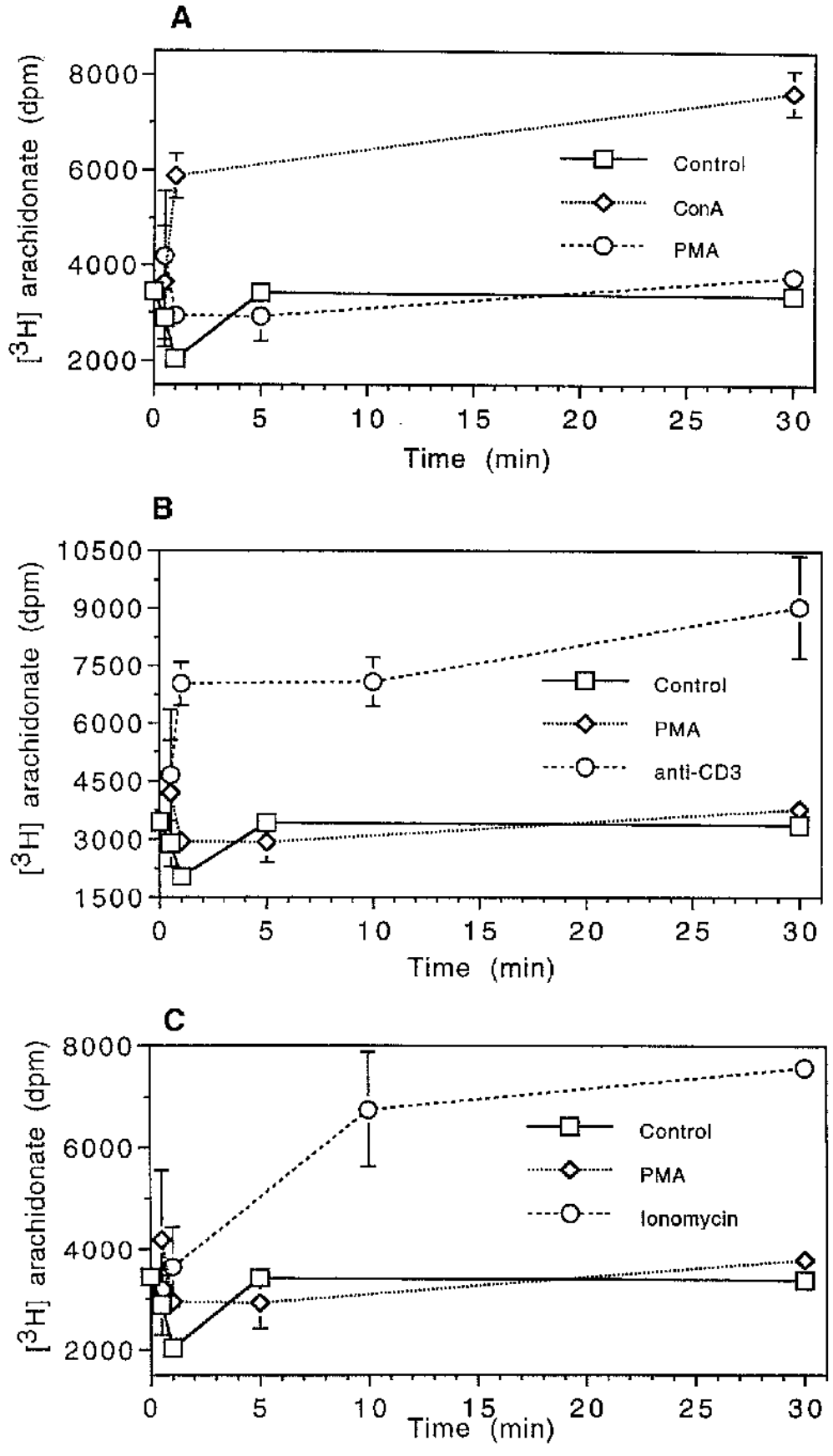

A
8000
6000
4000
2000
[3H] arachidonate (dpm)
0
5
10
15
20
25
30
Time (min)
Control
ConA
PMA
B
10500
9000
7500
6000
4500
3000
1500
[3H] arachidonate (dpm)
0
5
10
15
20
25
30
Time (min)
Control
PMA
anti-CD3
C
8000
6000
4000
2000
[3H] arachidonate (dpm)
0
5
10
15
20
25
30
Time (min)
Control
PMA
Ionomycin

Figure 48. Sustained intracellular arachidonate release is induced upon stimulation of T3 thymoma cells with Con A, anti-CD3, or ionomycin. Murine T3 thymoma cells, treated as described under "Materials and Methods", were radiolabelled with [^{3}H]arachidonic acid. Intracellular levels of [^{3}H]arachidonic acid were measured following stimulation, in *panel A* with with Con A (50 μg/ml) or PMA (100 ng/ml); in *panel B* with anti-CD3 (1 μg/ml) or PMA (100 ng/ml); and in *panel C* with ionomycin (1 μM) or PMA (100 ng/ml); for the indicated time. Arachidonic acid was measured by tlc analysis as described. The results are expressed as means ± SEM from single typical experiments where n=3.

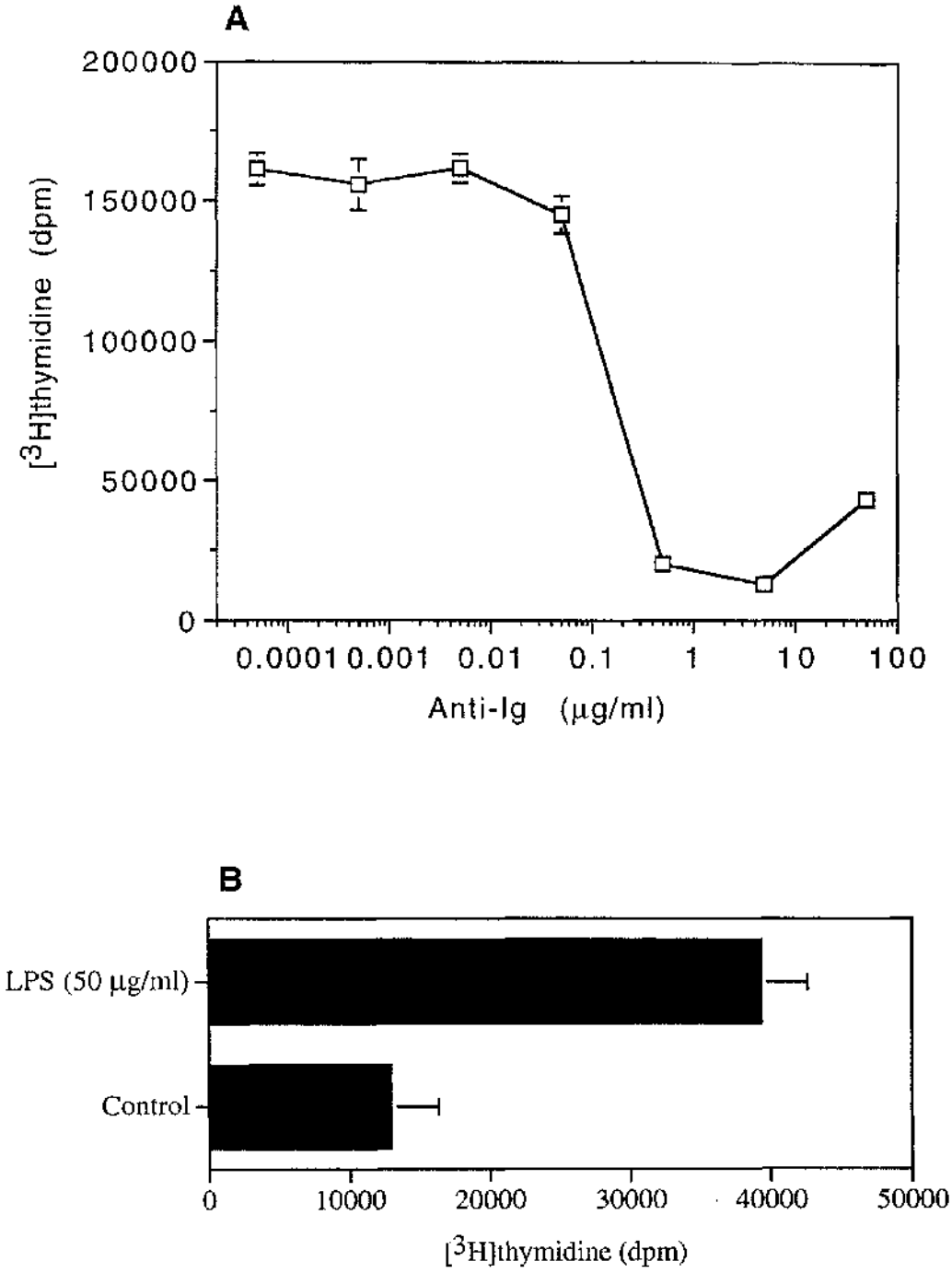

A
200000
150000
100000
50000
0
[3H]thymidine (dpm)
0.0001 0.001 0.01 0.1 1 10 100
Anti-Ig (µg/ml)
B
LPS (50 µg/ml)
Control
0 10000 20000 30000 40000 50000
[3H]thymidine (dpm)

Figure 49. Crosslinking of sIg on WEHI 231 immature B cells induces growth arrest. DNA synthesis is stimulated by LPS. In *panel A* WEHI 231 cells (5×10^4/ml) were cultured in the presence of anti-Ig (5×10^{-5} - 50 μg/ml); in *panel B* WEHI 231 cells (10^4/ml) were cultured in the presence of LPS (50 μg/ml). [^{3}H]thymidine uptake was determined after 72 h as described in the methods section. Data are presented as means ± SD.

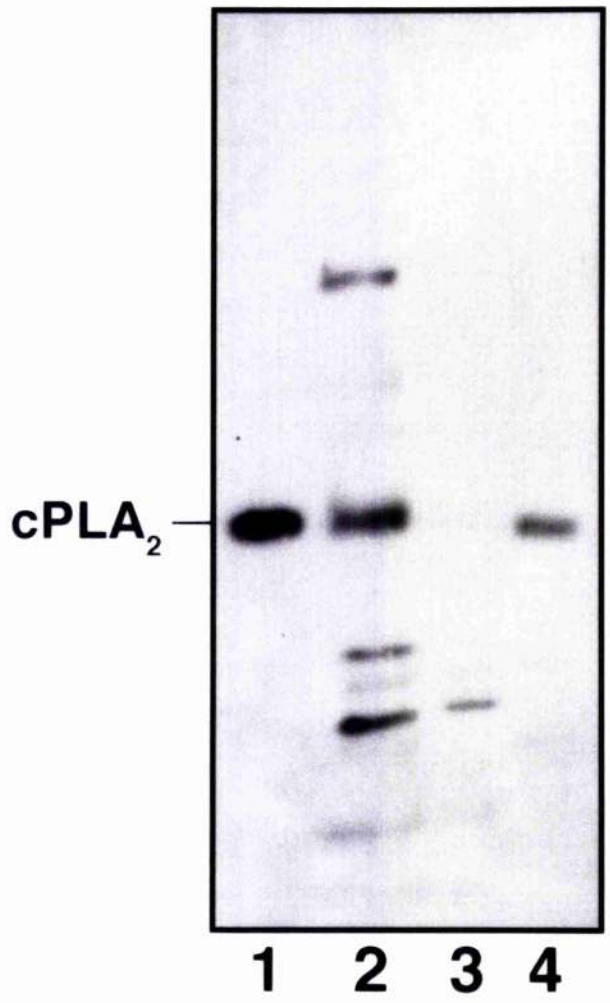

cPLA2
1
2
3
4

Figure 50. $cPLA_2$ is expressed in WEHI 231 immature murine B cells. Cell lysates were prepared and immunoblotted as described in "Materials and Methods". In lane 1: partially purified $cPLA_2$; lane 2: WEHI 231 immature murine B cells; lane 3: mature murine splenic B cells; lane 4: immature murine splenocytes. All lymphocyte cell lysates were loaded as 100 μg/lane whereas partially purified $cPLA_2$ samples were 5 μg/lane.

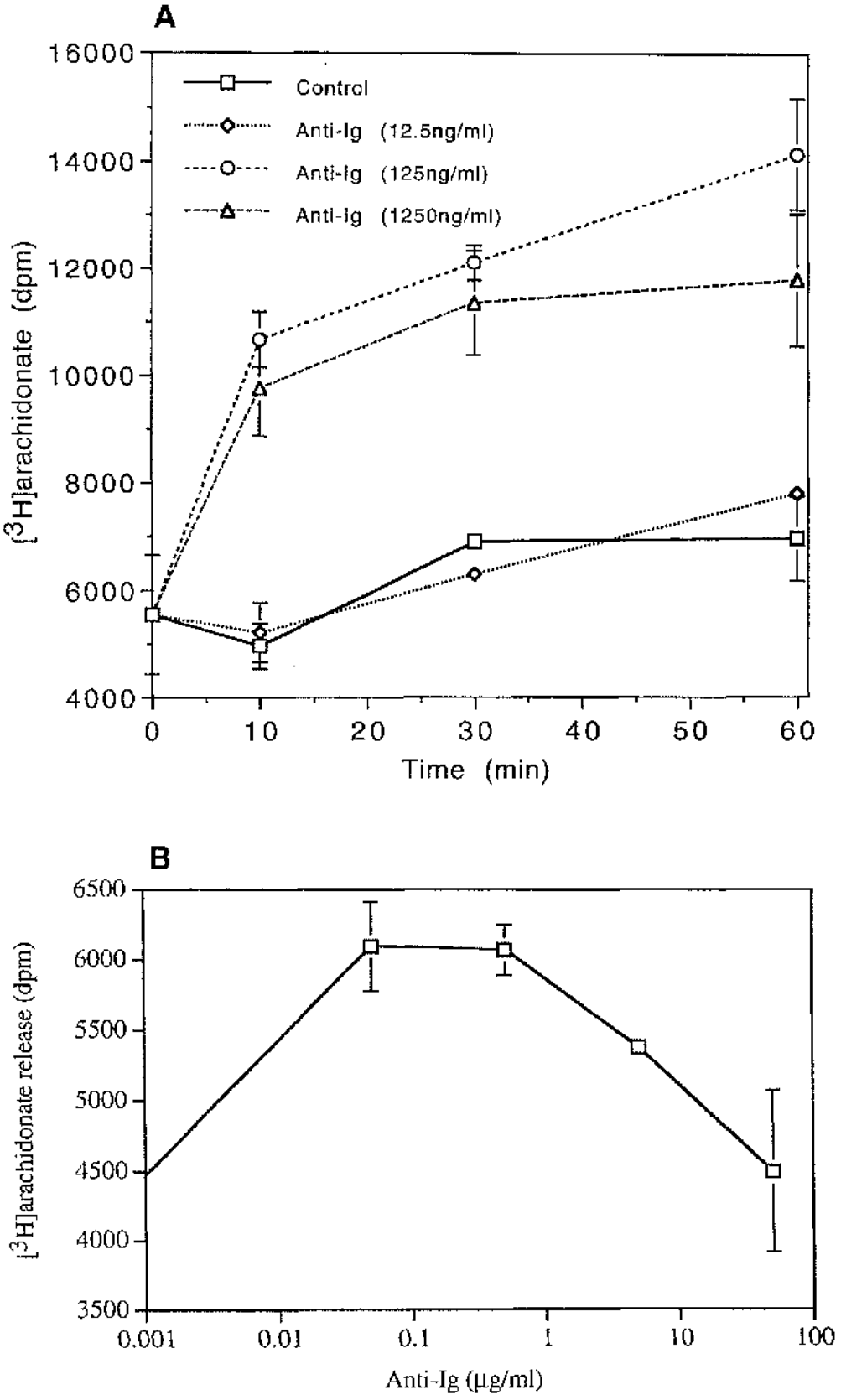
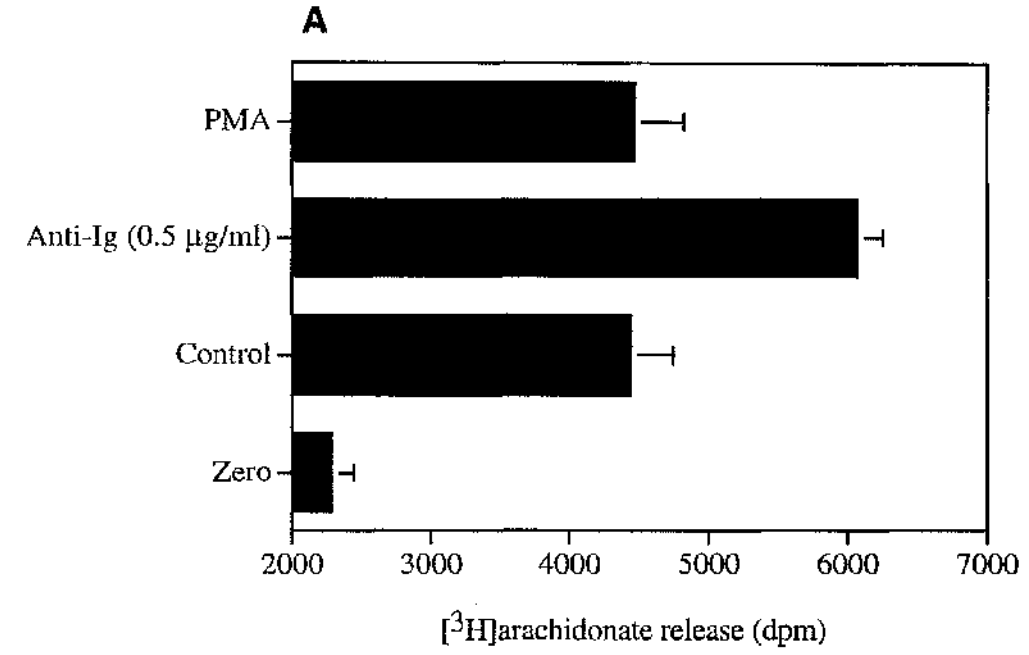

A
16000
14000
12000
10000
8000
6000
4000
[3H]arachidonate (dpm)
Control
Anti-Ig (12.5ng/ml)
Anti-Ig (125ng/ml)
Anti-Ig (1250ng/ml)
0
10
20
30
40
50
60
Time (min)
B
6500
6000
5500
5000
4500
4000
3500
[3H]arachidonate release (dpm)
0.001
0.01
0.1
1
10
100
Anti-Ig (µg/ml)

Figure 51. Crosslinking of the antigen receptors on WEHI 231 B cells stimulates the release of arachidonate. WEHI-231 cells were radiolabelled with [^{3}H]arachidonic acid, and stimulated [^{3}H]arachidonic acid generation was measured, in *panel A* following ligation of the antigen receptors with anti-Ig at final concentrations of 0.0125 μg/ml, 0.125 μg/ml or 1.25 μg/ml for the indicated times; in *panel B* following stimulation with anti-Ig (0.05-50 μg/ml) for 30 min. The results are expressed as means ± SEM from single typical experiments where n=3.


A
PMA
Anti-Ig (0.5 μg/ml)
Control
Zero
2000
3000
4000
5000
6000
7000
[3H]arachidonate release (dpm)

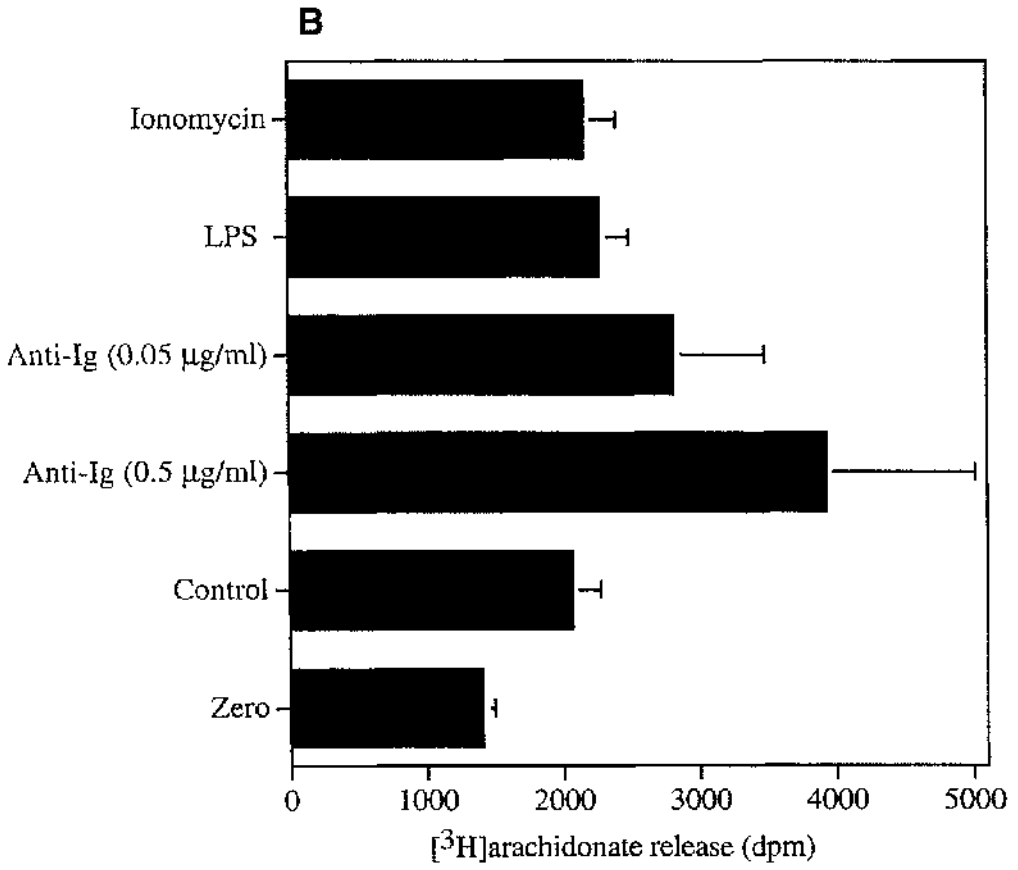

B
Ionomycin
LPS
Anti-Ig (0.05 μg/ml)
Anti-Ig (0.5 μg/ml)
Control
Zero
0
1000
2000
3000
4000
5000
[3H]arachidonate release (dpm)

Figure 52. Stimulation of WEHI 231 cells with PMA, ionomycin or LPS does not stimulate the release of arachidonate. WEHI-231 cells were radiolabelled with [^{3}H]arachidonic acid and stimulated, in *panel A* with anti-Ig (0.5 μg/ml) or PMA (100 ng/ml); in *panel B* with anti-Ig (0.05 or 0.5 μg/ml), LPS (50 μg/ml), or ionomycin (1 μM); for 30 min. Arachidonic acid was measured by tlc analysis as described. The results are expressed as means ± SEM from single typical experiments where n=3.

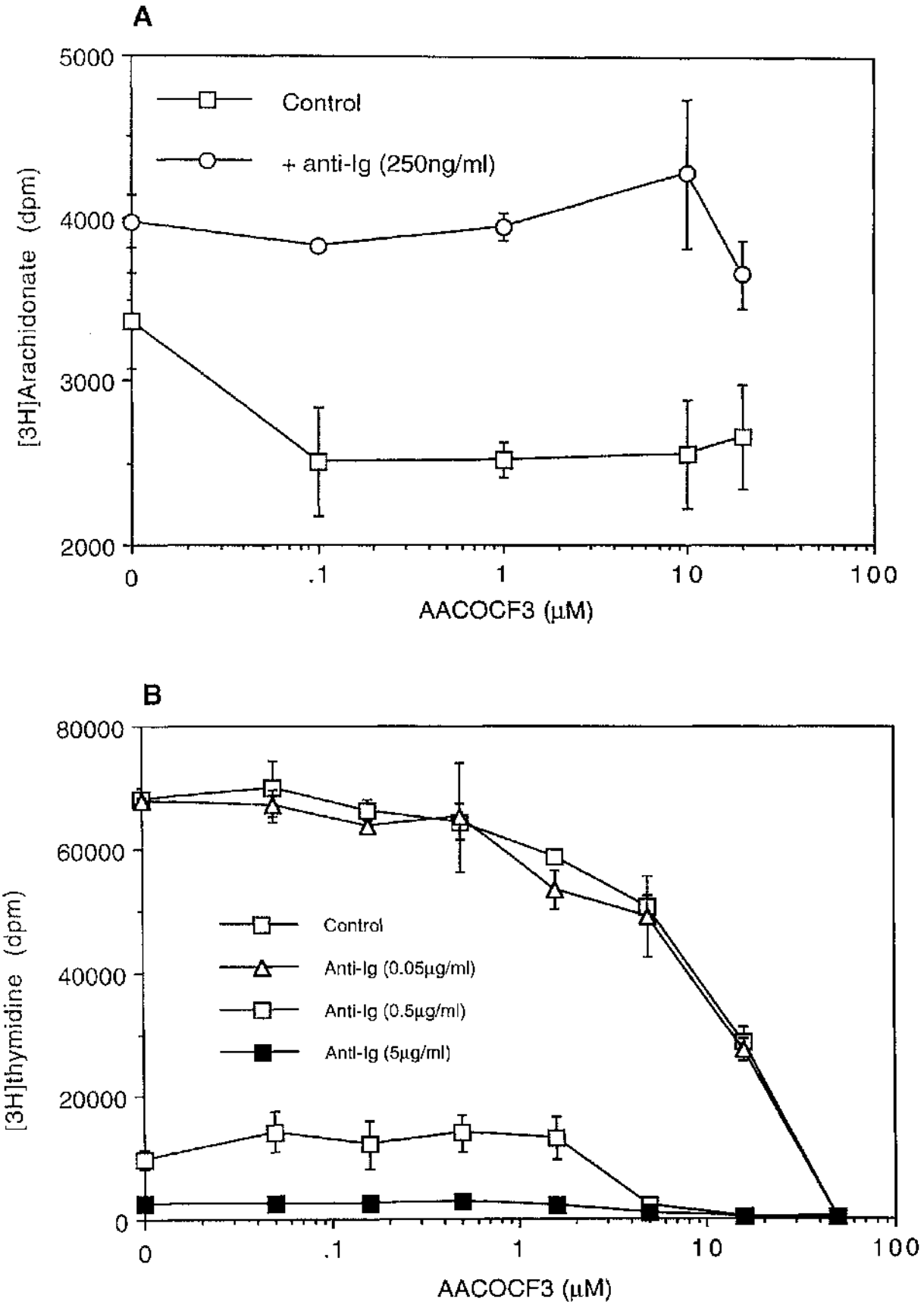

A
5000
4000
3000
2000
[3H]Arachidonate (dpm)
Control
+ anti-Ig (250ng/ml)
0
.1
1
10
100
AACOCF3 (μM)
B
80000
60000
40000
20000
0
[3H]thymidine (dpm)
Control
Anti-Ig (0.05μg/ml)
Anti-Ig (0.5μg/ml)
Anti-Ig (5μg/ml)
0
.1
1
10
100
AACOCF3 (μM)

Figure 53. The arachidonate analogue $AACOCF_3$ inhibits basal arachidonate generation and DNA synthesis in WEHI 231 B cells. In *panel A* WEHI-231 cells were radiolabelled with [^{3}H]arachidonic acid and stimulated, with anti-Ig (0.5 μg/ml) or medium in the presence of $AACOCF_3$ (0-20 μM) for 30 min. Arachidonic acid was measured by tlc analysis as described. The results are expressed as means ± SEM from single typical experiments where n=3. In *panel B* WEHI 231 cells (5 x 10^4/ml) were cultured, in the presence of anti-Ig (0, 0.05, 0.5 or 5 μg/ml) in the presence of $AACOCF_3$ (0-50 μM). [^{3}H]thymidine uptake was determined after 72 h as described in the methods section. Data are presented as means ± SD, n=3.

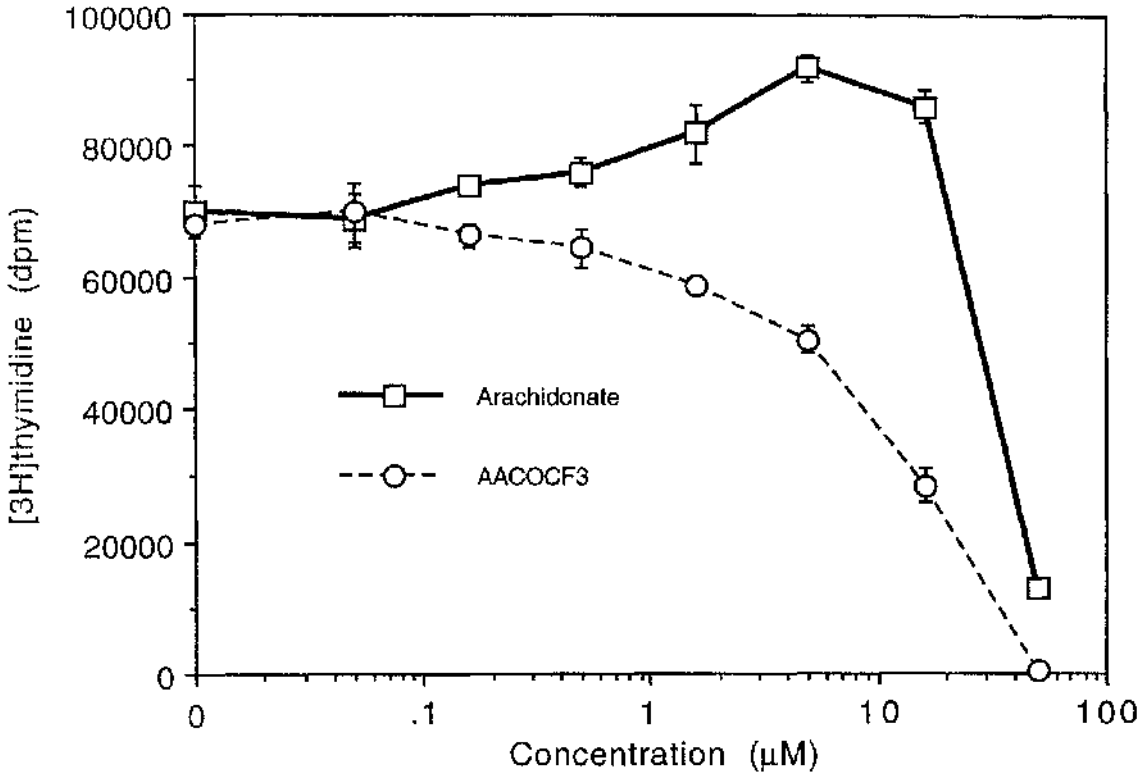
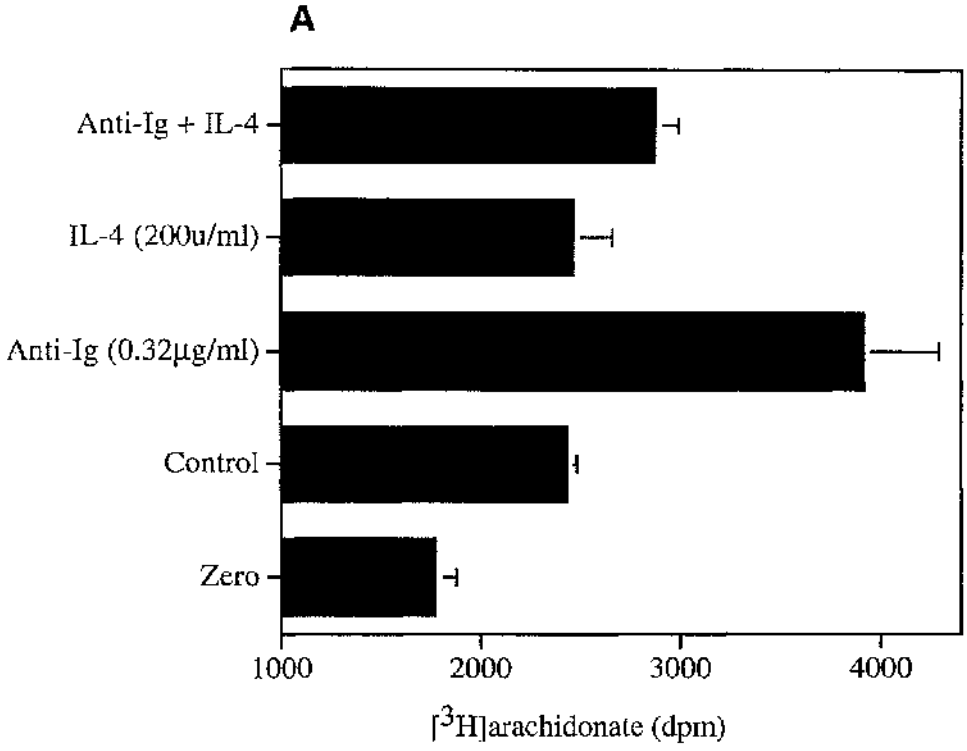

100000
80000
60000
40000
20000
0
[3H]thymidine (dpm)
Arachidonate
AACOCF3
0
.1
1
10
100
Concentration (µM)

Figure 54. Effects of arachidonate on DNA synthesis in WEHI 231 cells. WEHI 231 cells (5×10^4/ml) were cultured, in the presence of arachidonate (0-50 μM) or $AACOCF_3$ (0-50 μM). [^{3}H]thymidine uptake was determined after 72 h as described in the methods section. Data are presented as means ± SD, n=3.


A
Anti-Ig + IL-4
IL-4 (200u/ml)
Anti-Ig (0.32µg/ml)
Control
Zero
1000
2000
3000
4000
[3H]arachidonate (dpm)

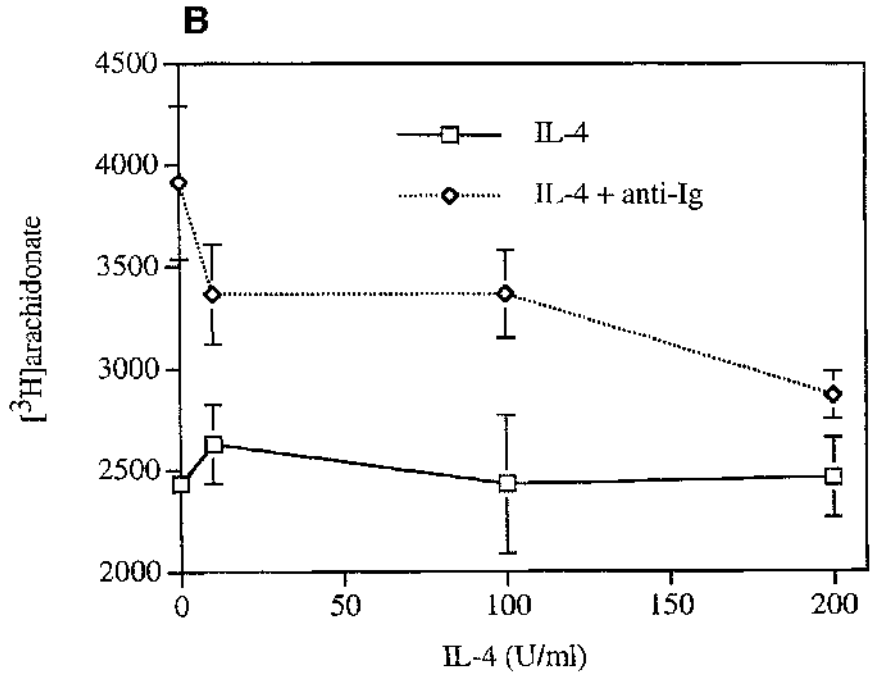

B
4500
4000
3500
3000
2500
2000
IL-4
IL-4 + anti-Ig
[3H]arachidonate
0
50
100
150
200
IL-4 (U/ml)

Figure 55. IL-4 inhibits the anti-Ig-mediated release of arachidonate in WEHI 231 B cells. In *panels A and B* WEHI-231 cells were radiolabelled with [^{3}H]arachidonic acid and stimulated, with anti-Ig (0.32 μg/ml) or medium in the presence of IL-4 (0-200 U/ml) for 30 min. Arachidonic acid was measured by tlc analysis as described. The results are expressed as means ± SEM from single typical experiments where n=3.

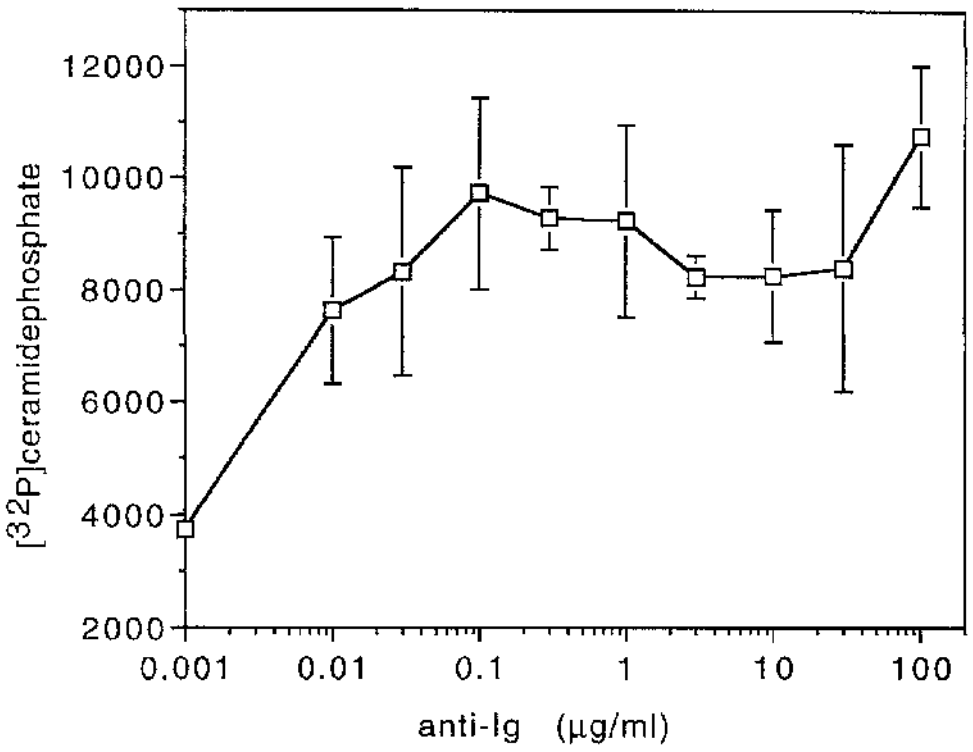

12000
10000
8000
6000
4000
2000
[32P]ceramidephosphate
0.001
0.01
0.1
1
10
100
anti-Ig (µg/ml)

Figure 56. Anti-Ig stimulates the generation of ceramide in WEHI 231 B cells. WEHI 231 B cells were stimulated with anti-Ig (0-100 μg/ml) for 30 min. Cellular lipids were extracted and ceramide levels were measured using the DAG-kinase assay and tlc analysis. The results are expressed as means ± SEM from single typical experiments where n=3.

5. Key roles for lipid signalling pathways in lymphocyte activation, maturation and cell death?

Lymphocytes respond to antigen receptor stimulation in a maturation stage-dependent manner. Thus, whereas mature lymphocytes become activated and proliferate in response to antigen receptor crosslinking, immature lymphocytes become unresponsive or undergo apoptosis. This investigation has focused on the role of phosphatidylcholine hydrolysis, and in particular on two key enzymes which mediate the hydrolysis of phosphatidylcholine, phospholipase D and phospholipase A_2, in the regulation of such maturation state-dependent lymphocyte responses. The findings presented in this thesis implicate key roles for these lipid signalling pathways in the regulation of lymphocyte activation, maturation and cell death.

PtdCho-PLD has been found to play a role in the transduction of intracellular signals initiated by mitogenic stimulation of a variety of cell types. However, it was found that stimulation of B cells, via sIg, did not induce activation of PtdCho-PLD, indicating that the B cell antigen receptor is not coupled to PtdCho-PLD in these cells. Moreover, PtdCho-PLD was not stimulated under conditions designed to mimic T cell-dependent B cell activation. In contrast, following mitogenic stimulation, a putative PtdIns-PLD could be stimulated via sIg on B cells. Interestingly, stimulation of B cells with ATP, arachidonate, ceramide and sphingosine induced PtdCho-PLD activation. As these reagents was found to suppress B cell proliferation, these findings suggest that PtdCho-PLD could be involved in the transduction of negative, anti-proliferative signals in B lymphocytes.

PLA_2 has been implicated in the control of proliferation and apoptosis in a number of cells types, and this investigation has generated a number of findings which indicate a key role for $cPLA_2$ in lymphocyte maturation. Firstly, $cPLA_2$ is expressed by B and T cells in a maturation state-specific manner: $cPLA_2$ is not expressed in mature B

and T cells but is expressed in immature murine splenocytes, murine thymocytes, T3 murine thymoma cells and WEHI 231 murine immature B cells, although not in the pre-B cell lines 697, 207 and REH. Secondly, the antigen receptors on immature B and T cells are coupled to $cPLA_2$ activation under conditions leading to the induction of growth arrest and/or apoptosis. Thirdly, stimulation of WEHI 231 immature B cells with specific stimuli known to rescue these cells from sIgM-mediated apoptosis, including IL-4, CD40 crosslinking and LPS, did not stimulate $cPLA_2$ activity. Moreover, IL-4 was found to block sIg-coupled arachidonate generation, suggesting that B cell rescue from apoptosis could be effected via inhibition of $cPLA_2$ activation. Furthermore, sphingomyelinase, a signal transducer which has been shown to be activated by arachidonate in other cell types, was found to be activated temporally downstream of $cPLA_2$ following crosslinking of the antigen receptors on WEHI 231 immature B cells. The findings of this investigation therefore strongly suggest that antigen receptor-mediated release of arachidonate is a key event in regulation and transduction of growth arrest and/or apoptosis in immature B cells.

In conclusion, the studies outlined above suggest that PtdCho-signalling pathways may transduce anti-proliferative and/or apoptotic signals throughout lymphocyte development: Models outlining the putative signalling mechanisms underlying the transduction of such antiproliferative signals in immature and B cells are presented in Figs. 57 and 58.

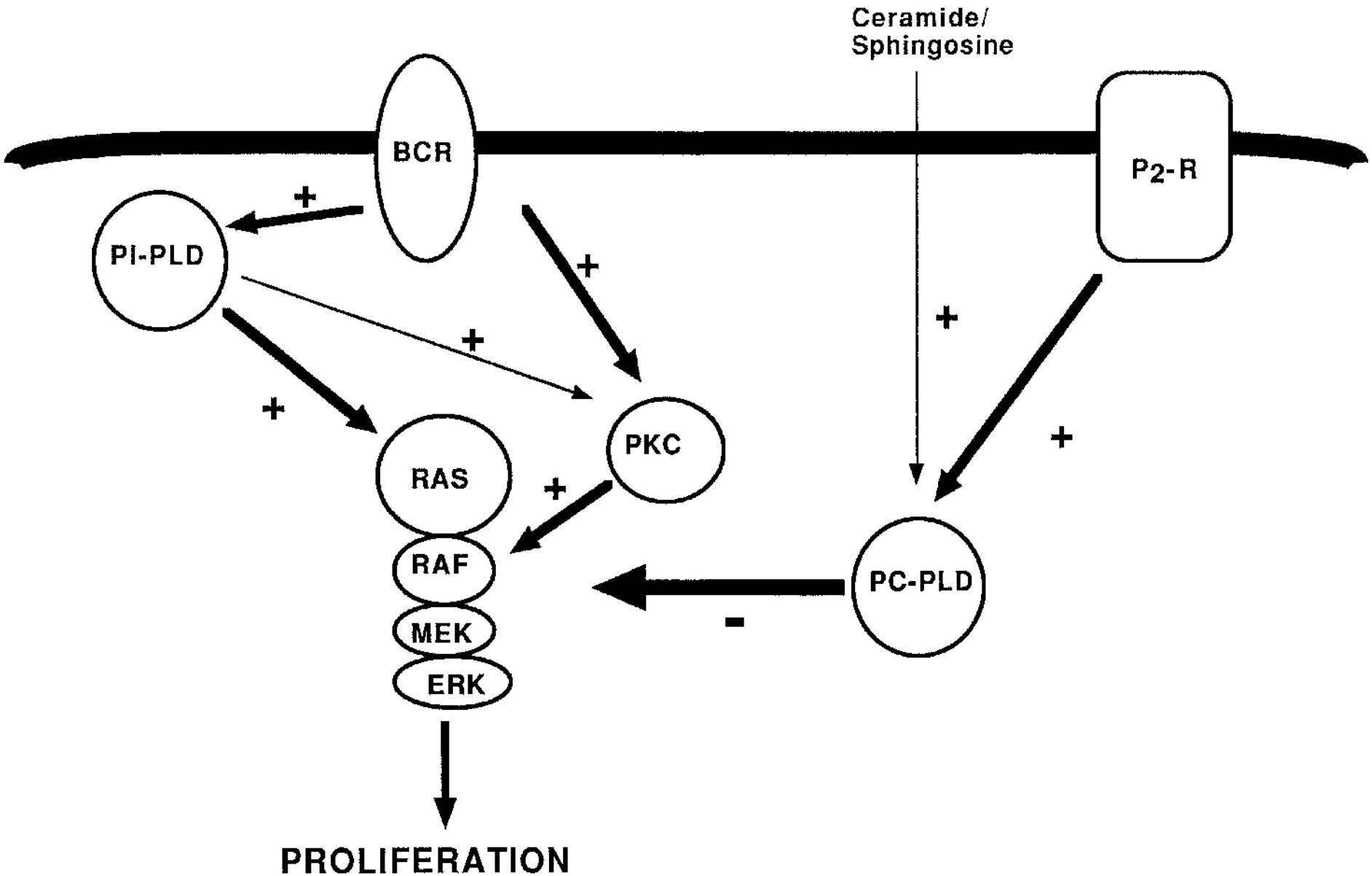

Ceramide/
Sphingosine
BCR
P2-R
PI-PLD
+
+
+
+
+
+
PKC
RAS
RAF
MEK
ERK
PC-PLD
-
PROLIFERATION

Figure 57. Model of phospholipase D signalling in mature B cells.

The antigen receptors on B cells are coupled to a putative PtdIns-PLD activity and potential consequent activation of PKC isoforms involved in the transduction of mitogenic signals. In addition, since the steroyl-arachidonoyl species of PtdOH (derived predominantly from PtdIns) has been found to induce the activation of Ras, BCR-coupling to PtdIns-PLD may play a role in the BCR-mediated activation of the RasMAPkinase pathway, and consequent downstream signalling events resulting in the induction of B cell proliferation. In contrast, stimulation of B cells with ATP, presumably via a P_2-purinoceptor, or by addition of exogenous ceramide or sphingosine, results in the activation of PtdCho-specific PLD (PC-PLD), suggesting that this enzyme may be involved in the transduction of negative, anti-proliferative signals in B cells. The downstream targets of PtdOH-derived from such PtdCho-PLD activity, are at present unknown, but, it is possible that PtdCho-PLD may play a directly antagonistic role to PtdIns-PLD-mediated signals by negatively modulating the activation of a number of mitogenic signals such as PKC and/or elements of the RasMAPkinase pathway.

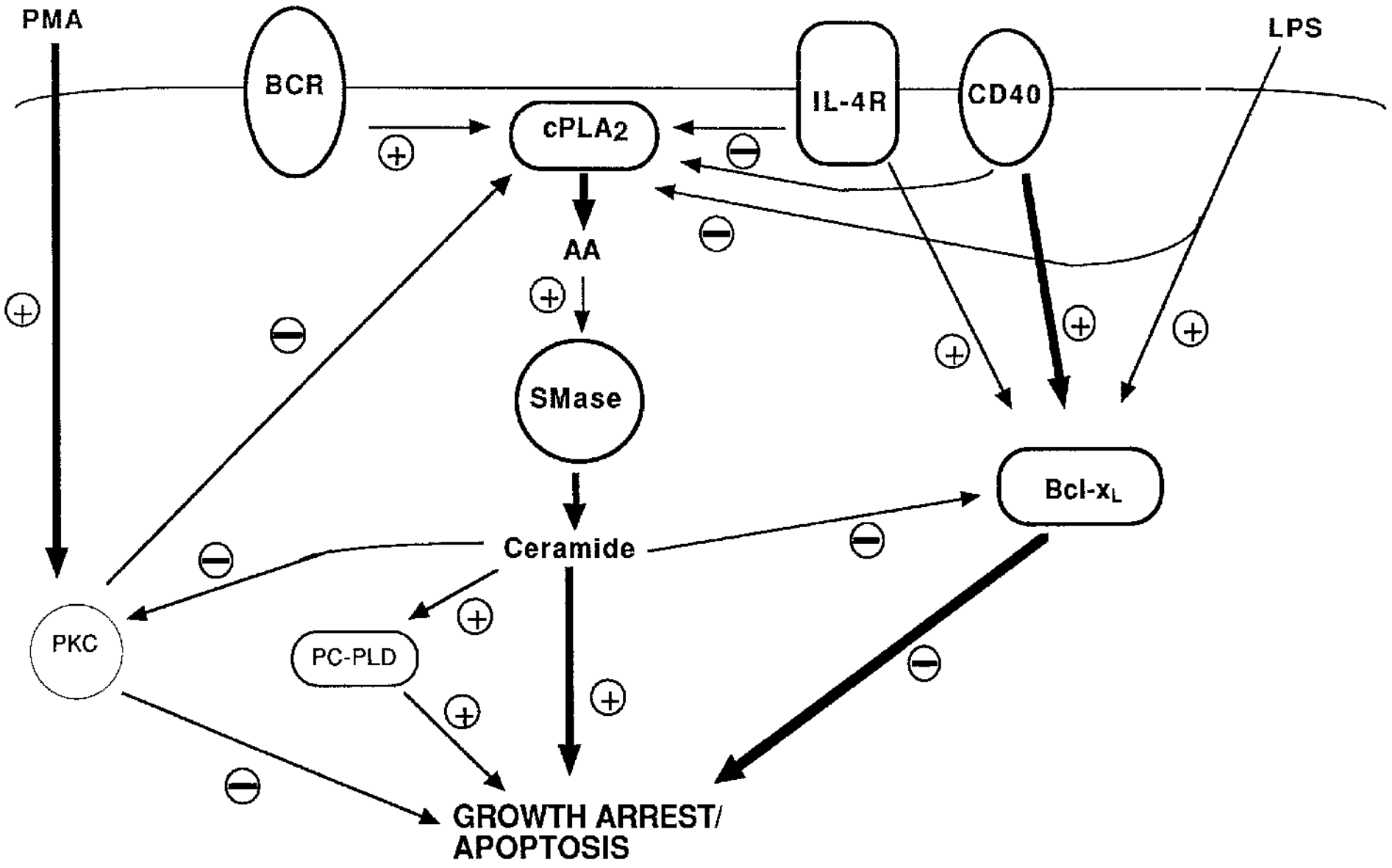

PMA
BCR
cPLA2
IL-4R
CD40
LPS
AA
SMase
Bcl-xL
Ceramide
PKC
PC-PLD
GROWTH ARREST/
APOPTOSIS

Figure 58. Model of anti-proliferative pathway transduced by $cPLA_2$ in immature B cells.

$cPLA_2$ activation is proposed to play a key role in the transduction of growth arrest and/or apoptosis in immature B cells. Crosslinking of the antigen receptors results in the activation of $cPLA_2$ and the release of arachidonate, which may induce the activation of downstream signalling elements including SMase. SMase is indeed activated following BCR crosslinking, and this results in the production of ceramide. Ceramide has been implicated in the regulation of a number of signalling elements including PtdCho-PLD, PKCζ, ceramide-activated protein phosphatase (CAPP) and ceramide-activated protein kinase (CAPK) and may also be involved in the modulation of Bcl-X_L expression and activity. Bcl-X_L protects immature B cells from BCR-mediated apoptosis and may regulate the activation of negative signalling elements such as $cPLA_2$, possibly by inhibiting the mobilisation of calcium which is required for the activation of this enzyme. This proposal is consistent with the finding that rescue from sIg-mediated growth arrest by coligation of receptors such as CD40 results in the upregulation of Bcl-X_L. Finally, signalling elements such as PtdCho-PLD, PKCζ, CAPP and/or CAPK may couple ceramide generation to downstream signalling elements such as NF-κB, Rb and Myc which have been proposed to play key roles in the regulation of cell differentiation, proliferation and apoptosis.

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