EARLY BIOCHEMICAL RESPONSES TO MITOGENIC STIMULATION OF T LYMPHOCYTES

by

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DECLARATION

This thesis is entirely the result of my own work, except where specific acknowledgement is made in the text. No part of it has been, or currently is being, submitted to any other Board for any other purposes.

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ABBREVIATIONS

Accepted abbreviations in this thesis are listed in Biochem. J. (1989) 257, 16-17. In addition the following abbreviations are used:

ACh	Acetylcholine
ATPγS	Adenosine 5'-O-(3-triphosphate)
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Intracellular free calcium concentration
$Ca^{2+}{}_o$	Extracellular calcium
CDTA	1,2-diaminocyclohexane-N, N, N', N'-tetraacetic acid
Con A	Concanavalin A
DAG	Diacylglycerol
EGF(r)	Epidermal growth factor (receptor)
FCS	Foetal calf serum
FI	Fluorescence intensity
GppNHp	Guanosine 5'-β,γ-imidotrisphosphate
GTPγS	Guanosine 5'-O-(3-thiotriphosphate)
IBMX	3-isobutyl-1-methyl-xanthine
IFN	Interferon
ILx	Interleukin x
IL2r	Interleukin 2 receptor
LiDS	Lithium dodecyl sulphate
LFA	Lymphocyte function associated antigen
MHC	Major histocompatibility complex
ODC	Ornithine decarboxylase
PBL	Peripheral blood lymphocyte
PDBu	Phorbol dibutyrate
PDGF(r)	Platelet-derived growth factor
PGE_1	Prostaglandin E_1

PHA	Phytohaemagglutinin
PPO	2,5-diphenyloxazole
TAP	T cell activating protein
T_c	Cytotoxic T cell
TCR	T cell receptor
TEN	Triethanolamine
T_H	Helper T cell
T_i	Idiotypic antigen receptor, expressed on T lymphocytes
TPA	12-O-tetradecanoyl 13-phorbol acetate

SUMMARY

The sequence of events between mitogen-receptor interaction and DNA synthesis was examined using T lymphocytes as a model system. The ability of four ligands (Concanavalin A (Con A), interleukin 2 (IL2), anti-T3 antibody and anti-Thy 1 antibody) to stimulate proliferation of two cell types (BALB/c thymocytes and a constitutive IL2r-expressing T cell line, clone 4) was determined. Clone 4 proliferated in response to IL2 and anti-Thy 1 antibody, but not Con A or anti-T3 antibody. BALB/c thymocytes responded strongly to Con A, but anti-Thy 1 antibody and anti-T3 antibody were only poor mitogens. IL2 alone was ineffective as a mitogen for thymocytes, but synergised with Con A, anti-Thy 1 antibody or anti-T3 antibody, suggesting that the failure of the latter two to activate significant DNA synthesis in thymocytes was due to the failure of these ligands to induce appreciable IL2 secretion. Anti-IL2 receptor (IL2r) antibody inhibited proliferation in response to all ligands tested, suggesting that ligand-induced proliferation required IL2-IL2r interaction.

The effect of each ligand on the intracellular free calcium concentration ($[Ca^{2+}]_i$) and the amounts of inositol phosphates and c-myc mRNA in both cell types was determined. Except for IL2 on thymocytes, all ligands stimulated c-myc mRNA accumulation in both cell types. Con A, anti-T3 antibody and anti-Thy 1 antibody stimulated increases in inositol phosphates in both cell types. Stimulation of thymocytes with Con A resulted in rapid, transient accumulation of $Ins(1,4,5)P_3$, rapid persistent elevation of $InsP_4$ and slow monotonic production of $Ins(1,3,4)P_3$. The amount of $InsP_5$ was not changed by Con A and $InsP_6$ was not detected. Calcium-depletion experiments and data from a permeabilised thymocyte system suggest that $Ins(1,4,5)P_3$ is phosphorylated to give $InsP_4$ by a Ca^{2+}-dependent enzyme. Activation of $InsP_3$ kinase by the Con A-stimulated $[Ca^{2+}]_i$ increases may be sufficient to account for the transience of $Ins(1,4,5)P_3$ accumulation.

Increases in $[Ca^{2+}]_i$ were stimulated by Con A and anti-Thy 1 antibody in both cell types, but interaction of IL2 with IL2r on clone 4 failed to induce $[Ca^{2+}]_i$ elevation or

inositol phosphate accumulation. The ability of anti-Thy 1 antibody and anti-T3 antibody to stimulate $PtdInsP_2$ hydrolysis and $[Ca^{2+}]_i$ elevation but not appreciable thymocyte proliferation in the absence of exogenous IL2 suggests that increases in inositol phosphates and $[Ca^{2+}]_i$ are insufficient to stimulate IL2 secretion, but may be required for induction of IL2r expression.

Several inhibitory receptor interactions were observed. IL2 and anti-Thy 1 antibody mutually antagonised clone 4 mitogenesis, and anti-CD4 antibody was a potent inhibitor of clone 4 proliferation induced by anti-Thy 1 antibody. The mechanism of antagonism of IL2 and anti-Thy 1 antibody was unclear; IL2-induced c-myc mRNA accumulation was unaffected by anti-Thy 1 antibody and IL2 caused only a small reduction in $[Ca^{2+}]_i$ and inositol phosphate responses to anti-Thy 1 antibody. The ability of anti-CD4 antibody to inhibit ligand-stimulated increases in $[Ca^{2+}]_i$, inositol phosphates and c-myc mRNA in clone 4 suggests that, although not sufficient to stimulate proliferation, these events may be important for the transition from quiescence to DNA synthesis.

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1.1 Mitogenic stimulation of T lymphocytes : a model for eukaryotic cell growth control

Mitotic cell division is an integral process both in growing and mature organisms, where cells divide to replace dying or damaged cells. Cell division is stimulated by the interaction of a ligand, or mitogen, with either a cell surface receptor or an intracellular receptor. Many mitogens and complementary receptors have been characterised, and selective expression of these allows rigorous control of the transition from quiescence to mitosis of individual cells and populations. Mitogen-receptor interaction triggers quiescent cells to pass through a defined sequence of stages prior to division (Pardee et al., 1978). DNA synthesis and mitosis are separated by two periods of macromolecular synthesis, G_1 and G_2 (Fig. 1.1). Cells entering G_1 after mitosis may arrest in response to nutrient deprivation or mitogen removal and are then said to be in G_0. Subsequent re-entry into the cell cycle requires renewed mitogen-receptor interaction. This interaction must trigger a primary response (or responses) capable of initiating a sequence of events culminating in DNA synthesis and cell division. The identity of this primary response and the subsequent sequence of events are currently the subjects of intense investigation.

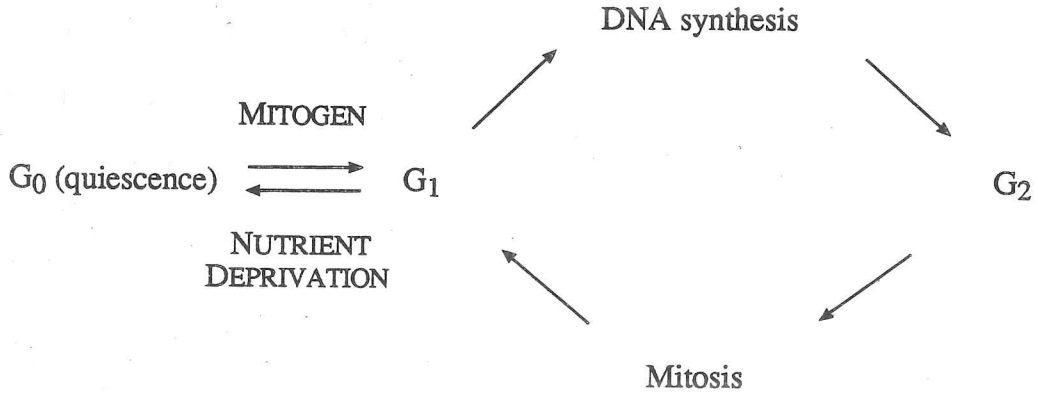

Fig. 1.1 The Cell Cycle

Lymphocytes, the effector cells of the immune system, are useful model cells for studying mitogenesis. Lymphocytes are small, non-adherent cells found in the bone marrow, thymus, spleen, lymph nodes and tonsils, in addition to those circulating in the blood (Ling and Kay, 1975). Lymphocytes are easily isolated from blood or other organs, and can be obtained in ample quantities for biochemical analysis. After isolation, lymphocytes grow readily but will remain quiescent until stimulated. Problems associated with the use of heterogeneous preparations such as thymocytes, splenocytes, or peripheral blood lymphocytes (PBL's) can be overcome by the use of monoclonal cell lines with defined growth requirements. In addition, lymphocytes are particularly suitable model systems for the analysis of mitogenic responses since clonal proliferation is an integral component of their physiological role in the immune response.

Two major classes of mature lymphocyte exist, distinguishable on the basis of their cell surface proteins (Ling and Kay, 1975), both originating from pluripotent haematopoietic stem cells in the bone marrow. B lymphocytes employ membrane-bound immunoglobulin molecules to recognise soluble foreign proteins (antigens). Immunoglobulin-antigen interaction results in secretion of immunoglobulins of identical specificity to the surface expressed-form and therefore capable of interaction with, and removal of, that antigen from the bloodstream. T lymphocytes respond to cell surface antigen displayed in association with major histocompatibility complex (MHC) proteins either by secreting lymphocyte growth factors (which facilitate the B cell response) or by lysing infected cells (Marrack and Kappler, 1986). T cells achieve antigen recognition through a polymorphic antigen receptor analogous to the B cell immunoglobulin molecule (reviewed in Clevers et al., 1988). Antigen recognition by either lymphocyte class stimulates proliferation; thus antigen-reactive clones are expanded as the need arises.

T lymphocytes originate as bone marrow stem cells. Pre-T cells developed from these migrate to the thymus, where the antigen receptor locus is progressively re-arranged and expressed (reviewed in Davis and Bjorkman, 1988). Subsequently mature antigen receptor-expressing cells are exported to the periphery. Thus antigen receptor is

expressed only by peripheral T cells and a small fraction of thymocytes, and in the latter may not be fully immuno-competent. Therefore immature T cell proliferation must be induced via additional mitogenic receptors. Several such receptors, physically distinct from the antigen receptor, have been identified (see below, also reviewed in Weiss and Imboden, 1987), although the functional independence of these from the antigen receptor is controversial (see Section 1.3). The presence of additional mitogenic receptors on T cells enhances their attractiveness as a model system for the study of eukaryotic cell growth. Not only can the sequence of responses to antigen receptor stimulation be elucidated, but this sequence can be compared with that obtained via stimulation of receptor molecules distinct from the antigen receptor.

1.2 Mitogenic stimulation of T lymphocytes through the antigen receptor

Antigenic stimulation of T cells can be mimicked by antibodies directed against various components of the antigen receptor, or by polyvalent plant lectins. The recognition sites for these ligands can best be understood by considering the structure of the antigen receptor. (Antigen receptor complexes isolated from human and murine lymphocytes are essentially similar, therefore all reports cited refer to the murine system unless otherwise stated in the text).

1.2.1 Structure of the antigen receptor

The antigen receptor consists of two major components T_i and T3 which themselves are composed of several protein chains. (T cell surface proteins, often assigned 'T' (human) or 'Ly' (murine) designations initially, are subsequently assigned to Clusters of Differentiation (CD) on the basis of common recognition by a panel of antibodies. T3 has been designated as CD3, and the acronyms T3 and CD3 are used interchangeably throughout this thesis.) T_i is a polymorphic disulphide-linked heterodimer M_r 80-90K (Meuer et al., 1983), composed of $T_{i\alpha}$, an acidic chain of 43-54K, and a less acidic $T_{i\beta}$ chain M_r 38-44K. $T_{i\alpha}$ and $T_{i\beta}$ are integral membrane proteins with 2-6 N-linked glycosylation sites within the extracellular domain, but both have only short cytoplasmic tails and thus are thought to be unlikely to transduce signals into the cytoplasm. This

function has therefore been ascribed to the monomorphic T3 complex, which co-modulates, co-cross-links and co-immunoprecipitates with T_i and is therefore assumed to be physically associated with T_i on the cell surface. The identified T3 proteins do have significant cytoplasmic domains, but cloning of the genes has so far not revealed any kinase domains or GTP-binding sites (Clevers et al., 1988). Five members of the T3 complex have been described; three of which - γ, δ, and ε - are present as single copies. While the 21kDa (γ) and 26kDa (δ) components are glycoproteins bearing N-linked carbohydrate side chains, the 25kDa chain (ε) is unglycosylated but may contain internal disulphide bonds (Samelson et al., 1985; Oettgen et al., 1986). All three chains contain conserved acidic residues of aspartic or glutamic acids within their hydrophobic putative transmembrane domains, which may stabilise the T3-T_i complex by interaction with corresponding basic lysine residues in the T_i protein transmembrane domains. The two additional T3 components ζ and η are non-glycosylated and are present in variable stoichiometry relative to the $\gamma\delta\varepsilon$ core. The 16kDa chain ζ is present as a homodimer in the majority of complexes, but may alternatively exist as a heterodimer linked to the 22kDa protein η (Oettgen et al., 1986; Baniyash et al., 1988a; Weissman et al., 1988a,b). A previously described protein, p21, of apparent M_r 21kDa (Samelson et al., 1986a,b) has been established as a phosphotyrosine-containing form of the 16kDa ζ peptide (Baniyash et al., 1988b). This peptide is apparently important in signal transduction (see below) and cell surface expression of the T cell receptor complex (TCR); a ζ^- mutant hybridoma line expresses only 5% cell surface receptor compared with the parental line; the other 95% being rapidly degraded in the lysosomes (Sussman et al., 1988a). However, the ζ protein is clearly not the only protein required for surface TCR expression since cell lines defective in the $T_{i\alpha}$ or $T_{i\beta}$ locus also fail to express surface TCR complexes (Saito et al., 1987a).

Recent studies focussing on the intracellular assembly of the T3-T_i complex suggest that assembly occurs within the endoplasmic reticulum where intermediate complexes of ζ_2 and $\gamma\delta\varepsilon$ bind independently to the α/β heterodimer (Bonifacino et al., 1988a; Alarcon et al., 1988). Sub-complexes consisting of $\gamma\delta$ or $\gamma\varepsilon$, with the capacity to bind α/β, have

also been observed (Berkhout et al., 1988; Bonifacino et al., 1988a). Correct TCR assembly may also require the involvement of auxiliary proteins, which dissociate before export of the complex to the Golgi apparatus. Two reports of such proteins in human cells identified one protein associated with the δ and ε chains (T3-p28; Pettey et al., 1987) and another associated with both CD3 components and free α and β chains (CD3-ω; Alarcon et al., 1988). A similar protein, the T cell receptor associated protein (TRAP), has also been identified in murine T cells (Bonifacino et al., 1988b).

A second form of the T_i heterodimer has been observed complexed with T3 in non-α/β-bearing cells (Brenner et al., 1986; Pardoll et al., 1987; Krangel et al., 1987; Reinherz, 1987). This γ/δ heterodimer is only expressed on a small percentage of T cells, which appear to arise by a distinct lineage compared with α/β-bearing cells; γ/δ^+ cells are first detectable in developing murine thymi 2 days prior to α/β^+ cells and the γ protein is transcribed from a re-arranged locus not observed in α/β^+ cells (Pardoll et al., 1987; Winoto and Baltimore, 1986). In addition to this early foetal subset, a minor subpopulation of peripheral T lymphocytes express γ/δ TCRs, including some natural killer cells (Alarcon et al., 1987), intestinal intraepithelial lymphocytes (Goodman and LeFrancois. 1988) and dendritic epidermal cells (Stingl et al., 1987). Despite differences in the carbohydrate processing of the T3δ oligosaccharide in some γ/δ^+ cells, T3 co-modulates with the γ/δ heterodimer and is implicated in functional activity mediated via this heterodimer (Alarcon et al., 1987; Krangel et al.,1987). No separate function for γ/δ-bearing cells has so far been identified which would account for the existence of this distinct lineage.

1.2.2 T3-T_i-stimulated proliferation of T lymphocytes

T cell proliferation, as measured by [^{3}H]thymidine incorporation, can be induced by antibodies directed against T3 or T_i. Anti-human T3 antibodies have been available for some time (OKT3, Van Wauwe et al., 1980; anti$T3_c$, Meuer et al., 1983; UCH-T1, Beverley and Callard, 1981), but an anti-murine CD3-ε hybridoma has only recently been cloned (Leo et al., 1987b). Since T3 is monomorphic these antibodies should recognise all T3-bearing cells. In contrast, T_i-directed antibodies, raised by immunisation with a

clonal T cell line, fall into 3 categories: those specific for the idiotypic receptor on the immunising clone (e.g. 1G3, Tomonari et al., 1985); those capable of recognising an element repeated in many receptors, such as the $V_{\beta 6}$ (Payne et al., 1988); $V_{\beta 8}$ (Staerz et al.,1985) or $V_{\beta 11}$ (Tomonari and Lovering, 1988) -encoded fragments; and those which interact with non-polymorphic determinants of T_i such as WT31 (Tax et al., 1983).

These antibodies are predominantly mitogenic for T3-T_i-bearing cells, although they are also capable of inhibiting antigen-directed T cell function, presumably by hindering interaction between antigen and receptor (e.g. Leo et al., 1987b). The T3-T_i complex expressed on γ/δ-bearing cells can also function to stimulate proliferation; both anti-$T_{i\delta}$ and anti-T3 antibodies stimulate proliferation of γ/δ^+ cells (Wu et al.,1988; Bluestone et al., 1987).

T cell proliferation can also be stimulated by lectins (e.g. Meuer et al., 1984b), as mentioned above. Lectins are glycoproteins, extracted from plants, which have the ability to bind specific saccharide groups on the cell surface. The most commonly used lectins Concanavalin A (Con A), from Canavalia ensiformis, and phytohaemagglutinin (PHA), from Phaseolus vulgaris, are both mitogenic for a wide range of T cells (Ling and Kay, 1975). Con A binds to α-D-mannopyranoside and α-D-glycopyranoside residues including the 3,6-di-o-(α-D-mannopyranosyl)-D-mannosyl moiety (Brewer and Bhattacharyya, 1986), and can therefore interact with any cell surface receptors bearing these residues. On the T cell surface, the T3-T_i complex is bound both by Con A and PHA (Chilson et al., 1984; Kanellopoulos et al., 1985), and both of these lectins also interact with a variety of other surface glycoproteins. PHA may also bind to CD45 (Bernabeu et al., 1987) and CD2 (O'Flynn et al., 1985), both of which may be involved in mitogenesis (see below). The interaction of Con A with CD45, LFA-1 (CD18), Lyt 2 (CD8), and at least five other unidentified proteins has been demonstrated (Sitkovsky et al., 1984). Thus, although lectins bind to the TCR complex, other interactions may also be important for their mitogenic action.

1.2.3 The requirement for accessory cells in T3-T_i-stimulated T cell proliferation

A common feature of T cell proliferation evoked by T3-T_i-directed antibodies and lectins is the requirement for accessory cells. In the absence of macrophages or monocytes putative mitogenic ligands provoke little or no proliferation. This requirement for accessory cells can be partially overcome by cross-linking the antibodies either with an anti-immunoglobulin antibody or by prior immobilisation of the antibodies on Sepharose beads (e.g. Meuer et al., 1984a,b), or on the surface of culture dishes. This suggests the accessory cells to be capable of cross-linking the antibodies, presumably via F_C receptors (Benichous et al., 1987; Ceuppens and Van Vaeck, 1989). It is likely that formation of a cross-linked matrix of antibody and T3-T_i more closely resembles the "in vivo" interaction of T cell with antigen-presenting cell, and the resultant stabilisation may be important for preventing premature ligand internalisation. However, even immobilised ligand is insufficient to stimulate highly purified resting T cells in the absence of accessory cells, suggesting the latter must supply an additional signal, possibly a soluble mediator. The best candidate for this function is the lymphokine interleukin 1, of which two forms exist, IL1α and IL1β, both of M_r 17kDa.

Receptors for IL1 have been identified on several T cell lines including LBRM-33-1A5, a murine T cell leukemic line (Dower et al., 1985) and the T cell lymphoma EL4 (Lowenthal and Macdonald, 1986). EL4 cells express 2 classes of IL1 receptor, distinguishable by their differing affinities for IL1. The majority are the lower affinity form (K_d~200-500pM), but 1-2% have a higher affinity (K_d=3.8pM) and are capable of internalising IL1 (Lowenthal and Macdonald, 1986).

Despite the presence of IL1 receptors on several T cell lines, IL1 alone has little effect on T cells. However, IL1 synergises with PHA (Hollander et al., 1987) or immobilised anti-T3 antibody (Scheurich et al., 1985) to activate T cells. Thus IL1 secretion may be the "missing" effector function supplied by accessory cells in addition to receptor cross-linking. However several lines of evidence suggest that accessory cells may also be required to secrete other lymphokines. Certain T cell subsets are unresponsive to IL1

(Lichtmann et al., 1988; Weaver et al.,1988). The synergistic effect of monocyte culture supernatant on PHA-stimulated PBL proliferation can be substituted for by IL6 and is inhibited by anti-IL6 but not anti-IL1β antibody (Ceuppens et al., 1988). Several murine T cell clones secrete and respond to IL1 (Tartakovsky et al., 1988). Taken together these data suggest accessory cells may produce several discrete lymphokines, and possibly that T cells can be subclassified according to their requirement for accessory cell-supplied lymphokines.

1.2.4 The requirement for interleukin 2 in T3-T_i-stimulated T cell proliferation

T lymphocytes can be broadly divided into two functional classes, as mentioned above. Cytotoxic T cells (T_C) lyse cells presenting foreign antigen, and are therefore implicated in the destruction of virally infected cells. Helper T cells (T_H) produce lymphokines, particularly interleukin 2 (IL2) and interferon-γ (IFN-γ), predominantly in response to foreign antigen presented by B lymphocytes or macrophages. Secretion of these lymphokines may occur only proximal to occupied antigen receptors (Janeway, 1986; Poo et al., 1988), thus facilitating specific activation of those cells interacting with the T cell (Malkovsky et al., 1987).

IL2 secretion is thus a characteristic T_H cell response to stimulation via the antigen receptor (Kronke et al., 1985), and is frequently used as a diagnostic marker for T cell activation. However, both immature and cytotoxic T cell populations also have the capacity to produce IL2, albeit a reduced capacity relative to T_H cells (McGuire et al., 1988; Lugo et al., 1986). This suggests a more general role for interleukin 2 and this is confirmed by the demonstration that interaction of IL2 with a T cell surface interleukin 2 receptor (IL2r) is required for T cell proliferation (Meuer et al., 1984a).

Two classes of IL2r have been observed, distinguishable on the basis of their affinity for IL2. The 55kDa human Tac antigen, cloned by two groups in 1984 (Nikaido et al., 1984; Leonard et al., 1984), and the non-Tac 75kDa (or 70kDa) IL2-binding peptide (Tsudo et al., 1987; Sharon et al.,1986) both have low affinities for IL2 (Tsudo et al.,1987). However, co-existence of p55 and p75 on the same membrane results in the

formation of high affinity IL2 binding sites (K_d=10-200pM), suggesting the high affinity receptor to be a p55-p75 complex (Tsudo et al., 1987; Robb, 1986; Hatakeyama et al., 1985; Dukovich et al., 1987). This conclusion is re-inforced by the fact that both p70 and Tac are labelled when IL2 is chemically cross-linked to the cell surface (Dukovich et al., 1987). Furthermore, mitogenic antibodies directed against a 75kDa peptide on YT natural killer cells cause down-regulation of the high affinity IL2r (Nakamura et al., 1989).

Early assays of IL2r expression employed anti-Tac antibody, and demonstrated that Tac expression is up-regulated by stimulation of the antigen receptor (Reem and Yeh, 1985; Hemler et al., 1984; Meuer et al., 1984a). Increased IL2r expression, like increased IL2 expression, is due to transcriptional activation of the IL2r gene; transcription is maximal 9h after receptor stimulation (Kronke et al., 1985). In contrast, two reports imply that the p75 protein may be constitutively expressed. Transgenic mice transfected with the gene for p55 express high affinity IL2r on unstimulated splenocytes and thymocytes implying the prior expression of p75 on these cells (Nishi et al., 1988). EL4 murine thymoma cells also apparently express p75 prior to stimulation, since p55 transfection results in the expression of high affinity IL2 receptors in EL4 cells but not L929 cells - a non-lymphoid lineage (Hatakeyama et al., 1985). However, the presence of p75 on unstimulated EL4 cells may be a phenotypic peculiarity due to their transformed state. Similarly, p55 transfection into developing mice may result in abnormal p75 expression. Thus the status of p75 expression in unstimulated cells is currently unclear.

The effect of IL2 on IL2r expression is complex. Interleukin 2 apparently stimulates enhanced Tac expression on IL2r-bearing T cells, but simultaneously reduces the number of high affinity IL2 binding sites (Depper et al., 1985; Reem and Yeh, 1985; Smith and Cantrell, 1985). No clear mechanistic implications can be derived from these data.

A further level of complexity has recently been revealed by the discovery that not all T_H cells secrete IL2; T_H1 cells secrete IL2, IFN-γ and lymphotoxin, but T_H2 cells secrete IL4 and IL5 (Cherwinski et al., 1987). The proportion of IL4-expressing foetal thymocytes declines throughout gestation and only one in 300 splenocytes express IL4 mRNA in response to Con A, suggesting IL4 might have some role in foetal ontogeny

(Sideras et al., 1988). However it is currently unclear whether IL2- and IL4-expressing populations overlap. Harel-Bellan and colleagues (1988a) demonstrated distinct IL2- and IL4-dependent T_H clones, antigenic activation of which was sensitive to inhibition by either antisense IL2 or IL4 oligonucleotides. But Pelkonen and co-workers (1987) describe eight $T_i\gamma$-expressing murine clones of which all eight respond to rIL4, but six also proliferate in response to IL2. Despite this apparent inconsistency, clear differences in effector function of the two subsets have been demonstrated, presumably using cells which are distinct IL2 or IL4 responders. Both subsets induce B cells to secrete IgM and IgG3, however IgG1 and IgG2a production are only stimulated by T_H2 and T_H1 cells respectively (Stevens et al., 1988). This may be due to differential stimulation of discrete B cell subsets. However such differential stimulation is apparently not mediated via restricted cell-cell interactions since a single B cell can bind to either T_H1 or T_H2 cells (Sanders et al., 1988).

1.2.5 The role of CD4 and CD8 molecules in T3-T_i-stimulated T cell proliferation

CD4 and CD8 are accessory molecules expressed on reciprocal subsets of mature peripheral T lymphocytes (originally designated respectively as T4 and T8 in human cells and L3T4 and Lyt2 in mouse). In contrast, 70-80% of thymocytes are $CD4^+8^+$ (Scollay et al., 1984) and four thymic phenotypes have been observed. These are generated sequentially in the order; $CD4^-8^-$ --> $CD4^+8^+$ --> $CD4^+8^-$ or $CD4^-8^+$ (Crispe et al., 1987; Fowlkes et al., 1985; Teh et al., 1988; Tamauchi et al., 1988), possibly with an additional immature $CD4^-8^+$ stage intermediate between double negative and double positive cells (Macdonald et al., 1988; Shortman et al., 1988). Mature T3-T_i-expressing cells are predominantly single positive, although a small subset of T3-T_i^+ double negative cells has also been identified (Ceredig et al., 1987).

The expression of CD4 or CD8 on peripheral T cells partially correlates with the functional division of T lymphocytes into helper and cytotoxic subsets, but is more stringently associated with the class of MHC protein recognised. CD4- and CD8-expressing T cells recognise antigen in the context of MHC class II and class I molecules

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The expression of CD4 or CD8 on peripheral T cells partially correlates with the functional division of T lymphocytes into helper and cytotoxic subsets, but is more stringently associated with the class of MHC protein recognised. CD4- and CD8-expressing T cells recognise antigen in the context of MHC class II and class I molecules

respectively. Since class II expression is restricted to immune lineage cells this provides a rationale for the partial correlation of CD4 expression with the T_H phenotype. Similarly, the ubiquitous expression of class I molecules facilitates cytolysis of infected cells by CD8-expressing T_C cells throughout the body.

The strict correlation between CD4/CD8 expression and MHC protein recognition has lead to the theory that these molecules physically interact thereby enhancing the avidity of intercellular binding. Several studies have demonstrated that CD4-transfectants show enhanced binding (Doyle and Strominger, 1987) and physiological responsiveness (Gay et al., 1987) to class II-bearing cells, and reciprocally that CD4-bearing cells respond more strongly to antigen co-expressed with class II than to antigen alone (Gay et al., 1988). T3-T_i and CD4 co-clustering has also been observed in the presence of specific but not non-specific antigen-presenting cells (Kupfer et al., 1987). Corresponding evidence for CD8/class I interaction has also been demonstrated (e.g. Gabert et al., 1987). These data imply that physical interaction between CD4/CD8 and non-polymorphic MHC protein determinants mediates MHC class restriction of T cells. However the demonstration that expression of murine T_i in human T_i^- cells is sufficient to confer murine MHC restriction (Saito et al., 1987), combined with evidence of direct interaction between T cell receptor and MHC protein (Ajitkumar et al., 1988) suggests minimally that the TCR complex is also involved in MHC protein recognition. The inability of other workers to confirm the binding studies of Doyle and Strominger (Q. Sattentau, personal communication) further questions the involvement of the CD4 molecule in MHC binding.

Early reports of anti-CD4 antibodies capable of antagonising T cell activation evoked by antigen presenting cells (Dialynas et al., 1983; Greenstein et al., 1984) were interpreted as evidence for the importance of class II/CD4 interaction. (Similar data have also been obtained using CD8-directed antibodies, thus to avoid repetition this account will be restricted to a consideration of CD4.) Subsequently, anti-CD4-mediated antagonism was demonstrated in systems in the absence of class II-bearing accessory cells. Anti-murine CD4 (L3T4) and anti-human CD4 antibodies inhibited T cell

proliferation and IL2 secretion stimulated by lectins or cross-linked anti-T3 antibodies (Tite et al., 1986; Wassmer et al., 1985; Bank and Chess, 1985) and also stimulation via non T3-T_i molecules (discussed below) such as CD2 (Carrera et al., 1987), Thy 1 (Pont et al., 1987), Tp44 and Tp103 (Schrezenmeier and Fleischer, 1988). In contrast, the ability of anti-CD4 antibodies to inhibit stimulation by IL2 or by a synergistic combination of phorbol ester and calcium ionophore is controversial (Blue et al., 1988; Moldwin et al., 1987; Carrera et al., 1987; Schrezenmeier and Fleischer, 1988); and anti-CD4 is apparently incapable of inhibiting Ly6.C-mediated T cell activation (Leo et al., 1987a). Nevertheless it is clear that anti-CD4 antibodies profoundly antagonise T cell activation induced by several ligands in the absence of class II-bearing accessory cells; and these findings have been taken as evidence that CD4 is capable of transmitting a negative signal. Consistent with this, anti-CD4 antibodies can also antagonise early metabolic responses to T cell activation (see below) such as increases in intracellular free calcium concentration ($[Ca^{2+}]_i$) and TCA3 transcription (Rosoff et al., 1987a; Blue et al., 1988; Wilson et al., 1988).

More recently however the ability of CD4 to transmit positive signals has been implied by several lines of evidence. Co-cross-linking of CD3 and CD4 molecules on the cell surface by using anti-CD3 and anti-CD4 antibodies immobilised on the same Sepharose bead results in enhanced responses compared with those evoked by anti-CD3-Sepharose alone whereas these responses are inhibited by soluble anti-CD4 antibody. Enhancement of several responses has been demonstrated, notably proliferation (Anderson et al., 1987; Eichman et al., 1987), lymphokine production (Owens et al., 1987; Kelso and Owens, 1988), and $Ca^{2+}{}_i$ mobilisation (Ledbetter et al., 1988a). These results imply a requirement for CD4:CD3 proximity in T cell activation. Consistent with this ~10% of surface CD4 molecules are associated with CD3 on human T_H cells (Anderson et al., 1988), and TCR triggering results in both T3-T_i and CD4 modulation (Acres et al., 1986; Saisawa et al., 1987; Anderson et al., 1988). Furthermore T cell activation enhances association between T3-T_i and CD4 (Rivas et al., 1988). Alternatively, the enhanced responses observed after CD4:CD3 co-cross-linking may be a more general phenomenon;

since co-cross-linking of CD3 with any of CD2, CD4, CD5, CD6 or CD8 enhances T cell proliferation relative to stimulation by cross-linked anti-CD3 antibody alone (Walker et al., 1987). However, the ability of the recently described anti-CD4 antibody B66.6 to evoke T cell proliferation in the absence of anti-CD3 antibody (Carrel et al., 1988) does suggest a specific role for CD4 in T cell proliferation. This is substantiated by the recent demonstration of tyrosine kinase activation by anti-CD4 antibody (Veillette et al., 1989; discussed below) and by the ability of human immunodeficiency virus I envelope protein gp 120, which interacts with CD4 on human T_H cells (Sattentau and Weiss, 1988), to stimulate increases in $[Ca^{2+}]_i$ and IL2r expression in peripheral blood T cells (Kornfeld et al., 1988). Consistent with its possible role as a positive signal-transducing receptor, CD4 is phosphorylated in response to a variety of ligands including antigen, anti-CD3 antibody, and anti-CD2 antibody combinations (Acres et al., 1986; Blue et al., 1987), in an analogous manner to various growth factor receptors. Thus, far from being merely an adhesion molecule involved in intercellular association, CD4 appears to be actively involved in the generation of mitogenic responses to ligands directed at T3-T_i and alternative receptors.

The antagonistic effects of some CD4 antibodies in the absence of class II-bearing accessory cells may be due to their interaction with inactivating epitopes distinct from that recognised by B66.6. More likely however, given that the same antibody (e.g. Gk1.5) may have antagonistic or stimulatory effects depending on whether it is used to cross-link CD4 alone or to co-cross-link CD4 and CD3 (see above), is that negative effects are caused by destabilisation of activating receptor:CD4 complexes. This in turn suggests that CD4 is required to covalently modify the TCR, most probably by phosphorylation. Finally, despite mounting evidence that CD4 is intimately involved in signal transduction, these results do not exclude a role for CD4 in MHC class II protein recognition. Indeed given the almost total coincidence between CD4 expression and MHC class II restriction, CD4 is highly likely to function as a recognition molecule for class II proteins. CD8 in $CD8^+$ cells has substantially similar effects to CD4 in $CD4^+$ cells, including the ability to enhance T3-induced responses when co-cross-linked to T3, and a demonstrated

association with the tyrosine kinase $p56^{lck}$ (Veillette et al., 1988). Thus CD8 probably interacts with MHC class I proteins, and functions as a signal transducer in a manner analogous to CD4.

1.3 Mitogenic stimulation of T lymphocytes through alternative cell surface receptors

T cell proliferation may be stimulated not only by T3-T_i-directed ligands, but also by antibodies directed against distinct extracellular receptors. Of these Thy 1 and CD2 (T11) are the best characterised, but information is also rapidly accumulating concerning CD28 (T44) and products of the Ly6 locus including T cell activating protein (TAP) and Ly6 protein. While some of these have been detected only in murine or human systems, others occur in both species but have only been extensively characterised in one. Comparison of stimulation via each receptor suggests they may have differential requirements for accessory cells, but may all require the presence of T3-T_i. A dependence on T3-T_i expression would preclude the involvement of these receptors in immature T cell proliferation, and suggest them to be accessory molecules analogous to CD4/CD8 required in antigen-mediated rather than antigen-independent mitogenic stimulation. However, their dependence on T3-T_i is controversial (see below) and the widespread expression in both mature and immature T cells of such molecules as Thy 1 suggests a function independent of antigenic stimulation.

1.3.1 Thy 1

The Thy 1 antigen is a 25-30kDa glycoprotein, which may also exist as a 50kDa dimer unsusceptible to reduction (Gunter et al., 1984, 1986). Human Thy 1 expression is restricted to immature T cells; 0.1-0.5% of bone marrow cells, and 0.2-10% of thymocytes, but no PBL's are Thy 1^+ (McKenzie and Fabre, 1981; Ritter et al., 1983). In contrast, Thy 1 is widely expressed on murine T cells including thymocytes, splenocytes, peripheral T cells and hybridomas and is also expressed in nervous tissue (Reif and Allen, 1964; Gunter et al., 1984). Thy 1 expression is not obligatory for murine T cell proliferation (Gunter et al., 1988; Wadsworth et al., 1989), but some anti-

Thy 1 antibodies activate T cells to proliferate and express IL2 and IL2r in the presence of accessory cells (Gunter et al., 1984; MacDonald et al., 1985; Pont et al., 1985). Accessory cells can be substituted for by phorbol ester and a second, cross-linking antibody (Gunter et al., 1986). Anti-Thy 1 antibodies can also induce IL2 secretion from Thy 1-transfected Jurkat cells (a human leukemic T cell line), implying that no murine T cell-specific molecules are required for Thy 1 function. However, several experiments suggest T3-T_i expression may be obligatory for Thy 1-mediated T cell activation. The anti-Thy 1 antibody G7 fails to stimulate IL2 secretion from T3-T_i^- mutant Jurkats (Gunter et al., 1987) or hybridomas (Sussman et al., 1988b); but transfection of the missing T_i chain restores both surface T3-T_i expression and the response to G7. The required T3-T_i chain is clearly not $T_{i\alpha}$ or $T_{i\beta}$, since γ/δ-bearing cells proliferate in response to anti-Thy1 antibody (MacDonald et al., 1987), but may be ζ, since mutants deficient in ζ expression do not secrete IL2 in response to anti-Thy 1 or antigen (Sussman et al., 1988a).

In contrast, Thy 1-mediated $[Ca^{2+}]_i$ increases can be observed in T3-T_i^- Jurkats (Gunter et al., 1987) and Thy 1-transfected B cells (Kroczek et al., 1986a), although not in a $T_{i\alpha}^-$ T_C clone (Schmitt-Verhulst et al., 1987) implying that Thy 1 may elevate $[Ca^{2+}]_i$ independently of T3-T_i, but that $[Ca^{2+}]_i$ elevation is not sufficient for IL2 expression and is therefore not a reliable indicator that a T cell will progress to DNA synthesis. Gunter and colleagues (1986) have provided the only clear demonstration of Thy 1-mediated activation independent of T3-T_i using an unusual Thy 1^+ Jurkat transfectant. This cell line secreted IL2 in response to anti-Thy 1 antibody but not T3 or T_i-directed antibodies. Loss of Thy 1 resulted in resumption of T3/T_i reactivity, implying Thy 1 expression had suppressed the ability to respond through T3-T_i. Thus it is clear that Thy 1 can generate signals independently of T3-T_i, but that these may or may not be sufficient to fully activate T cells. Consistent with this, distinct patterns of protein phosphorylation can be generated in response to anti-TCR or anti-Thy 1 antibodies, presumably due to independent early response activation (Peyron et al., 1988). It should also be noted that

since all of these studies have been performed on T3-T_i^- mature cells, no conclusions can be drawn about the role of Thy 1 in immature T cell proliferation.

1.3.2 Ly6

The murine Ly6 locus encodes a family of proteins differentially expressed in various T cell populations; Ly6.2A is expressed in peripheral tissues, Ly6.2B in bone marrow, Ly6.2C in bone marrow and peripheral cells, Ly6.2D as Ly6.2C plus thymocytes, and Ly6.1E in lymphoblasts (Kimura et al., 1984). Several mitogenic anti-Ly6 antibodies have been described, almost all of which appear to require cross-linking and co-addition of IL2 or the phorbol ester tetradecanoyl phorbol acetate (TPA). D7, which recognises a non-polymorphic Ly6 specificity present on Ly6.1E$^+$ and Ly6.2A$^+$ cells, stimulates IL2/IL2r expression and proliferation of splenic T cells in the presence of TPA plus accessory cells (Malek et al., 1986). The accessory cells may be substituted by a second cross-linking antibody implying that IL1 and other accessory cell secretions are not required. Splenic T cells also proliferate in response to anti-Ly6.2C antibody 143-4-2 plus accessory cells and IL2 or TPA (Leo et al., 1987c). Peripheral T cell mitogenesis is stimulated by 34-2-11 antibody combined with TPA and a cross-linking agent (Yeh et al., 1987). In contrast, HK1.4 antibody stimulates a T_C clone to proliferate in the absence of accessory cells or phorbol ester (Havran et al., 1988). Ly6 antibodies appear to preferentially stimulate CD8$^+$ cells rather than CD4$^+$ cells (Toulon et al., 1988; Leo et al., 1987c), which may explain the failure of anti-CD4 antibody to inhibit anti-Ly6.2C-stimulated T cell proliferation (Leo et al., 1987a). IFN-γ up-regulates expression of Ly6.2A/E (Dumont et al., 1987; Toulon et al., 1988) suggesting a role for Ly6 receptors in post-antigenic or non-specific proliferation. However, Ly6 fails to mediate T cell activation in T3-T_i^- hybridomas (Sussman et al., 1988c) or T3-modulated T_C clones (Leo et al., 1987c) implying that Ly6-mediated activation may require T3-T_i expression.

1.3.3 TAP

TAP (T cell activating protein) is a 12kDa glycoprotein often found associated with a 16kDa protein TAPa, which may be a reduced form of TAP (Rock et al., 1986; Reiser et al., 1986). Although encoded in the Ly6.2 locus, TAP has a different pattern of

expression from any of the previously described Ly6 proteins, and is therefore assumed to be a distinct protein (Reiser et al., 1988). TAP is expressed on 70% of peripheral T lymphocytes and on 10-20% of thymocytes, predominantly mature medullary cells, but is not expressed on B cells or on the T cells of several mice strains, including BALB/c, C3H and A/J mice (Yeh et al., 1986; Rock et al., 1986). Anti-TAP antibodies plus TPA are mitogenic for peripheral blood cells (Yeh et al., 1987) and mature thymocytes (Yeh et al., 1986). Splenic T cells proliferate in response to anti-TAP antibody only in the presence of accessory cells or IL1 implying that accessory cell secretions are required, rather than merely cross-linking (Rock et al., 1986).

TAP-directed antibodies are similar to Ly6-directed antibodies in that they fail to induce proliferation in T3-T_i^- mutants or T3-T_i-modulated cells, implying a requirement for T3-T_i expression (Bamezai et al., 1988). Conversely, TAP expression appears to enhance T3-T_i-mediated T cell activation. TAP^+ thymocytes are required for responsiveness in mixed lymphocyte reactions (i.e. antigenic stimulation) or Con A (Yeh et al., 1986), although this may simply reflect the correlation between TAP and T3-T_i expression. The demonstration that TAP^- mutants are less responsive to antigen (Yeh et al., 1988) constitutes more powerful evidence for the involvement of TAP in antigenic stimulation. Similarly unresponsive mutants have also been observed which are deficient in the ability to make phosphatidylinositol- (PtdIns-) linked proteins, and the effect of this mutation can be mimicked by treatment of cells with phosphatidylinositol-specific phospholipase C (Yeh et al., 1988). Since PtdIns-specific phospholipase C treatment releases TAP from T cells (Reiser et al., 1986), these data taken together imply that PtdIns-linked TAP is involved in antigenic T cell activation. However since several other T cell antigens are also PtdIns-linked (e.g. Thy 1, Homans et al., 1988; Ishihara et al., 1987; LFA-3 (lymphocyte function associated antigen-3), Dustin et al., 1987), the inhibitory effects of PtdIns-phospholipase C cannot be attributed specifically to effects on TAP. In contrast, the ability of PtdIns-specific phospholipase C to cause cellular activation (Depper et al., 1984) suggests cleavage of PtdIns-linked proteins might be an

integral component of T cell stimulation. However there is no evidence that mitogenic anti-Thy 1 antibodies stimulate cleavage of Thy 1 (Peyron et al., 1988).

1.3.4 CD2

In contrast to Thy 1, TAP and Ly6; CD2 (T11) is well characterised on human T cells but a murine homologue has only recently been cloned (Sewell et al., 1987; Clayton et al., 1987). Human CD2 is a non-polymorphic 50kDa glycoprotein expressed on all T cells from the earliest T lineage precursor through thymocytes to peripheral T cells and T cell clones. Three epitopes are distinguishable on the basis of reactivity to antibodies; $T11_1$ and $T11_2$ are expressed on all T lymphocytes, but $T11_3$ is generated by conformational change in response to activation (Meuer et al., 1984b). Macrophage-depleted peripheral T cells proliferate in response to combinations of anti-$T11_3$ with anti-$T11_1$ or anti-$T11_2$ antibodies, via expression of IL2 and IL2r (Meuer et al., 1984b; Siciliano et al., 1985). However the ability of anti-CD2 combinations to stimulate IL2 secretion from thymocytes and thus thymocyte proliferation is controversial (Fox et al., 1986; Reem et al., 1987).

O' Flynn and colleagues (1985) concluded that CD2 was responsible for the effects of PHA, since anti-CD2 antibodies could block $[Ca^{2+}]_i$ elevation induced by PHA, but not UCH-T1 (anti-T3) or Con A. However, PHA- and CD2-mediated T cell proliferation differ in their requirements for macrophages (Meuer et al., 1984b) and T3-T_i expression (Moretta et al., 1987) implying that CD2 is not the sole PHA effector molecule. This is consistent with the observed interaction between PHA and T3-T_i (Chilson et al., 1984; Kanellopoulos et al., 1985).

Recently, LFA-3 has been proposed as the in vivo ligand for CD2, due to the observed ability of CD2 to bind LFA-3 (Takai et al., 1987; Selvaraj et al., 1987). A physiological role for this interaction has been inferred since anti-CD2 antibodies activate T cells synergistically with purified LFA-3 or LFA-3-transfected cells (Bierer et al., 1988a; Bockenstedt et al., 1988). CD2 stimulation during intercellular interaction may contribute to antigen-evoked proliferation since CD2-transfected murine T cell hybridomas show enhanced responses to antigen, and this augmentation is reversed by anti-CD2 or anti-

LFA-3 antibodies (Bierer et al., 1988b). At least in mature cells, CD2 may be incapable of transducing signals in the absence of T3-T_i.

Several investigators have observed the failure of CD2-directed ligands to evoke $[Ca^{2+}]_i$, IL2 or proliferative responses in T3-modulated or mutant T3-T_i^- cell lines, but found that T3-T_i re-expression restores CD2-responsiveness (Meuer et al., 1984b; Pantaleo et al., 1987c; Bockenstedt et al., 1988; Breitmeyer et al., 1987). In contrast, other groups have observed CD2-stimulated $[Ca^{2+}]_i$ and IL2 increases in apparently T3-T_i^- cell lines (Pantaleo et al., 1988; Moretta et al., 1987; Alcover et al., 1986), but these studies may have failed to detect the low level of T3-T_i expression required for CD2-responsiveness (<1,000 molecules/cell; Alcover et al., 1988a). Thus it appears likely that T3-T_i expression is obligatory for CD2 responsiveness in cells normally T3-T_i^+. However, in non-T3-T_i-expressing cells such as immature thymocytes and natural killer cells, CD2 stimuli may be sufficient to induce proliferation (Siciliano et al., 1985; Fox et al., 1986; Toribio et al., 1989). Thus, CD2-mediated proliferation may have a role in human T cell maturation.

The murine CD2 homologue is expressed in thymus and spleen (Sewell et al., 1987) and may also be expressed on B cells (Yagita et al., 1989). The cloned gene appears to encode a transmembrane protein with a large, proline-rich cytoplasmic domain, possibly with a cation binding site (Clayton et al., 1987). No mitogenic antibodies have so far been cloned. However, anti-rat CD2 antibodies are mitogenic suggesting that CD2 may have similar function in humans and rodents (Clark et al., 1988).

1.3.5 CD28

CD28 (T44) is a glycoprotein of similar size (40-45kDa reduced or 80-85kDa non-reduced) but distinct from T_i, expressed on both α/β^+ and γ/δ^+ human T cells (Moretta et al., 1985; Poggi et al., 1987; Testi and Lanier, 1989). Anti-CD28 antibodies are mitogenic for peripheral blood T cells in the presence of accessory cells or TPA (Moretta et al., 1985; June et al., 1987; Baroja et al., 1988), but T3-T_i-modulated and T3-T_i-deficient cells are unresponsive to CD28, although CD28 does not co-modulate with T3-T_i or CD2 (Moretta et al., 1985, 1987). However, synergistic combinations of CD2-

and CD28-directed ligands stimulate PBL proliferation independent of accessory cells or T3-T_i modulation (Van Lier et al., 1988; Pierres et al., 1988), implying that these receptors may enable non-specific involvement of T cells in immune reactions.

1.3.6 Additional molecules

An ever increasing number of additional molecules also appear to be implicated in T cell activation including THAM, a 110-128kDa molecule expressed on immature thymocytes (Naquet et al., 1988), and Tp90 and Tp45, both expressed on mature $CD8^+$ cells (Carrel et al., 1987a,b). CD45, a 180-220kDa glycoprotein, may also be important, particularly since it has tyrosine phosphatase activity (Charbonneau et al., 1988). Anti-CD45 antibodies may antagonise or enhance stimulation via other antigens depending on whether or not they are used to cross-link CD45 to the other antigen (Martorelli et al., 1987; Bernabeu et al., 1987; Ledbetter et al., 1988a). Doubtless other molecules remain to be identified, many of which may function primarily during antigenic stimulation as, for example, lymphokine receptors and adhesion molecules, possibly with considerable overlap with molecules already characterised functionally but not at the molecular level.

1.4 Early responses to receptor stimulation in T lymphocytes

1.4.1 Phosphatidylinositol 4,5-bisphosphate metabolism

Phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) metabolism is one of the earliest detected events consequent on T3-T_i-mediated T lymphocyte stimulation. Receptor-stimulated inositol phospholipid turnover, first demonstrated in the 1950s by the Hokins (Hokin and Hokin, 1953) and subsequently observed in response to a wide variety of hormones and mitogens (reviewed in Michell, 1975; Berridge and Irvine, 1984; Abdel-Laitif, 1986), comprises several components. Synthesis of the phosphoinositides (phosphatidylinositol, phosphatidylinositol 4-phosphate (PtdInsP) and phosphatidylinositol 4,5-bisphosphate) is accompanied by rapid phosphodiesteratic cleavage of $PtdInsP_2$, and possibly PtdIns and PtdInsP (Durell et al., 1968; Downes and Wusteman, 1983). Phosphoinositide breakdown has been assayed both as loss of pre-labelled inositol-containing lipids and accumulation of inositol phosphates, but neither of

these techniques distinguish between hydrolysis of $PtdInsP_2$ and that of PtdInsP or PtdIns. Inositol mono- and bisphosphates (InsP and $InsP_2$) may be directly generated by hydrolysis of PtdIns and PtdInsP respectively, but are also dephosphorylation products of inositol trisphosphate ($InsP_3$). Conversely, decreases in PtdIns and PtdInsP may be the result of phospholipase or kinase action, since $PtdInsP_2$ is synthesised from PtdIns via phosphorylation. Therefore, although the term "$PtdInsP_2$ breakdown" is used in this thesis to mean phospholipase C-catalysed $PtdInsP_2$ hydrolysis, its usage is not intended to imply that other phosphoinositides are not also cleaved.

In T cells, increases in inositol phosphate accumulation and phosphoinositide synthesis are detectable within 1 min of Con A or PHA addition (Taylor et al., 1984, Grier and Mastro, 1985; Sasaki and Hasegawa-Sasaki, 1985). Inositol phosphate accumulation also occurs in response to T3-T_i-directed antibodies. In Jurkats, anti-T3 antibody (OKT3) and anti-T_i antibody (C305) stimulate $InsP_2$ and $InsP_3$ accumulation within 30s, followed by a slower accumulation of InsP (Imboden and Stobo, 1985). More recently cloning of 145-2C11, an antibody-producing hybridoma specific for murine T3-ε (Leo et al., 1987b), has facilitated observation of similar T3-mediated inositol phosphate increases in murine T cells (Klausner et al., 1987). Such responses do not require $T_{i\alpha/\beta}$ expression, since anti-T3 antibody has a similar effect in γ/δ-expressing T cells (Pantaleo et al., 1987a). However, interaction of ligand with T_i is sufficient to induce phosphoinositide metabolism; antigen presentation to α/β^+ cells stimulates inositol phosphate accumulation (Klausner et al., 1987; Treves et al., 1987) and anti-$T_{i\gamma}$ antibody stimulates increases in PtdIns and phosphatidic acid (PA) in a γ/δ^+ T cell clone (Bismuth et al., 1988).

These results suggest that phosphoinositide metabolism may occur during antigenic stimulation of T cells. However stimulation of non-T3-T_i receptors may also result in inositol phosphate accumulation. The anti-Thy 1 antibody G7 stimulates small inositol phosphate increases (relative to those induced by 145-2C11) in a murine T cell hybridoma (Klausner et al., 1987). Anti-CD2 antibody combinations stimulate $PtdInsP_2$ breakdown in α/β^+ and γ/δ-bearing cells (Pantaleo et al., 1987a,b; Gardner et al., 1989). The ability

of purified LFA-3 plus anti-CD2 antibody to evoke an inositol phosphate response (Bockenstedt et al., 1988) implies that this may reflect a physiological event since LFA-3 is thought to be the physiological ligand for CD2. The absence of this response in mutant T3-T_i^- cells has been suggested as evidence that T3-T_i is required for transduction of signals from non T3-T_i receptors (as discussed above), particularly since $T_{i\beta}$ transfection simultaneously restores T3-T_i surface expression and the ability to respond to LFA-3/anti-CD2 antibody. The substantial cytoplasmic domains of several T3 components render the T3 complex a suitable candidate for "universal transducer" for all surface receptors. However only molecular characterisation of the mechanism of receptor coupling to phospholipase C can validate this hypothesis.

1.4.2 Receptor coupling to $PtdInsP_2$ breakdown

Several lines of evidence suggest that receptor-mediated phospholipase C activation involves an intermediary GTP-binding protein, or G-protein. Strategies employed to evaluate G-protein involvement reflect current knowledge of adenylate cyclase modulation by G-proteins, and can therefore be best explained by a brief review of receptor coupling to adenylate cyclase (reviewed in Neer and Clapham, 1988; Fain et al., 1988; Levitski, 1988).

Cyclase activity is regulated by two classes of G-protein, G_s (M_r 40kDa) and G_i (M_r 41kDa), which respectively couple cyclase to activatory and inhibitory cell surface receptors. Both proteins are coupled to $\beta\gamma$ subunits when inactive, but stimulation of a positive (or negative) receptor by its agonist results in dissociation of $\beta\gamma$ from G_s (or G_i) and exchange of GDP for GTP in the guanine nucleotide binding site. Cyclase is activated by the GTP-bound form of G_s (G_s-GTP), and can be permanently activated by non-hydrolysable GTP analogues such as GTPγS and GppNHp. Conversely these analogues prevent cyclase inactivation by G_i, since this requires hydrolysis of G_i-bound GTP. Cyclase activity can also be modulated by some bacterial toxins, which covalently modify specific G proteins by the addition of an ADP-ribose group. ADP-ribosylation of G_s by cholera toxin inhibits G_s GTPase activity thereby stabilising G_s-GTP and enhancing cyclase activation. Pertussis toxin-mediated ADP-ribosylation of G_i abrogates

G_i-mediated cyclase inactivation by inhibiting GTP entry into the G_i guanine nucleotide binding site. Adenylate cyclase activity may also be modulated independently of surface receptors by fluoride ions complexed with aluminium. AlF_4^- appears to mimic the role of the γ phosphate of GTP and thus modulates G protein activity (Bigay et al., 1987).

The above results for the cyclase system suggested three obvious methods for probing the involvement of G-proteins in receptor-mediated phospholipase C activation.

i The effect of guanine nucleotides and analogues on $PtdInsP_2$ breakdown in permeabilised cell systems

Activation of $PtdInsP_2$ hydrolysis in permeabilised cells or membrane preparations by GTP, GTPγS, or GppNHp, first observed in mast cells transiently permeabilised with ATP^{4-} (Cockroft and Gomperts, 1985), has now been demonstrated in a wide variety of cell types including rat hepatocytes (Wallace and Fain, 1985), blowfly salivary glands (Litosch et al., 1985), platelets (Baldassare and Fisher, 1986), rat pituitary cells (Straub and Gershengorn, 1986), and chinese hamster lung fibroblasts (Magnaldo et al., 1987). Lo and Hughes (1987b) demonstrated that although similar inositol phosphate responses could be evoked in permeabilised human pituitary tumour cells by guanine and non-guanine nucleotides (e.g. CTP, ATP, UTP, ATPγS) only guanine nucleotides could significantly enhance cholecystokinin-stimulated $PtdInsP_2$ breakdown. Similarly, guanine nucleotides and $PtdInsP_2$-cleaving ligands synergistically stimulate $PtdInsP_2$ hydrolysis in many other systems (e.g. Rebecchi and Rosen, 1987; Hrbolich et al., 1987; Dunlop and Larkins, 1986), suggesting a possible G-protein involvement in receptor-stimulated $PtdInsP_2$ hydrolysis. GTPγS-activated $PtdInsP_2$ hydrolysis has also been demonstrated in Jurkat membranes and murine thymocytes, implying that a similar mechanism operates in T lymphocytes, although synergistic activation with Con A or anti-T3 antibody could not be demonstrated (Sasaki and Hasegawa-Sasaki, 1987; Zilberman et al., 1987).

ii The effect of fluoride ions on $PtdInsP_2$ breakdown

$PtdInsP_2$ breakdown stimulated by fluoride and fluoroaluminate ions has been demonstrated in several cell types including hepatocytes, rat brain, and T lymphocytes

(Blackmore et al., 1985; Litosch, 1987; Sasaki and Hasegawa-Sasaki, 1987; O'Shea et al., 1987). Similarly to guanine nucleotide-evoked stimulation, AlF_4^- stimulation could be reversed by GDPβS in hepatocytes (Cockroft and Taylor, 1987), implying that both operate via a G-protein. However, in T3-modulated T cells AlF_4^- was not competent to induce $InsP_3$ increases (Pantaleo et al., 1987c), implying that a component of the T3-T_i complex, or a T3-T_i-associated peptide, is required at a stage between AlF_4^- function and phospholipase C function. The required T3-T_i component is clearly not CD3-ζ, since ζ- mutants retain the capacity to accumulate $InsP_3$ in response to anti-T3 antibody (Sussman et al., 1988a), but may be a T3-T_i-associated G-protein (see below). Furthermore, CD3-η may also be required for T3-T_i-mediated (although not AlF_4^--induced) $PtdInsP_2$ hydrolysis. Mutants deficient in CD3-η give poor inositol phosphate responses to anti-CD3-ε antibody, but normal responses to AlF_4^- (Mercep et al., 1988).

iii The effect of bacterial toxins on $PtdInsP_2$ breakdown

Pertussis toxin-mediated inhibition of phospholipase C activation has been demonstrated in hamster fibroblasts (Paris and Pouyssegur, 1987), rabbit neutrophils (Volpi et al., 1985) and human polymorphonuclear leukocytes (Smith et al., 1985) among other cell types; suggesting that G_i or a G_i-like protein is involved in phospholipase C activation. Similar results cannot be demonstrated in T lymphocytes, since the non-ADP-ribosylating β subunit of pertussis toxin is mitogenic for T cells, and stimulates similar early responses to T3-T_i-directed T cell mitogens (Rosoff et al., 1987b; Strnad and Carchman, 1988; Thom and Casnellie, 1989; Y. Zilberman and J.P. Moore, unpublished data). However cholera toxin pre-treatment which inhibits pituitary cell $InsP_3$ responses to cholecystokinin, acetylcholine, and bradykinin via alteration of receptor-G protein coupling (Lo and Hughes, 1987a) inhibits T3-T_i-mediated inositol phosphate increases in Jurkats via a mechanism distinct from cAMP elevation (Imboden et al., 1986). Cholera toxin effects imply that G_s may be involved in phospholipase C activation.

Taken together these results imply that distinct G proteins may be responsible for receptor coupling to phospholipase C in different cell types, or even that distinct proteins couple to different receptors within the same cell. The latter situation has been suggested

by the demonstration that $PtdInsP_2$ hydrolysis stimulated via different receptors (in the same cell population) is differentially sensitive to inhibition by pertussis toxin (Taylor et al., 1988; Ashkenazi et al., 1989; Johnson and Garrison, 1987). However, the identities of the relevant G-proteins are unclear. Effects of cholera and pertussis toxins suggest the involvement of G_s and G_i (or G_s- and G_i-like proteins) respectively, but other data suggest the involvement of a distinct class of G-proteins, G_p, which have not yet been cloned. It is probable that different G-proteins including G_i and G_s may be utilised in different cell types. This apparent complexity contrasts sharply with the earlier hypothesis that many growth factors might couple through a single GTP-binding protein $p21^{ras}$, the cellular homologue of transforming oncoproteins Harvey-, Kirsten- and N-ras. Initial reports described elevated basal and stimulated amounts of inositol phospholipids and phosphates in cells transformed with oncogenic ras proteins, and suggested that the transforming potential of ras might be due to an enhancement of phosphoinositide metabolism (Chiarugi et al., 1985, 1986; Wakelam et al., 1986; Fleischman et al., 1986). However, subsequent investigations failed to find increases in phosphoinositide metabolism in ras-transformed cells (Seuwen et al., 1988; Huang et al., 1988; Wolfman and Macara, 1987; Lacal et al., 1987b), and demonstrated that previously reported increases in phosphoinositide metabolism might be artefacts associated with cell density or receptor number effects (Wakelam, 1988; Downward et al., 1988). Furthermore, direct introduction of oncogenic $p21^{ras}$ into 3T3 fibroblasts by scrape-loading does not stimulate inositol phosphate accumulation despite protein kinase C activation (Morris et al., 1989). Activation of protein kinase C has also been demonstrated in ras-transformed and ras-injected cells, and apparently precedes, rather than being a consequence of, phosphatidylcholine hydrolysis (Lacal et al., 1987a; Wolfman and Macara, 1987; Huang et al., 1988; J.D.H. Morris, personal communication). Therefore $p21^{ras}$ is unlikely to be the G-protein responsible for phospholipase C activation, although it is almost certainly involved in mitogenesis.

In murine thymocytes, phospholipase C-receptor coupling may be achieved by a 25kDa G-protein recently described by Wang and colleagues (1989). This protein,

together with G_i, was found to have affinity to a Con A-Sepharose column, implying it to be associated with Con A receptor(s). G_i apparently dissociated from the cell membranes during Con A stimulation, since no G_i was found associated with Con A-Sepharose after passage of membranes prepared from Con A-stimulated thymocytes. G_i dissociation may reflect a role for G_i in cAMP suppression during Con A stimulation of thymocytes. The other receptor-associated G protein (25kDa) was found to enhance $PtdInsP_2$ hydrolysis by PtdIns-phospholipase C, suggesting it might be the protein responsible for receptor-phospholipase C coupling in murine thymocytes. Despite the demonstrated requirement for T3-T_i expression in AlF_4^--stimulated $InsP_3$ accumulation (Pantaleo et al., 1987c), and the coincidence of molecular weight of this G-protein and the CD3-ε chain, the failure to find a GTP-binding domain in the CD3-ε protein (Clevers et al., 1988) suggests that the two proteins are not identical.

1.4.3 Novel inositol polyphosphates

In addition to inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), the direct product of phospholipase C-catalysed $PtdInsP_2$ breakdown, several additional inositol polyphosphates have now been characterised. The production of inositol 1:2-cyclic phosphate from PtdIns in stimulated tissues was described in the 1970s (Michell and Lapetina, 1972, 1973), but more recently Majerus and colleagues have suggested that cyclic inositol 1:2,4,5-trisphosphate ($cIns(1:2,4,5)P_3$) may be a significant breakdown product of $PtdInsP_2$ in platelets and sheep seminal vesicles (Wilson et al., 1985; Ishii et al., 1986; Connolly et al., 1986b). This contrasts with data from Hawkins and co-workers (1987) who could find no evidence of $cIns(1:2,4,5)P_3$ accumulation in carbachol-stimulated parotid gland. It has been suggested independently that both the presence and absence of $cIns(1:2,4,5)P_3$ are artefacts of the protocols employed for reaction termination and inositol phosphate extraction (Hawkins et al., 1987; Majerus et al., 1988). However, the demonstration that $Ins(1,4,5)P_3$ and $cIns(1:2,4,5)P_3$ accumulate with distinct kinetics and in varying amounts relative to each other implies that both may be genuine products of $PtdInsP_2$ hydrolysis (Dixon and Hokin, 1987; Hughes et al., 1988).

The existence of a distinct $InsP_3$ isomer inositol 1,3,4-trisphosphate ($Ins(1,3,4)P_3$) was demonstrated in carbachol-stimulated rat parotid glands in 1984 (Irvine et al., 1984). The identity of the precursor for $Ins(1,3,4)P_3$ was at first unclear due to the failure to identify a parent lipid (i.e. $PtdIns(3,4)P_2$), but was apparently resolved by the isolation of inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$) from carbachol-stimulated rat brain and the subsequent demonstration of sequential $Ins(1,4,5)P_3$ / $Ins(1,3,4,5)P_4$ / $Ins(1,3,4)P_3$ conversion in parotid homogenates and $Ins(1,4,5)P_3$-microinjected Xenopus oocytes (Irvine et al., 1985; Batty et al., 1985; Irvine et al., 1986a; Downes et al., 1986; Hawkins et al., 1986). More recently, the identification of a PtdIns 3'-kinase and the isolation of phosphatidylinositol 3-phosphate (PtdIns 3-P), $PtdIns(3,4)P_2$, and phosphatidylinositol 3,4,5-trisphosphate ($PtdIns(3,4,5)P_3$) has suggested alternative mechanisms of $Ins(1,3,4)P_3$ and $Ins(1,3,4,5)P_4$ generation (Whitman et al., 1987, 1988; Traynor-Kaplan et al., 1988; Auger et al., 1989). PtdIns(3)P has now been identified in human astrocytoma cells (Stephens et al., 1989), fibroblasts (Whitman et al., 1988), and smooth muscle cells (Auger et al., 1989). In contrast, $PtdIns(3,4)P_2$ and $PtdIns(3,4,5)P_3$ have not been found in quiescent cells, but appear rapidly on agonist addition (Traynor-Kaplan et al., 1988; Auger et al., 1989). PtdIns 3'-kinase associates with $p60^{v\text{-}src}$ in Rous sarcoma virus-infected chick embryo fibroblasts, and is also found in anti-phosphotyrosine immunoprecipitates from platelet-derived growth factor (PDGF)-stimulated smooth muscle cells, suggesting that PtdIns 3'-kinase activity may be involved in mitogenesis and cell transformation (Fukui and Hanafusa, 1989; Auger et al., 1989).

The possible origins and observed interconversions of inositol polyphosphates (excluding possible cyclic derivatives) are summarised in Figure 1.2. Despite the possibility of $PtdIns(3,4,5)P_3$ cleavage, it seems clear that $Ins(1,4,5)P_3$ phosphorylation is a major synthetic route for $Ins(1,3,4,5)P_4$, since a specific kinase has been identified in turkey erythrocytes (Morris, A.J. et al., 1987), RINm5F cells (Biden and Wollheim, 1986), pig aortic smooth muscle (Yamaguchi et al., 1987), rat brain (Johanson et al., 1988), platelets (Daniel et al., 1988) and murine thymocytes (Zilberman et al., 1987). Cyclic $Ins(1:2,4,5)P_3$ is not a substrate for this kinase (Connolly et al., 1987).

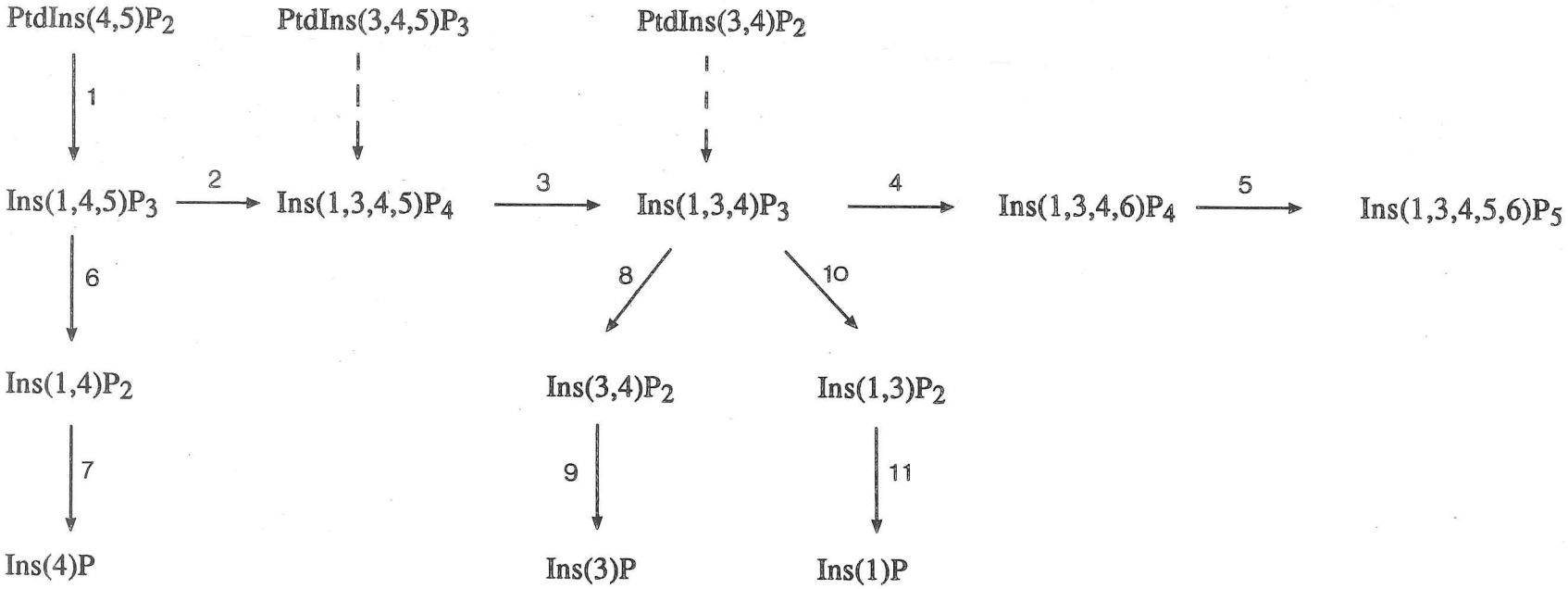

Figure 1.2 Inositol polyphosphate generation and metabolism. This reaction scheme shows both reactions which have been demonstrated to occur (⟶) and possible, but as yet unreported, reactions (- -➝). Reactions 1-5 are discussed in the text. Reactions 3 and 6 are both catalysed by a Mg^{2+}-dependent Ins(1,4,5)P_3 5'-phosphomonoesterase (Downes et al, 1982; Shears et al, 1985, 1987). Reactions 7 and 8 are catalysed by inositol polyphosphate 1-phosphatase in calf brain (Inhorn and Majerus, 1987), adrenal glomerulosa cells (Balla et al, 1988), rat pituitary tumour cells (Dean and Moyer, 1988), and rat brain (Ernaux et al, 1987; Delvaux et al, 1987). Reaction 9 has been observed in hepatocytes, calf brain extracts, and rat basophilic leukemia 2H3 cells (Hansen et al, 1986; Bansal et al, 1987; Cunha-Melo et al, 1988). Reactions 10 and 11 have been demonstrated in calf brain extracts (Bansal et al, 1987; Inhorn et al, 1987). All inositol monophosphates may be degraded to inositol and subsequently recycled to inositol phospholipids. Several of these reactions are inhibited by LiCl.

The demonstration that Ins(1,4,5)P_3 kinase is calcium-sensitive forms part of this thesis, and is discussed later. Ins(1,3,4)P_3, probably formed by Ins(1,3,4,5)P_4 dephosphorylation, can be rephosphorylated to Ins(1,3,4,6)P_4 in adrenal glomerulosa cells and hepatocytes (Balla et al., 1987, 1988; Shears et al., 1987; Hansen et al., 1988). In rat brain, Ins(1,3,4,6)P_4 can be further phosphorylated to Ins(1,3,4,5,6)P_5 (Stephens et al., 1988), which may be the $InsP_5$ detected in GH_4 cells (Heslop et al., 1985). Thyrotropin-releasing hormone causes little peturbation of $InsP_5$ and $InsP_6$ in GH_4 cells (Heslop et al., 1985), implying that neither has a role as a rapid second messenger. Indeed $InsP_5$ and $InsP_6$ are better known respectively as an allosteric regulator of oxygen binding to haemoglobin in avian erythrocytes and a major plant phosphate store (phytic acid). However, local infusion of $InsP_5$ and $InsP_6$ into a discrete brain stem nucleus implicated in cardiovascular regulation results in dose-dependent changes in heart rate and blood pressure, implying that $InsP_5$ and $InsP_6$ may function as neurotransmitters (Vallejo et al., 1987). A clearer picture exists of the functional role of Ins(1,4,5)P_3, and possibly Ins(1,3,4,5)P_4. Both may be involved in the rapid agonist-induced rise in $[Ca^{2+}]_i$ observed in many cell systems (see Section 1.4.5).

1.4.4 Ligand-stimulated $[Ca^{2+}]_i$ increases

T cell $[Ca^{2+}]_i$ is increased several-fold above the resting level (approximately 100nM) by ligands directed against a variety of surface receptors including T3-T_i. $[Ca^{2+}]_i$ increases within 30s of lectin or T3-T_i-directed antibody addition both in human and murine T cells, and reaches a maximum of 0.5-1μM within 60-120s (Tsien et al., 1982; Hesketh et al., 1983b; Weiss, M.J. et al., 1984; O' Flynn et al., 1984; Oettgen et al., 1985). Subsequently, $[Ca^{2+}]_i$ rapidly decreases to about 200nM then gradually declines to basal level over 24h (Hesketh et al., 1983a). In Ca^{2+}-depleted medium, ligands stimulate $[Ca^{2+}]_i$ increases of identical magnitude, but only 5-10 minutes duration, implying that the initial increase due to mobilisation of intracellular calcium stores is succeeded by influx of extracellular calcium (Hesketh et al., 1985; Imboden and Stobo, 1985). These responses have been observed predominantly in $T_{i\alpha/\beta}$-expressing cells such as Jurkats and thymocytes. However, similar increases have also been observed in

γδ-bearing T cells in response to PHA, or antibodies directed against T3, $T_{i\delta}$, or $T_{i\gamma}$ (Pantaleo et al., 1987a; Krangel et al., 1987; Wu et al., 1988; Bismuth et al., 1988). Similar responses have been observed in T cells challenged with antigen-presenting cells, implying that $[Ca^{2+}]_i$ elevation is a physiological response to antigen-T cell receptor interaction (Nisbet-Brown et al., 1985; Treves et al., 1987; Poenie et al., 1987). However, $[Ca^{2+}]_i$ elevation is also evoked by antibodies directed at receptors other than T3-T_i.

Anti-CD2 antibody combinations stimulate similar $[Ca^{2+}]_i$ responses to T3-T_i-directed ligands in peripheral blood lymphocytes and Jurkats (Breitmeyer et al., 1987; Pantaleo et al., 1987a,b,c; Weiss, M.J. et al., 1984). The initial failure to find an intracellular component of the CD2 response led to the suggestion that CD2 might function as a Ca^{2+}-channel (Alcover et al., 1986). However, despite the requirement for the cytoplasmic CD2 domain in CD2-mediated $[Ca^{2+}]_i$ responses (He et al., 1988), anti-CD2 antibody failed to evoke $[Ca^{2+}]_i$ elevation in CD2-transfected gut epithelial SF9 cells, implying that additional molecules are required to generate responses to CD2 (Alcover et al., 1988b). In contrast, cross-linking of anti-Thy 1 antibodies on both T cells and Thy 1-transfected B cells results in increased $[Ca^{2+}]_i$ implying that no other T cell-specific molecules are required (Kroczek et al., 1986a,b; Gunter et al., 1986).

Cross-linking of several other surface receptors also generates $[Ca^{2+}]_i$ responses. Anti-TAP and anti-Ly6 antibodies are effective in murine T cells (Malek et al., 1986; Yeh et al., 1987), while in human T cells small responses can be generated by cross-linking of any of CD4, CD5, CD6 or CD8 (Ledbetter et al., 1987b; Carrel et al., 1988). The ability of these molecules to stimulate $[Ca^{2+}]_i$ elevation may be important to their function as accessory molecules in antigenic stimulation.

1.4.5 The mechanism of ligand-stimulated $[Ca^{2+}]_i$ increases

The mechanism by which ligands induce calcium release from intracellular stores remained a mystery until the demonstration that $Ins(1,4,5)P_3$ could release calcium from a non-mitochondrial intracellular calcium store in permeabilised parotid acinar cells (Streb et al., 1983). Subsequently $Ins(1,4,5)P_3$-mediated Ca^{2+} release has been demonstrated in a

variety of cells including T lymphocytes (Imboden and Stobo, 1985; Eberl and Schnell, 1987; Gukovskaya et al., 1986), neutrophils (Prentki et al., 1984), hepatocytes (Joseph et al., 1984), sea urchin egg homogenates (Clapper and Lee, 1985) and platelets (Wilson et al., 1985). Although clearly non-mitochondrial, the precise identity of the Ins(1,4,5)P_3-sensitive calcium store is not known, although it may be an intracellular membrane system related to, but distinct from, the endoplasmic reticulum (Volpe et al., 1988). Ins(1,4,5)P_3 receptors have been characterised in microsomal fractions from adrenal cortex, anterior pituitary and liver (Baukal et al., 1985; Guillemette et al., 1987), but have also been found associated with a plasma membrane fraction (Guillemette et al., 1988). In the brain Ins(1,4,5)P_3 binding to the cerebellar receptor is antagonised by calcium due to the action of a Ca^{2+}-binding protein, calmedin (Worley et al., 1987; Supattapore et al., 1988; Danoff et al., 1988). In contrast, Ins(1,4,5)P_3-mediated calcium release is enhanced by GTP, via a mechanism involving GTP hydrolysis (and thus distinct from G-protein mediated phospholipase C activation), in a variety of permeabilised cell systems (Guillemette et al., 1987; Wolf et al., 1987; Gill et al., 1986; Ueda et al., 1986, 1988). It is not clear whether the in vivo function of GTP in this context is the synergistic activation of Ca^{2+} release with Ins(1,4,5)P_3, or as a component required for store refilling.

Thus there is strong evidence that intracellular Ca^{2+} release is mediated at least in part by Ins(1,4,5)P_3. However the role, if any, of Ins(1,3,4,5)P_4 as a mediator of $[Ca^{2+}]_i$ responses is unclear. Ins(1,3,4,5)P_4 at concentrations up to 20μM is ineffective at releasing Ca^{2+} from permeabilised Swiss 3T3 cells (Irvine et al., 1986b), but two Ins(1,3,4,5)P_4 receptors of differing affinity (k_d $4x10^{-9}$M and $9x10^{-8}$M) have been observed in adrenal cortical microsomes (Enyedi and Williams, 1988). These may be involved in Ca^{2+} re-uptake into intracellular stores since Ins(1,3,4,5)P_4 apparently causes Ca^{2+} sequestration into the Ca^{2+} stores of electropermeabilised neoplastic rat liver epithelial cells, via a mechanism not involving binding to the Ins(1,4,5)P_3 receptor (Hill et al., 1988b). This work contrasts with previous data suggesting that Ins(1,3,4,5)P_4 mediates influx of extracellular Ca^{2+} (Ca^{2+}_o). Irvine and Moor (1986) demonstrated that

$Ins(2,4,5)P_3$, a non-metabolisable but Ca^{2+}-mobilising $InsP_3$, was only capable of causing Ca^{2+}_o entry in the presence of $Ins(1,3,4,5)P_,$, using the raising of a fertilisation envelope in sea urchin eggs as a diagnostic assay for Ca^{2+}_o entry. However, Crossley and co-workers (1988) were unable to find a requirement for either $Ins(1,3,4,5)P_4$ or for external Ca^{2+} ions for the sea urchin egg fertilisation envelope to be raised by $Ins(2,4,5)P_3$ and Irvine et al. (1988) subsequently reported that the validity of their earlier finding was restricted to a single species of sea urchin (Lytechinus variegatus) at a particular season. Several groups have shown synergistic effects of $Ins(1,3,4,5)P_4$ and $Ins(1,4,5)P_3$ on intracellular Ca^{2+} release (Spat et al., 1987; Joseph et al., 1987). However since Ca^{2+} re-uptake does not occur until $Ins(1,4,5)P_3$ has been degraded, these effects may simply be due to $Ins(1,3,4,5)P_4$-mediated inhibition of $Ins(1,4,5)P_3$ hydrolysis via competition for the same enzyme (Joseph et al., 1987). This explanation may also apply to the apparent synergistic effect of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ on Ca^{2+}_o influx in lacrimal acinar cells (Morris, A.P. et al., 1987).

Consistent with this lack of hard evidence for a role for $Ins(1,3,4,5)P_4$ in Ca^{2+}_o entry, two recent pieces of work imply that $Ins(1,3,4,5)P_4$ is neither necessary nor sufficient to mediate Ca^{2+}_o entry. Substance P fails to stimulate $Ins(1,3,4,5)P_4$ accumulation in a pancreatoma cell line, apparently due to a deficiency in $Ins(1,4,5)P_3$ kinase activity, but nevertheless induces Ca^{2+}_o entry (Horstman et al., 1988). In contrast Ca^{2+} mobilisation in Jurkats is accompanied by $Ins(1,3,4,5)P_4$ accumulation (Stewart et al., 1986). However, examination of the decline in $Ins(1,3,4,5)P_4$ and $[Ca^{2+}]_i$ in Con A-treated Jurkats after the addition of α-methyl mannoside (which displaces Con A from its receptors) shows that $Ins(1,3,4,5)P_4$ is insufficient to mediate Ca^{2+}_o entry, since the onset of $[Ca^{2+}]_i$ decline precedes that of $Ins(1,3,4,5)P_4$ by at least 60s (Imboden and Weiss, 1987). These workers also examined the time course of $Ins(1,4,5)P_3$ decline, and found it to be co-incident with that of $[Ca^{2+}]_i$, implying a possible role for $Ins(1,4,5)P_3$ in receptor-stimulated Ca^{2+}_o influx. Furthermore, Kuno and Gardner (1987) have shown that in T cell membrane patches $Ins(1,4,5)P_3$ increases the opening probability of a voltage-insensitive Ca^{2+} channel, which may be identical to that activated by PHA, and

T3- and CD2-directed antibodies (Kuno et al., 1986; Pecht et al., 1987; Gardner et al., 1989). Taken together, these data imply that Ins(1,4,5)P_3 may be responsible for both components (i.e. intracellular Ca^{2+} release and extracellular Ca^{2+} entry) of the Ca^{2+} response evoked in T cells by lectins and T3-T_i- and CD2-directed antibodies.

1.5 Receptor-mediated protein kinase activation in T lymphocytes

1.5.1 Protein kinase C activation

Ligand-induced PtdInsP_2 hydrolysis causes $[Ca^{2+}]_i$ elevation via production of Ca^{2+}-mobilising inositol phosphate(s) (see Section 1.4.5). However, PtdInsP_2 cleavage may also activate a Ca^{2+}/phospholipid-dependent protein kinase, protein kinase C, via production of diacylglycerol (Kishimoto et al., 1980). The early measurements of protein kinase C activity as a calcium/phospholipid dependent increase in histone phosphorylation have now been extended by assaying the phosphorylation of distinct characteristic proteins, particularly an acidic 80kDa protein, distinct from protein kinase C (Rodriguez-Pena and Rozengurt, 1986; Blackshear et al., 1986). In addition to the effect of diacylglycerol (DAG), protein kinase C is also activated by phorbol esters (Parker et al., 1984; Castagna et al., 1982) and possibly by PtdInsP_2 (Chauhan and Brockerhoff, 1988). Since phorbol esters stimulate protein kinase C translocation from the cytosol to the plasma membrane (Kraft and Anderson, 1983), protein kinase C translocation is often used as a marker for protein kinase C activation. However, some mitogens apparently activate protein kinase C without causing translocation, implying that protein kinase C translocation may not be necessary for activation, and therefore that failure to observe translocation does not necessarily equate with an absence of protein kinase C activation (Halsey et al., 1987).

Seven subspecies of protein kinase C have so far been identified; α, βI, βII, γ, δ, ε and ζ (Kikkawa et al., 1987; Ono et al., 1987, 1988; Ohno et al., 1988). These are differentially expressed; α is most widely distributed, the β subspecies are fairly widespread, and γ expression is confined to nervous tissue in the brain and spinal cord (reviewed in Nishizuka, 1988); but most cell types contain more than one subspecies.

Investigation of the subspecies in T lymphocytes has so far revealed that human peripheral blood T cells contain α and β subspecies (Shearman et al., 1988). As yet no specialised functions have been ascribed to individual sub-species. Attempts to identify the function of protein kinase C have focussed on identification of proteins which undergo stimulated serine or threonine phosphorylation and might be physiologically relevant substrates. Alternatively, the effects of phorbol esters have been determined, although these are unlikely to reflect accurately the effects of DAG, since DAG undergoes rapid metabolism and is therefore only transiently elevated, but phorbol esters are not significantly metabolised and thus cause irreversible protein kinase activation, succeeded by down-regulation, possibly via calpain-mediated cleavage (Nishizuka, 1988).

In T lymphocytes, several serine phosphorylation events occur in response to T3-T_i stimulation, and these may be important for T cell activation. Lectins, antigen and T3-T_i-directed antibodies all stimulate serine phosphorylation of two murine T3 components; the 21kDa γ chain, and to a lesser extent the 25kDa ε glycoprotein (Oettgen et al., 1986; Samelson et al., 1985, 1986a,b; Klausner et al., 1987). Analogous ligands also stimulate phosphorylation of human T3 components, but of the γ and δ, rather than γ and ε, chains (Cantrell et al., 1987; Davies et al., 1987; Nel et al., 1987). Identical phosphorylations are also evoked by phorbol esters, implying that T3-T_i-evoked phosphorylations may be due to protein kinase C activation (Davies et al., 1987; Cantrell et al., 1985; Klausner et al., 1987). Phorbol esters also induce phosphorylation of IL2r, CD45, CD4, CD8 and the β-chain of LFA-1 (Autero and Gahmberg, 1987; Shackelford and Trowbridge, 1986; Chatila and Geha, 1988; Acres et al., 1986).

Further evidence for T3-T_i-mediated protein kinase C activation is provided by experiments demonstrating anti-CD3 antibody-stimulated protein kinase C translocation in Jurkats and human peripheral blood T cells (Ledbetter et al., 1987a; Nel et al., 1987) and anti-CD3 antibody-evoked 80kDa protein phosphorylation in human T cell clones (Friedrich et al, 1989). Manger et al. (1987) have observed enhanced protein kinase C translocation in Jurkats stimulated with immobilised compared with soluble anti-CD3 antibody, and suggest this may provide at least a partial explanation for the requirement

for antibody immobilisation for proliferation (see Section 1.2.3). Consistent with this, Davis and Lipsky (1989) find that T cell proliferation evoked by soluble anti-CD3 antibody plus phorbol dibutyrate (PDBu) may be reversed by removal of PDBu at any stage up to 24h after ligand addition. In contrast to these data demonstrating T3-T_i-mediated protein kinase C activation in mature T cells, neither protein kinase C translocation nor 80kDa protein phosphorylation can be observed in Con A-stimulated BALB/c thymocytes (T.R. Hesketh, personal communication). This suggests that the antigen receptor may be in differing functional states in mature (peripheral) and immature (thymic) T cells. Consistent with this, Finkel and colleagues (1987) have reported smaller anti-T_i antibody-induced $[Ca^{2+}]_i$ increases in thymocytes compared with lymph node cells, and Havran et al. (1987) have found that splenocytes but not thymocytes respond to anti-CD3 antibody by expressing IL2 and IL2r, although a $[Ca^{2+}]_i$ response is generated in both.

Protein kinase C may also be activated through cell surface receptors other than T3-T_i. Anti-CD2 antibodies stimulate phosphorylation of 80kDa protein and the CD3-γ chain in human T cells (Friedrich et al., 1989; Breitmeyer et al., 1987); and anti-Thy 1 antibody stimulates phosphorylation of murine CD3-γ (Klausner et al., 1987). Murine CD3-γ phosphorylation stimulated by antigen or T3- or Thy 1-directed antibodies, but not by AlF_4^-, is inhibited by cAMP or analogue elevation, implying that both T3- and Thy 1-evoked responses channel through a common step, upstream of G protein activation by AlF_4^- (Klausner et al., 1987; O'Shea et al., 1987).

The widespread ability of ligands to activate protein kinase C both in T lymphocytes and other cells suggests protein kinase C activity may be central to mitogenic stimulation. Furthermore, mitogens can be substituted for by the synergistic combination of calcium ionophore and phorbol ester (Truneh et al., 1985; Guy et al., 1985) implying that $[Ca^{2+}]_i$ elevation and protein kinase C activation, the "signals" generated by the cleavage products of $PtdInsP_2$, may be sufficient to trigger cellular transition from G_0 to DNA synthesis (Dual Signal Hypothesis, e.g. Nishizuka, 1984). However, more recent demonstrations both of synergistic mitogen combinations which fail to elicit $PtdInsP_2$ hydrolysis (e.g.

epidermal growth factor and insulin on fibroblasts; L'Allemain and Pouyssegur, 1986; Besterman et al., 1986) and of an additional response evoked by both $PtdInsP_2$-cleaving and non-$PtdInsP_2$-hydrolysing ligands by a mechanism independent of $PtdInsP_2$ hydrolysis (tyrosine kinase activation, see below) suggests the actions of DAG and $Ins(1,4,5)P_3$ to be insufficient to induce mitogenesis. Despite this, the failure of T3-T_i-directed ligands to induce mitogenesis in protein kinase C down-regulated cells (Kim et al., 1989; Valge et al., 1988), suggest protein kinase C activation is important in T cell activation. Furthermore, combination of any of a variety of antibodies directed against T cell surface receptors with TPA synergistically stimulates T cell proliferation (see Section 1.3). However, such synergistic combinations may stimulate proliferation via pathways other than those evoked by in vivo intercellular interactions.

1.5.2 Consequences of protein kinase C activation

In addition to the protein phosphorylations described above, protein kinase C may activate a plasma membrane-associated Na^+/H^+ exchanger, although this may also be activated independently of protein kinase C (Grinstein et al., 1987). In HCO_3^--free medium, both TPA and T3-T_i-directed ligands stimulate intracellular alkalinisation via activation of Na^+/H^+ exchange (Hesketh et al., 1985; Rosoff and Cantley, 1985; Rogers et al., 1983). However, in the presence of HCO_3^- the overall intracellular pH (pH_i) change evoked by ligands is an acidification (Gelfand et al., 1988; Ganz et al., 1989). These results are in apparent conflict with data from several groups implying a role for intracellular alkalinisation in the stimulation of DNA synthesis (Pouyssegur et al., 1985; Ober and Pardee, 1987; Perona and Serrano, 1988; Chambard and Pouyssegur, 1986); but are consistent with the idea, suggested by the reported failure of amiloride analogues to inhibit DNA synthesis at concentrations greater than or equal to those which effectively antagonise Na^+/H^+ exchange, that Na^+/H^+ exchanger-mediated alkalinisation is not required for mitogenesis (Pennington et al., 1989; Mills et al., 1986). Taken together these data suggest that although intracellular alkalinisation may not be an obligatory step in mitogenesis, pH_i may be required to remain within a certain permissive range for DNA synthesis to occur.

Activation of protein kinase C may also result in phosphorylation of the S6 protein, a component of the 40S ribosomal subunit, although S6 phosphorylation may also be stimulated by a variety of other agents including cAMP (Parker et al., 1985; Rance et al., 1985; Pelech and Krebs, 1987). An apparent causal link between Na^+/H^+ exchange and S6 phosphorylation in Chinese hamster lung fibroblast cells (Pouyssegur et al., 1982) may actually have been an artefact due to the use of amiloride, since dimethylamiloride (a more specific Na^+/H^+ exchange inhibitor) blocks pH_i increases without affecting S6 phosphorylation in 3T3 fibroblasts (Pennington et al., 1989). Several pieces of data suggest a causal link between mitogen-induced S6 phosphorylation and increases in protein synthesis (Thomas et al., 1982; Duncan and McConkey, 1982). Thus the general mitogen-stimulated increase in protein synthesis may be at least partially caused by protein kinase C, via S6 phosphorylation.

In contrast to these positive effects of protein kinase C, it may also have a role in both long and shorter term negative feedback. The ability of phorbol esters to cause down-modulation of a variety of cell surface molecules including in T cells the T3-T_i complex and CD4 (Cantrell et al., 1985; Davies et al., 1985; Wang et al., 1987) may mimic a protein kinase C-mediated receptor down-regulation necessary to terminate response to a particular stimulus. Protein kinase C activation may also modulate $Ins(1,4,5)P_3$ accumulation, both by activating $Ins(1,4,5)P_3$ 5'-phosphomonoesterase (Connolly et al., 1986a; Molina y Vedia and Lapetina, 1986) and by inhibiting $PtdInsP_2$ breakdown. Phorbol ester-mediated antagonism of $PtdInsP_2$ hydrolysis has been observed in a wide range of cells including platelets (Tohmatsu et al., 1986), B cells (Bijsterbosch and Klaus, 1987; Harnett and Klaus, 1988), fibroblasts (Jamieson Jr. and Villereal, 1985; Brown et al., 1987) and vascular smooth muscle cells (Brock et al., 1985; Takata et al., 1988). Thus the early activatory effects associated with protein kinase C activation, which include transcriptional activation of a variety of genes (see below), may precede, or even occur simultaneously with, protein kinase C-mediated negative feedback control.

1.5.3 Tyrosine kinase activation

In addition to serine/threonine phosphorylation, the generation of phosphotyrosine residues is also associated with mitogenic stimulation. Several oncoproteins have tyrosine kinase activity (e.g. src, abl, fes), implying a role for tyrosine phosphorylation in cell transformation, and receptors for several growth factors (including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin) have an intrinsic tyrosine kinase domain, capable of mediating phosphorylation of both themselves and other cellular proteins (Cooper et al., 1982; Ek et al., 1982; Zippel et al., 1986; Kasuga et al., 1982; Klein et al., 1986; Yu and Czech, 1986).

In T cells, despite the absence of intrinsic tyrosine kinase domains in any of the T3-T_i complex components, CD3-ζ is phosphorylated on tyrosine in response to mitogenic stimulation via T3-T_i or Thy 1 (Baniyash et al., 1988b; Samelson et al., 1986b, 1987; Klausner et al., 1987; Patel et al., 1987), and IL2 stimulates tyrosine phosphorylation of several proteins distinct from CD3-ζ (Saltzmann et al., 1988). Several tyrosine kinases have been identified in T cells, including a 40kDa enzyme generated proteolytically from a 72kDa protein isolated from bovine thymus (Zioncheck et al., 1988), and two human enzymes of M_r 70-100kDa and 35-40kDa which may be homologous to the bovine proteins (Hall et al., 1987). However, $pp56^{lck}$ is the best characterised T cell tyrosine kinase, and the most likely candidate for mediator of CD3-ζ phosphorylation.

Originally identified in a murine T cell lymphoma LSTRA, $pp56^{lck}$ is a 56-58kDa, phosphotyrosine-containing, membrane-associated tyrosine kinase of T lymphoid lineage, expressed in both murine and human systems (Casnellie et al., 1983; Marth et al., 1985; Trevillyan et al., 1986). Over-expression of $pp56^{lck}$ in Thy19 and LSTRA cells is due to insertion of the murine Moloney leukemia virus upstream of the $pp56^{lck}$ gene (Voronova and Sefton, 1986; Voronova et al., 1987; Marth et al., 1988b). However, expression of $pp56^{lck}$ is insufficient to transform fibroblasts since phosphorylation of Tyr-505 down-regulates the kinase activity of $pp56^{lck}$, in a manner analogous to the down-regulation of $pp60^{c\text{-}src}$ by Tyr-527 phosphorylation. Therefore Tyr-505 mutation is necessary for $pp56^{lck}$-mediated transformation of fibroblasts in vitro (Marth et al., 1988a; Amrein and

Sefton, 1988). However, in LSTRA cells Tyr-505 of $pp56^{lck}$ is intact, but hypophosphorylated (Marth et al., 1988a).

In murine T cell clones, $pp56^{lck}$ may be physically associated with CD4 or CD8, since it co-modulates and co-immunoprecipitates with these molecules (Veillette et al., 1988). Recent data suggest that CD4-associated $pp56^{lck}$ may mediate CD3-ζ tyrosine phosphorylation, since cross-linking of CD4 causes simultaneous phosphorylation of $pp56^{lck}$ and CD3-ζ (Veillette et al., 1989). In vivo, $pp56^{lck}$ activation may be achieved by interaction of CD4 with Class II MHC proteins on the surface of antigen-presenting cells (see Section 1.2.5). The failure of antigen-presenting cells in the absence of appropriate antigen to stimulate CD3-ζ phosphorylation (e.g. Klausner et al., 1987) presumably reflects a requirement for a T3-T_i-directed ligand to stimulate association of CD3 with CD4 (Rivas et al., 1988), thereby bringing substrate and enzyme into close proximity. However, the involvement of $pp56^{lck}$, and therefore CD4:class II interaction, in CD3-stimulated CD3-ζ phosphorylation is apparently contradicted by the demonstration by Veillette et al. (1989) that anti-CD3-ε antibody 145-2C11 stimulates tyrosine phosphorylation of CD3-ζ but not $pp56^{lck}$. However, this discrepancy may be due to the use of different cross-linking agents in the two experiments, Rabbit anti-hamster Ig plus 145-2C11 fails to stimulate $pp56^{lck}$ autophosphorylation, but 145-2C11 plus B cells (presumably bearing MHC class II molecules) induces CD3-ζ phosphorylation. Thus, these results are consistent with the idea that class II:CD4 interaction activates CD4-associated $pp56^{lck}$, which in turn phosphorylates CD3-ζ.

The causal relationship, if any, between tyrosine kinase activation and $PtdInsP_2$ breakdown is currently unclear. Since $PtdInsP_2$ is not hydrolysed in response to tyrosine kinase activating ligands such as EGF and insulin (Besterman et al., 1986; L'Allemain and Pouyssegur, 1986), activation of tyrosine kinase is clearly insufficient to trigger $PtdInsP_2$ breakdown, and conversely tyrosine kinase activation is not always a consequence of $PtdInsP_2$ hydrolysis. This latter is also suggested by the observations that AlF_4^- stimulates inositol phosphate formation, but not CD3-ζ phosphorylation in murine T cells (O'Shea et al., 1987) and dibutyryl CAMP inhibits anti-CD3 antibody-

stimulated inositol phosphate formation but not CD3-ζ phosphorylation (Klausner et al., 1987). However, experiments with EGF receptors deficient in tyrosine kinase activity due to mutation of the residue required for phosphate transfer (Lys-721) suggest that tyrosine kinase activity is obligatory for EGF-induced mitogenesis, $Ca^{2+}{}_o$ influx, and inositol phosphate accumulation in EGFr-transfected fibroblasts (Chen et al., 1987; Honnegger et al., 1987; Moolenaar et al., 1988). Furthermore, tyrosine kinase activating ligands enhance inositol phosphate accumulation evoked by $PtdInsP_2$-hydrolysing ligands in Chinese hamster lung fibroblasts (Paris et al., 1988). Taken together, these results suggest that although tyrosine kinase activation is insufficient alone to stimulate $PtdInsP_2$ hydrolysis, activation of the latter may require tyrosine phosphorylation of an unidentified protein.

The possible requirement for tyrosine kinase activity for $PtdInsP_2$ hydrolysis may be linked to the association and phosphorylation of an 81-85kDa PtdIns kinase with/by tyrosine kinases including $pp60^{c\text{-}src}$ and PDGFr (Courtneidge and Heber, 1987; Kaplan et al., 1987). However, the relationship between this and $PtdIns(4,5)P_2$ breakdown or accumulation is not immediately obvious, since this lipid kinase is a PtdIns 3'-kinase rather than a 4'- or 5'-kinase (Auger et al., 1989; Fukui and Hanafusa, 1989). Furthermore, experiments with mutant PDGF receptors suggest that the PDGFr-associated PtdIns 3'kinase mediates events distinct from $PtdIns(4,5)P_2$ breakdown, but required for mitogenesis. Interaction of PDGF with PDGF receptors lacking the region necessary for association with PtdIns 3' kinase induces tyrosine kinase activation, and $PtdIns(4,5)P_2$ hydrolysis, but fails to stimulate DNA synthesis (Escobedo and Williams, 1988; Coughlin et al., 1989).

1.5.4 The role of cAMP-dependent protein kinase in mitogenic stimulation of T cells

Serine/threonine phosphorylation is catalysed not only by protein kinase C, but also by cAMP-dependent protein kinase (protein kinase A). The latter is activated by a variety of hormones, including β-adrenergic agonists, via activation of adenylate cyclase, as described in Section 1.4.2. Activation of cAMP-dependent protein kinase may also be

required during mitogenic stimulation of some cell types (reviewed by Boynton and Whitfield, 1983; Dumont et al., 1989). However the role of protein kinase A in T cell activation is controversial. Observed increases in cAMP during the T cell cycle suggest a role for cAMP in mitogenic stimulation of T lymphocytes (Boynton and Whitfield, 1983) but cAMP analogues and agents which raise cAMP are capable of antagonising the proliferative response of T cells to T3-T_i-directed ligands (Ling and Kay, 1975; Novogrodsky et al., 1983; Nel et al., 1988) and IL2 (Knudsen et al., 1987; Farrar et al., 1987; Beckner and Farrar, 1988; Johnson et al., 1988). Cyclic AMP-mediated inhibition of T3-T_i-stimulated proliferation is apparently due to an effect on early responses; cAMP elevation or cAMP analogues diminish or abolish stimulated increases in inositol phosphates, $[Ca^{2+}]_i$, and protein kinase C-mediated phosphorylation (Taylor et al., 1984; Takayama et al., 1988; Klausner et al., 1987; Rincon et al., 1988; Lerner et al., 1988) and also inhibit transcriptional activation of the IL2 and IL2r genes (Novogrodsky et al., 1983; Yamamoto et al., 1986; Rincon et al., 1988). The event inhibited by cAMP presumably lies between ligand-receptor interaction and G protein activation, since AlF_4^--induced responses are not sensitive to cAMP (O' Shea et al., 1987). This is consistent with the absence of rapid increases in cAMP in murine and porcine lymphocytes stimulated with mitogenic lectins (Moore et al., 1983). In contrast, Shirakawa et al. (1988) reported that IL1 caused transient cAMP accumulation, and that the role of IL1 in the synergistic proliferative response of murine thymocytes to IL1 plus PHA could be substituted for by cAMP elevating agents. Wickremashinge et al. (1987) have suggested that IL2 may also be capable of elevating cAMP, but several other reports imply that IL2 actually inhibits both basal and stimulated adenylate cyclase activity, possibly via activation of protein kinase C (Knudsen et al., 1987; Beckner and Farrar, 1986, 1987, 1988).

Taken together these data suggest that cAMP elevation is unlikely to be a rapid response to T cell stimulation via T3-T_i or the IL2 receptor. However, accessory cell secretions such as interleukin 1 may mediate cAMP increases at discrete points in the cell cycle, presumably regulated by the time courses of both secretion and receptor

expression, and these transient increases may be an obligatory component of the T cell transition from quiescence to DNA synthesis.

1.6 Receptor-mediated activation of gene transcription in T lymphocytes

Over 60 distinct cDNA clones have been derived from immediate early mRNA species that are induced on addition of PHA and TPA to quiescent human peripheral blood T cells Zipfel et al., 1989) but only a handful of mitogen-activated genes have so far been characterised. Numbered among these are some key metabolic enzymes, the cellular homologues of some oncogenes, and the IL2 and IL2r genes, expression of which is confined to T cells, and possibly only to a subset thereof (see Section 1.2.4). Transcriptional activation of the IL2 and IL2r genes is apparently dependent on different antigen-induced responses. IL2r expression is stimulated by either phorbol ester alone or a synergistic combination of phorbol ester and calcium ionophore implying that T3-T_i-induced IL2r expression may be achieved by a combination of protein kinase C activation and increased $[Ca^{2+}]_i$ (McCrady et al., 1988; Shackelford and Trowbridge, 1984; Weiss, A et al., 1984b; Kumagai et al., 1988). In contrast, although phorbol ester plus ionomycin stimulates IL2 mRNA accumulation in resting PBLs (McCrady et al., 1988), events other than those consequent on $PtdInsP_2$ breakdown may be sufficient to induce IL2 secretion since anti-Thy 1 antibody stimulates IL2 secretion from a murine T cell mutant in the absence of increases in $[Ca^{2+}]_i$ or inositol phosphates (Sussman et al., 1988b).

The transcriptional activation of genes encoding various key metabolic enzymes may account in part for the enhanced metabolic activity detectable in mitogen-stimulated T cells. However activation of pre-existing enzymes also occurs, and some control points may be regulated by both modulation of existing proteins and induction of gene transcription. A prime example of both phenomena is ornithine decarboxylase (ODC), the rate limiting enzyme in polyamine biosynthesis. Rapid ODC induction occurs within minutes of ligand addition via a Ca^{2+}-dependent mechanism with no requirement for de novo protein biosynthesis, implying activation of pre-existing enzyme (Scott et al., 1985)

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and is succeeded by a slower increase in activity which may be due to transcription of the ODC gene (Fidelus et al., 1984). ODC activation may in turn lead to the induction of several glycolytic enzymes (Brand, 1987), and may also be required for induction of loci unrelated to metabolism, such as the proto-oncogene c-myc (Celano et al., 1988).

Proto-oncogene transcription is a mitogen-evoked response common to T cells and many fibroblastic cell lines, with transcription of the c-myc and c-fos loci (cellular counterparts of the transforming genes of avian myelocytomatosis virus and FBJ murine osteosarcoma virus respectively) the best characterised responses. Rapid transient increases in c-fos mRNA precede more prolonged increases in c-myc mRNA in lectin-stimulated T cells and growth factor-stimulated 3T3 fibroblasts (Kelly et al., 1983; Moore et al., 1986; Kronke et al., 1985; Reed et al., 1985; Muller et al., 1984), and IL2-IL2r interaction induces a second wave of c-myc and c-fos transcription in T cells (Farrar et al., 1987; Depper et al., 1985; Reed et al., 1985). Increased levels of c-myb mRNA are also observed in activated T cells challenged with IL2 (Farrar et al., 1987; Pauza, 1987; Stern and Smith, 1986), but as c-myb mRNA is maximal subsequent to maximal DNA synthesis, c-myb expression may not be required for DNA synthesis (Lipsick and Boyle, 1987). In contrast, experiments utilising antisense myc and fos oligonucleotides and anti-myc and fos antibodies suggest that both c-myc and c-fos proteins are required for mitogenic stimulation (Holt et al., 1986, 1988; Freytag, 1988; Yokayama and Imamoto, 1987; Wickstrom et al., 1988; Heikkila et al., 1987; Harel-Bellan et al., 1988b; Riabowol et al., 1988; Iguchi-Ariga et al., 1987). While the precise function of c-myc is not known, c-fos appears to act as a transcription factor when complexed with AP-1 (c-jun) (Rauscher et al., 1988; Schuermann et al., 1989; Chiu et al., 1988; Neuberg et al., 1989).

Fos protein regulates expression of several genes, including itself and collagenase (Schonthal et al., 1988). However, the mechanism by which lectins or IL2 activate c-fos transcription in T cells is unclear. Lectin-stimulated fos transcription is independent of $[Ca^{2+}]_i$ increases, protein kinase C activation, or Na^+/H^+ exchange (Moore et al., 1986, 1988; Grinstein et al., 1988). Furthermore, although c-fos expression can be regulated by cAMP (e.g. 8BrcAMP induces c-fos expression in Swiss 3T3 fibroblasts; Tsuda et

al., 1986) cAMP is not an early response to T cell activation (see Section 1.5.4), and is therefore unlikely to be responsible for c-fos induction in T cells. Thus despite copious observations of mitogen-stimulated gene expression in T cells, the causal relationship between transcriptional activation of many of these loci and earlier biochemical responses remains unclear.

1.7 Aims of this thesis

I have attempted to identify important events in T lymphocyte mitogenesis by examining the responses of two murine T cell populations to a panel of ligands. The study has been undertaken in two cell populations not only to allow comparisons, but also to facilitate identification of phenotypic peculiarities. The cells and ligands used are described below.

1.7.1 Cell types

The two cell populations used are thymocytes derived from BALB/c mice, and clone 4, a monoclonal helper T cell line cloned from a CJ3/H mouse by Dr. K. Tomonari (Clinical Research Centre, Harrow). BALB/c thymocytes are a heterogeneous cell population comprised of several cell types including T lymphocytes (85%), B lymphocytes (<5%), erythrocytes (1-3%), and monocytes and macrophages (Corps, 1982). As described above, thymic T cells are a mixture of cells in varying stages of maturity, with different stages distinguishable by their array of surface-expressed proteins. Of these, only the more mature cells express a T cell receptor. In contrast, Thy 1 is expressed on all T lymphocytes in the thymus.

Thymocytes are a useful system to work on as they are easily prepared and, despite individual preparation for each experiment, exhibit reproducible responses. In addition, thymocytes are not subject to the phenotypic alterations often induced by long term cell culture. However, the heterogeneity of thymocytes is undesirable for several reasons;

i Only a small proportion of thymocytes may respond to a given stimulus.

ii Cell types other than T lymphocytes may respond to mitogenic ligands.

iii Mitogenic stimulation of T lymphocytes may be facilitated (for instance by accessory cell-secreted lymphokines) or hindered (e.g. accessory cells may adsorb/degrade ligands) by other cell types within the thymocyte population.

These considerations make thymocytes a complex system in which to analyse the requirements for activation of DNA synthesis.

In contrast, clone 4 is a murine monoclonal cell line which stably expresses CD4, Thy 1, LFA 1, IL2r and a T cell receptor specific for the MHC class II molecule E^k. Clone 4 may be mitogenically stimulated by cells bearing E^k antigen in the context of MHC class II proteins, or specific anti-T_i antibodies 1G3 and 2H11 (Tomonari, 1985). The homogeneity of clone 4 should result in similar responses to a particular ligand in every individual cell. Thus the problems associated with heterogeneous cell populations do not arise. However, there are problems associated with the use of monoclonal cell lines for analysis of the mitogenic pathway. Many such cell lines have phenotypic abnormalities which prevent them from generating "normal" responses to ligands. For example, the interaction of EGF with receptors on 3T3 fibroblasts results in no inositol phosphate accumulation (Besterman et al., 1986), but inositol phosphates are generated by the abnormal cell line A431 in response to EGF (Pike and Eakes, 1987; Hepler et al., 1987).

By study and comparison of the responses to ligands of two cell types, one a partially activated (i.e. IL2r-expressing) monoclonal cell line and the other a heterogeneous primary cell population, it should be possible to avoid some of the difficulties associated with study of a single cell population.

1.7.2 Ligands

Four potentially mitogenic ligands have been used in this work; Con A, anti-Thy1 antibody (KT16; Tomonari, 1988), anti-T3 antibody (145-2C11; Leo et al., 1987b), and recombinant IL2. The effect of a novel anti-CD4 antibody KT6 has also been determined. KT6, raised by Dr. K. Tomonari, antagonises stimulation of clone 4 through the T cell receptor (K. Tomonari, personal communication), and may also be expected to have some effect on thymocyte mitogenesis as 80-90% of thymocytes bear CD4. Therefore it is of interest to determine the effect of KT6 on ligand-stimulated mitogenesis of both cell types.

1.7.3 Experimental Programme

Using the described ligands, the following programme of research has been undertaken:

1 Characterisation of the effects of the described panel of ligands on mitogenesis in the two cell types, and definition of interactive effects of the ligands (particularly anti-CD4 antibody) on mitogenesis.

2 Characterisation of some early responses of the two cell types to each of these ligands. Effects of the ligands on $[Ca^{2+}]_i$, inositol phosphates, and c-myc mRNA have been determined.

3 Investigation of the effect on early responses to mitogens of the antagonistic receptor interactions identified in 1.

The results of these experiments are described in Chapters 3-5. These results have been analysed to assess the importance of these responses for mitogenesis Questions of particular interest include:

i Are there differences in early responses to the same ligand in two different cell populations, and do any differences correlate with mitogenic stimulation?

ii Do mitogenic and non-mitogenic ligands evoke the same early responses in a single cell type?

iii Can ligands stimulate mitogenesis in a single cell type by evoking different sets of early responses?

iv Are early responses to mitogenic ligands inhibited by ligands which antagonise mitogenesis?

Such analysis may facilitate identification of responses obligatory for the transition of lymphocytes from quiescence to DNA synthesis. To explain the sequence of experiments it is necessary to discuss results as they arise. Therefore all discussion and analysis of results is included within the results chapters, rather than being presented in separate sections.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

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2.1 Materials

2.1.1 Chemicals

AMP, Antifoam A, cyclic AMP (cAMP), dextran sulphate, EGTA, forskolin, herring sperm DNA, 3-isobutyl-1-methyl-xanthine (IBMX), N-lauroyl sarcosine, octanoic acid, mannitol, sodium azide, streptomycin sulphate (750U/mg), 12-O-tetradecanoyl 13-phorbol acetate (TPA), and tris[hydroxymethyl]aminoethane (Trizma base) were purchased from Sigma Chemical Company Ltd. ADP (disodium salt), ATP (disodium salt), DNAse I, and tRNA, were obtained from Boehringer Mannheim. Agarose, DNA polymerase I (125U/ml), and restriction endonucleases were supplied by Pharmacia. Guanidinium isothiocyanate was obtained from Bethesda Research Laboratories, USA. Triton X-100 and 2,5-diphenyloxazole (PPO) were purchased from Koch-Light. Safe fluor S scintillant was obtained from May and Baker. Concanavalin A was from Miles-Yeda. The calmodulin antagonist CGS9343B was a gift of Dr. A.M.Tolkovsky but was originally obtained from Ciba-Geigy. Interleukin 2 was generously provided by Dr. E. Simpson (Clinical Research Centre, Harrow). All other chemicals used were obtained from British Drug Houses, Fisons Scientific Apparatus, May & Baker or Merck.

2.1.2 Radiochemicals

Myo-[2-^{3}H]inositol (10-20Ci/mmol, 1mCi/ml), D-myo-[2-^{3}H]inositol 1,4,5-trisphosphate (1.0Ci/mmol, 10mCi/ml), [6-^{3}H]thymidine (5.0Ci/mmol, 1mCi/ml), [2,8-^{3}H]adenosine 3',5'-cyclic phosphate (30-50Ci/mmol, 1mCi/ml), and [α-^{32}P]dATP (~3,000Ci/mmol, 10mCi/ml) were purchased from Amersham International. [^{3}H]inositol 1,3,4,5-tetrakisphosphate was kindly supplied by Dr. R.F. Irvine, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge. [^{3}H]quin

2-acetoxymethyl ester (5.4 Ci/mol) was synthesised by Dr. G.A.Smith (Hesketh et al., 1983b).

2.1.3 Tissue culture equipment and reagents

Falcon 96 well microtitre plates were purchased from Becton Dickinson. L-glutamine (200mM), 2-mercaptoethanol (50mM), Penicillin G (sodium salt, 1,625U/mg), and RPMI 1640 were obtained from Gibco Ltd (Scotland). Flow Laboratories (Scotland) supplied gentamycin (10mg/ml), Linbro 24 well culture plates and Basal Medium Eagle (BME) Diploid. Mycoplasma-free foetal calf serum (FCS) was obtained from Biological Industries (Scotland), and was heat-inactivated by incubation at 60°C for 1h.

2.1.4 Chromatography equipment and reagents

HPLC columns, guard columns and packing materials were all obtained from HPLC Technology. AG1-X8 anion exchange resin (200-400mesh, formate form) was supplied by Bio-rad (USA). Sephacryl HR400 gel and Protein A-Sepharose (CL-4B) were obtained from Pharmacia, as were the FPLC equipment and Phast microgel electrophoresis apparatus used in antibody purifications. The FPLC and Phast equipment employed were the property of the Ludwig Institute for Cancer Research, Cambridge and were used with the kind permission of Dr. G.I. Evan.

2.1.5 Additional material

Phytic acid hydrolysate (Wreggett et al., 1987) was a gift from Dr. R.F.Irvine. Nitrocellulose membrane was obtained from Schleicher and Schuell. X-omat x-ray film and Tri-X pan black and white film were from Kodak. A pUC8 plasmid containing exons 2 and 3 of the mouse c-myc gene was provided by Dr. M.S. Neuberger, Medical Research Council Laboratory of Molecular Biology, Cambridge. All water used was purified first by distillation, and subsequently by passage through a Milli Q water purification system (Millipore Corporation).

2.2 Antibodies and antibody purification

All antibodies used were obtained from Dr.K.Tomonari, Clinical Research Centre, Harrow. Table 2.1 shows the source and isotype of the antibodies used. Of these,

KT16, KT6 and 15D5 were established by Dr.K.Tomonari (1988) from a fusion of NSO myeloma cells with Sprague-Dawley rat splenocytes, after immunisation of the rats with clone 4 (KT16, KT6) or clone C6 (15D5). PC61-53 (Lowenthal et al., 1985) and 145-2C11 (Leo et al., 1985) were originally obtained from Dr. H.R. MacDonald, and Dr. J. Bluestone respectively.

Antibody	Target	Source/Isotype		Method of purification
KT6	CD4	Rat IgG2a	}	Octanoic acid, then Mono Q
15D5	CD8	Rat IgG2a	}	FPLC
KT16	Thy1	Rat IgG2c		Protein A or Hypotonic Pptn
PC61-53	IL2 receptor	Rat IgG1		Octanoic acid
145-2C11	T3	Hamster IgM		Gel filtration

TABLE 2.1 Antibody source, type, and purification

All antibodies were purified from serum or ascites to apparent homogeneity, as assayed by SDS microgel electrophoresis (see section 2.2.7). The choice of purification method depended on the antibody source and isotype (see Table 2.1). Some antibodies were treated with octanoic acid as described below.

2.2.1 Octanoic acid treatment

Ascites fluid or serum was placed in a Corex tube, to minimise antibody adsorption (G.I.Evan, personal communication), and pH adjusted to 5.0 with 3M acetic acid/1M sodium citrate pH 5.2. Octanoic acid was then added (5% volume). Lipids were precipitated by stirring for 30 mins at 25°C, then pelleted by centrifugation (15 mins, 25°C, 12,000xg). Antibody was precipitated from the supernatant by addition of an equal volume of saturated ammonium sulphate, then resuspended in approximately $^1/_{10}$th the original volume in inorganic salts solution (125mM NaCl, 5.2mM KCl, 1mM Na_2HPO_4 , 0.5mM $MgCl_2$, 0.43mM $CaCl_2$, 10mM HEPES, 11mM glucose, at pH

7.2). Ammonium sulphate traces were removed by dialysis against 2x500ml of salts solution.

This protocol substantially depleted the sera of lipids, and was therefore useful for cleaning up sera before subjecting them to ion-exchange FPLC. In this case, ammonium sulphate-precipitated protein was resuspended in the FPLC starting buffer; 20mM triethanolamine (TEN) HCL, pH 7.5.

One antibody, PC61-53, appeared as a single band after octanoic acid treatment, and was therefore not further purified.

2.2.2 Anion exchange FPLC

Rat IgG2a antibodies were separated from contaminating albumin and transferrin by anion exchange FPLC. After octanoic acid treatment, aliquots containing 50-500μg protein were centrifuged (1 min, 13,000xg) to remove large particles, then loaded onto a Mono Q column. This was protected by 2-3 in-line prefilters, changed whenever increasing back-pressure could not be reversed by thorough column washing. Proteins were eluted by increasing the sodium chloride concentration from 0 to 1M, using a flow rate of between 0.5 and 1.0ml/min (all eluents contained 20mM TEN HCl, pH 7.5). A typical solvent regime was: 5ml of 0M NaCl, pH7.5, to load and equilibrate the column; linear increase over 1ml to 100mM NaCl; linear increase over 15ml to 300mM NaCl; rapid linear increase (in 3 ml) to, and maintenance (for 3ml) at, 1M NaCl; return over 3ml to 0M NaCl; and finally 5ml of 0M NaCl to re-equilibrate the column. Protein was detected by measuring A_{280} of the eluate. Protein peaks were collected as 1ml fractions, and subsequently analysed by microgel electrophoresis (see Section 2.2.7, below). Antibody eluted between 140 and 160 mM NaCl. Relevant fractions were pooled, ammonium sulphate precipitated, resuspended in and dialysed against inorganic salts solution.

2.2.3 Protein A affinity chromatography

Octanoic acid treatment was apparently unsuitable for rat IgG2c antibodies, since no antibody could be recovered from portions of KT16 serum thus treated. Nor was untreated KT16 serum amenable to Mono Q (anion exchange) or Mono S (cation

exchange) FPLC. Therefore, KT16 (rat IgG2c) was purified on a Protein A-Sepharose affinity column, or by hypotonic precipitation. Prior to column preparation, Protein A-Sepharose was swelled by shaking gently in 20mM Tris-HCl, pH 8.0, for 10 mins, then washed 3-4 times to remove fines and preservative (sodium azide). Antibody serum was dialysed against 20mM Tris-HCl / 5mM NaCl, pH 8.0, to reduce the salt concentration and thereby facilitate interaction of the immunoglobulin with Protein A, and then loaded onto the column. The column was washed twice with 10ml of 20mM Tris-HCl, pH 8.0, reversing it between washes. Antibody was eluted with 10ml of 0.1M sodium acetate, pH 4.5, and the column washed into 20mM Tris-HCl, pH 8.0, containing 0.1% sodium azide as preservative. The antibody fraction was concentrated by ammonium sulphate precipitation and resuspension/dialysis in inorganic salt solution.

2.2.4 Hypotonic Precipitation

Rat IgG2c antibodies tend to precipitate at low salt concentrations (Steve Cobbold, personal communication). The necessity for dialysis against low salt concentrations prior to Protein A purification therefore resulted in a low KT16 yield due to loss by precipitation. Higher yields were achieved simply by hypotonically precipitating the antibody and resuspending it in the desired buffer. In practice this was achieved by dialysing KT16 serum against water, collecting both precipitate and supernatant, and washing the precipitate by repeated centrifugation and suspension in water. After 5-6 centrifugations, the supernatant was then resuspended/dialysed in inorganic salts solution.

2.2.5 Gel Filtration

IgM serum contains large, multivalent molecules not amenable to purification by similar techniques to IgG molecules. Therefore 145-2C11 was purified by gel filtration using Sephacryl S-400 HR resin. An FPLC column with bed volume 100ml was prepared, and the void volume determined to be 30ml by eluting dextran blue with 20mM Tris HCl / 100mMNaCl, pH 7.5. 145-2C11 serum was diluted with eluent, and run on the Sephacryl column. Protein elution was observed by monitoring A_{280} , and fractions were collected between 26 and 130 mins. Three peaks eluted at 55mins, 63mins, and 82mins. The first and second peaks were identified as albumin and immunoglobulin

respectively by microgel-electrophoresis (Section 2.2.7). The relevant fractions were pooled, ammonium sulphate precipitated, and resuspended/dialysed in inorganic salts solution.

2.2.6 Protein assay

After resuspension in and dialysis against inorganic salts solution, protein concentration was assayed using a microbiuret assay as described by Goa (1953). A standard curve was constructed using bovine serum albumin (BSA; 0-500µg), and small aliquots (10-100µl) of antibody solutions were spiked with 150µg BSA. Protein was precipitated from each sample by addition of 1ml of 10% trichloroacetic acid and incubation for 5-10 mins at 25°C. Protein was pelletted by centrifugation (3s, 13,000xg), the supernatant aspirated, and the protein resuspended in 950µl 3% sodium hydroxide by incubation at 37°C for 3-12h. After resuspension, tubes were incubated for a further 15-30mins at 37°C with 50µl of Benedicts reagent (1.73% copper (II) sulphate, 10% sodium carbonate, 17.3% sodium citrate). A_{330} was measured spectrophotometrically against a water blank, and protein concentrations calculated from the standard curve. Concentrations were found to be between 1 and 5mg/ml.

2.2.7 Microgel electrophoresis

Electrophoresis was used at several stages to confirm the presence and ultimately the purity of immunoglobulin. Fractions from the FPLC were fractionated electrophoretically to identify those containing immunoglobulin, and thereby facilitate selection of the relevant fractions. After ammonium sulphate precipitation and resuspension in salts solution, antibody stocks were once more fractionated to assess their purity. SDS microgels were run on a Phast system, and stained in the Phast staining chamber. Gels were stained either using Coomassie or silver stain depending on the sensitivity required. Both staining protocols were as described in the Phast User Handbook.

2.3 Cell culture and preparation

2.3.1 Thymocyte preparation

Thymocytes were prepared by the method of Hesketh et al. (1983b).Thymi were isolated from 4-6 week old BALB/c mice after killing by cervical dislocation. The thymi were teased into RPMI 1640, supplemented with 20mM Tris, 24mM $NaHCO_3$, 2mM glutamine, and 20µg/ml gentamycin, pH 7.2. Connective tissue was pelleted by gravity, and the supernatant suspension decanted to a fresh tube, washed once by centrifugation (3 mins, 250xg), and resuspended at a density of $1x10^7$ cells/ml. Cell number was determined by viewing an aliquot of the final cell preparation on a haemocytometer under a Nikon binocular microscope, and viability, assayed by exclusion of 0.05% sodium eosin, was found to be greater than 95%. Cells were used immediately for proliferation assays, but stirred in a siliconised vial in a humidified gassed incubator (95% air, 5% CO_2, 37° C) for 2-4h to allow endogenous cAMP levels to fall (J.P. Moore et al., 1983) before use in $[Ca^{2+}]_i$ assays.

2.3.2 Clone 4 culture

Clone 4, a T_H cell line, was obtained from Dr. K. Tomonari, Clinical Research Centre, Harrow. Clone 4 stably expresses a variety of surface proteins which include a T cell receptor, IL2 receptors, CD4, LFA 1, and Thy 1. The TCR recognises the E^k antigen, and clone 4 division is stimulated by cells bearing E^k in the context of class II MHC proteins (Tomonari, 1985). Clone 4 was therefore stimulated using irradiated E^k-bearing splenocytes from CBA mice combined with recombinant IL2 (Tomonari, 1985). Cultures were grown in RPMI 1640 supplemented with 10mM HEPES, 24mM $NaHCO_3$,10% FCS, $5x10^{-5}$ M 2-mercaptoethanol, 12µg/ml Penicillin G and 100µg/ml Streptomycin, pH 7.2. Cells were restimulated every 2-4 weeks by washing into fresh medium containing 50U/ml IL2, and replating in Linbro 24 well plates (2ml/well) at 1-$2.5x10^4$ cells per well, with $4x10^6$ irradiated splenocytes per well. Splenocytes were obtained from 3-16 CBA mice, and prepared as follows. Cells were teased into RPMI 1640 supplemented with HEPES, $NaHCO_3$ and antibiotics; and pelletted by centrifugation (3 mins, 250xg). The cell pellet was resuspended in 400-800µl 0.83%

NH_4Cl, and incubated for 9 mins. The incubation was terminated by re-addition of medium, and the cells were washed into fresh medium. This NH_4Cl "shock" destroyed erythrocytes without significantly affecting lymphocyte viability (Agostini and Ideo, 1965; Ling and Kay, 1975). The splenocytes were exposed to 3,500 rads γ-irradiation using the irradiation facilities of the Radiotherapy Department, Addenbrookes Hospital, Cambridge (with the assistance of D. Adams).

Antigen alone stimulated little DNA synthesis, but in combination with IL2 was a potent stimulus (Fig. 2.1, assayed using the protocol described in Section 2.4.2). IL2 plus antigen caused maximal [^{3}H]thymidine incorporation at 96h and no incorporation was seen on or after day 6. The response to IL2 alone, at 96h, was ~50% of that stimulated by antigen plus IL2 (37,786$^+$/-855 and 82,682$^+$/-2,277 cpm respectively). However, long term culture with IL2 alone was not used, to avoid possible alterations in surface phenotype (K. Tomonari, personal communication). Single cultures using IL2 alone were, however, used to grow cells for $[Ca^{2+}]_i$ measurements (see Section 2.6.2, below).

The maximal cell number reached was 1-2x10^6 per well, suggesting 5-6 cycles of division had occurred, and that the time taken for one cycle was approximately 20h.

Cells were used a fixed number of days after stimulation in order to obtain optimal responses; 7-9 days for assays of KT16-induced proliferation, 11-16 days for all other experiments (K. Tomonari, personal communication).

2.4 Proliferation assays

The ability of various ligands to stimulate lymphocyte proliferation was examined by determining the ability of each ligand to stimulate incorporation of [^{3}H]thymidine into DNA. Lymphocyte cultures were established in Falcon flat-bottomed 96 well plates (final volume 200µl/well), and incubated in a gassed incubator (as above). The peripheral wells of the plate were not used for lymphocyte culture but were filled with medium to reduce evaporation from the central wells (Ling and Kay, 1975). Cultures were exposed to a brief [^{3}H]thymidine pulse prior to termination of the reaction.

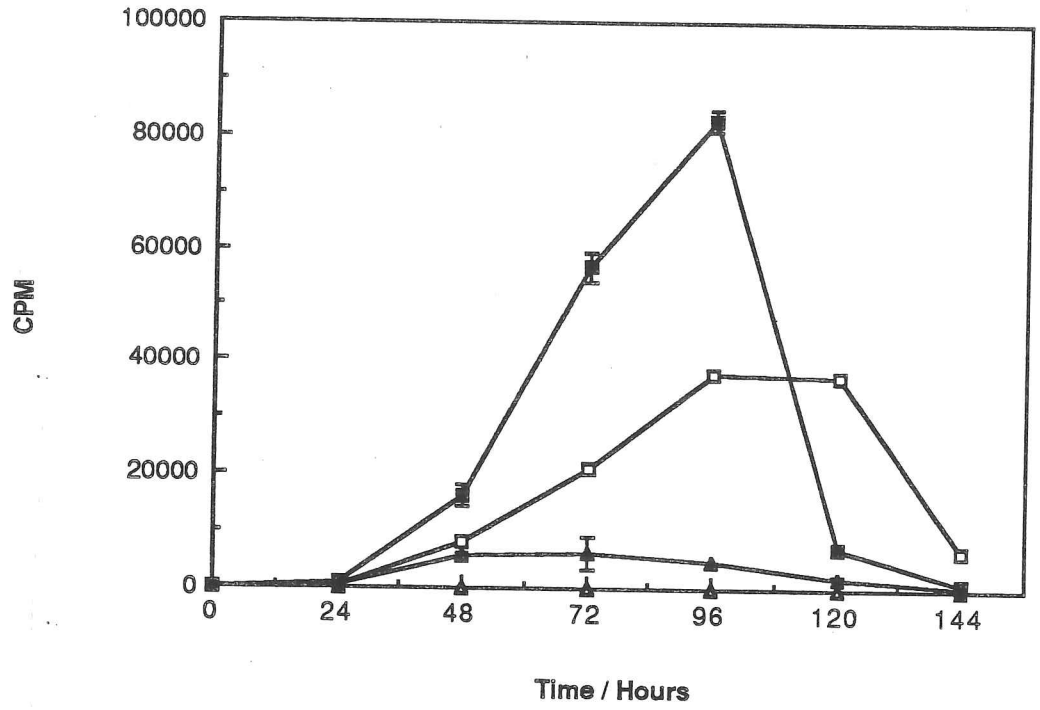


FIGURE 2.1 Time course of clone 4 proliferation stimulated by antigen-presenting cells and/or IL2.
Clone 4 cells were cultured in flat-bottomed microtitre wells as described in Section 2.4 with medium (△), $8x10^5$ CBA splenocytes/well (▲), 50U/ml IL2 (□), or CBA splenocytes plus IL2 (■). Cultures were harvested at 24h intervals up to 10 days, but data are only shown up to 144h, as there was no [^{3}H]thymidine incorporation after 144h. The y axis shows cpm of [^{3}H]thymidine incorporated.

Incubation time was chosen such that thymidine pulses were administered at the time of maximal DNA synthesis. Incubations were terminated by cell disruption and macromolecules were collected on glass microfibre filters (Whatman) using a Titertek Cell Harvester (Skatron A.S., Norway). Filters were dried and counted for ^3H in 5ml of scintillant (see Section 2.8).

2.4.1 Assay of thymocyte proliferation

Thymocyte proliferation was assayed using a similar protocol to Hesketh et al. (1983b). Thymocytes were prepared as described above (Section 2.3.1). $1.4x10^6$ cells per well were incubated for 48h, and pulsed for the final 6h with 0.3μCi [^3H]thymidine/well . Longer incubations resulted in progressively lower indices of stimulation correlating with decreases in cell viability (L. Kean, personal communication).

2.4.2 Assay of clone 4 proliferation

Stimulated clone 4 proliferation was assayed using a protocol similar to that of Tomonari (1985). Clone 4 cells were harvested and washed into medium identical to the culture medium above, except for the FCS concentration which was increased to 15%. $1x10^4$ cells per well were incubated for 96h, to observe maximal stimulation (Fig. 2.1), or at 24h intervals up to 10 days to investigate time courses of stimulation. In all experiments, cultures were exposed to a [^3H]thymidine pulse of 0.5μCi/well for the final 12h.

Both for clone 4 and thymocyte assays, all ligands were added to the wells prior to cell addition. Ligand combinations were therefore added simultaneously to the cells.

2.5 Photomicrography

Incubations of lymphocytes with different ligands caused markedly different patterns of cell aggregation. These were recorded by photomicrography using a Combiphot automatic camera system (Leitz). A Leicaflex camera was mounted on a Diavert Inverted Microscope, and pictures taken of cell cultures, established in 96 well microtitre plates for proliferation assays, immediately prior to cell harvesting. Photographs were taken of

thymocytes and 48 after stimulation. Exposed films (Kodak Tri-X Pan, black and white) were processed by the photographic department, Biochemistry Department.

2.6 Measurement of intracellular free calcium concentration

Intracellular free calcium concentration ($[Ca^{2+}]_i$) was measured using the fluorescent Ca^{2+} indicator quin 2. The protocol used was similar to that described by Hesketh et al. (1985).

2.6.1 Thymocyte loading

Thymocytes were prepared as described above (Section 2.3.1), and aliquots ($2x10^7$ cells) washed into salts solution immediately prior to loading with indicator. Thymocytes (4ml) were incubated with 1.5μM quin 2-acetoxymethylester (AME) for 40 mins at 37° C, with regular inversion, then washed into 4.1ml of salts solution and divided between two 3ml quartz cuvettes to allow simultaneous $[Ca^{2+}]_i$ measurements in two fluorimeters. 0.1ml was retained to count for ^3H. Aliquots thus counted showed the intracellular quin 2 concentration to be around 1mM, taking 104fl as the intracellular volume of a thymocyte (Hesketh et al., 1983b). The final cell density was $2.5\text{-}5x10^6$ cells/ml.

2.6.2 Clone 4 loading

Clone 4 cells ($0.5\text{-}1x10^7$) were loaded by incubation under identical conditions to thymocytes, but for only 30 mins, then washed as thymocytes (final cell density: $1\text{-}2x10^6$ cells/ml).

After incubation with quin 2-AME, cells cultured with both antigen and IL2 exhibited large fluorescence intensities (FI) but poor responses to ligands, with an emission maximum around 450nm. This suggested quin 2-AME was partitioning into the feeder cell membranes during loading, but not being hydrolysed due to the lack of viable esterase. This ester was not removed by cell washing, and its fluorescence masked the signal due to hydrolysed quin 2 in the clone cells. To avoid this clone cells for $[Ca^{2+}]_i$ measurements were grown with IL2 alone.

To control for effects of growing cells differently for Ca^{2+} and inositol phosphate experiments, inositol phosphate responses to anti-Thy 1 and Con A were compared in cells grown both with and without CBA cells. The index of stimulation (relative to control) evoked by anti-Thy 1 was very similar in the two cell populations (Table 2.2).

	Inositol phosphate fraction			
Growth stimulus	InsP	$InsP_2$	$InsP_3$	$InsP_4$
IL2 alone	1.6	2.4	1.7	2.1
CBA + IL2	1.7	2.1	1.5	1.8

TABLE 2.2 Stimulation index of inositol phosphate fractions with KT16
Clone 4 cells cultured by stimulation with $IL2^+$/-CBA splenocytes were labelled with [^{3}H]inositol, washed and pre-incubated as described in Section 2.7.1. Cells were challenged with anti-Thy 1 (20μg/ml) for 10 minutes. The reactions were quenched, and samples processed as described in Section 2.7.3. Inositol phosphate fractions were separated, dried and counted for ^{3}H (Section 2.8). The stimulation index is the ratio cpm in stimulated cells / cpm in unstimulated cells.

Con A caused massive increases in cpm in inositol phosphates in IL2-grown cells, similar to those seen in other experiments in CBA-grown cells. These results suggest that both Con A and anti-Thy 1 evoke responses of similar magnitude in cells grown +/- CBA cells, and therefore that comparisons between Ca^{2+} and inositol phosphate responses to anti-Thy 1 and Con A are valid.

In some experiments, clone 4 cells were washed into RPMI 1640 ([inositol] = 0.19mM) or the inositol-free medium, BME Diploid (supplemented with 2.4μM inositol) used for labelling with [^{3}H]-inositol (see Section 2.7.1, below), and incubated for 7h prior to use in $[Ca^{2+}]_i$ measurement experiments. The responses to anti-Thy 1 were identical in cells pre-incubated in either medium, suggesting that incubation at low [inositol] does not modify responses to KT16.

2.6.3 $[Ca^{2+}]_i$ measurement

Quin 2 fluorescence was continuously recorded using Perkin Elmer MPF 44B and 44E fluorescence spectrophotometers (λ_{ex} = 339nm, λ_{em} = 492nm). Cells were maintained in

suspension using a magnetic stirring and heating block. Cells were incubated in the fluorimeter for 10 mins prior to ligand addition to allow temperature equilibration. Anti-CD4 (KT6) and isotype control antibody (15D5) were added 5 mins prior to Con A / KT16 to check their lack of effect on $[Ca^{2+}]_i$. In some experiments, the extracellular free Ca^{2+} concentration, ($[Ca^{2+}]_o$), was reduced to 0.1μM (i.e. equal to $[Ca^{2+}]_i$) by 5 min preincubation with 1mM EGTA. EGTA was added from a 100mM stock, pH adjusted such that addition to salts solution changed the pH less than 0.02 units. By reducing $Ca^{2+}{}_o$ entry this permitted measurement of Ca^{2+} release from intracellular stores.

Ca^{2+} responses were calibrated as described by Hesketh et al. (1983b). The fluorescence intensities (FI) of 100% saturated quin 2 and 0% saturated quin 2 were determined by lysing the cells with 0.02% Triton X-100 (thereby exposing the quin 2 to 0.43mM Ca^{2+}) then displacing Ca^{2+} from quin 2 by addition of 0.25mM $MnCl_2$. Using these values, the FI values observed at 60s intervals were converted to percentage quin saturation using the formula:

$$\%\text{Ca-quin 2} = \frac{(a-c) - 0.16(b-c)}{0.84(b-c)}$$

where; a = fluorescence intensity of intact cells

b = fluorescence intensity at 100% saturation

c = fluorescence intensity at 0% saturation

This formula corrects for the effect of Mg^{2+}-binding to quin 2 (Hesketh et al., 1983b). $[Ca^{2+}]_i$ was then calculated from %Ca-quin 2 using the relation:

$$[Ca^{2+}]_i = \frac{\%\text{Ca-quin 2}}{100-\%\text{Ca-quin 2}} \times \frac{100}{1.122} \text{ nM}$$

Data are presented as intracellular free calcium concentration (nM) against time, plotted at 1 min intervals. Data are the means of duplicate or triplicate determinations. Where the effects of inhibitors were determined, control and inhibited responses were measured simultaneously in two fluorimeters with cells from a single loading.

2.7 Inositol phosphate assays

Ligand-stimulated inositol phosphate accumulation was measured in lymphocytes pre-labelled with [^{3}H]inositol, using an assay derived from that of Taylor et al. (1984). Reactions were performed in salts solution. Inositol phospholipids were separated from inositol phosphates in the reaction mixture by extraction into an organic phase. Individual inositol phosphate fractions were separated by anion exchange chromatography on Partisil 10 SAX (HPLC) or AG1-X8 resin, and quantitated by counting for ^{3}H.

2.7.1 Labelling of lymphocytes with [^{3}H]inositol

Thymocytes from 2-3 BALB/c mice were prepared in BME Diploid medium, supplemented with 20mM Tris, 24mM $NaHCO_3$, 2mM glutamine, penicillin and streptomycin, pH 7.3. Cells ($2\text{-}3\times10^8$) were continuously stirred for 16-18h with 20μCi/ml [^{3}H]-inositol (or 23μCi/ml for HPLC experiments), then washed twice by centrifugation into salts solution. Cells were resuspended to give different densities for different experimental procedures; $2\text{-}3\times10^7$ cells/ml for HPLC experiments (0.6ml aliquots), and $5\text{-}10\times10^6$ cells/ml for non-HPLC experiments (0.5ml aliquots) and incubated 20-30 mins at 37°C.

A permeabilised thymocyte system, developed by Dr. Y. Zilberman, was employed to investigate the [Ca^{2+}] dependence of inositol 1,4,5 trisphosphate phosphorylation. Cells for these experiments were not labelled with [^{3}H]inositol but were prepared in RPMI 1640 as described (Section 2.3.1), washed twice into salts solution , and resuspended at $5\text{-}10\times10^7$ cells/ml.

Clone 4 cells were labelled by incubation with 40μCi/ml [^{3}H]inositol for 8-9h in BME Diploid, supplemented with 10mM HEPES, 24mM $NaHCO_3$, Penicillin and Streptomycin, pH 7.3. Cells were washed twice into salts solution, and 0.5ml aliquots incubated 15-30 mins at 37°C ($4\text{-}9\times10^6$ cells/ml).

2.7.2 Cell experiments

Intact cell reactions were initiated, after 10 mins preincubation with 5mM LiCl, by addition of ligand. To avoid possible Li^+-mediated alterations of inositol phosphate interconversions, samples for HPLC analysis were not pre-incubated with LiCl. Where

the effects of anti-CD4 or isotype control 15D5 were examined, anti-CD4 or 15D5 was added either simultaneously (time course experiments) or 5 mins prior to ligand addition (titration experiments). Addition at either time gave equivalent effects on ligand-stimulated inositol phosphate accumulation. Other additions were as described in the Figure legends.

Permeabilised cell reactions were initiated by dilution of 40µl of cells into 380µl of a hypo-osmotic medium containing 5.5mM $MgCl_2$, 5.5mM Na_2ATP, 1.3mM EGTA, 0.1mM $CaCl_2$ and 12.5nM [^{3}H]inositol 1,4,5-trisphosphate, pH 7.1. At 2 mins, 80µl of buffer solution containing 5.5mM $MgCl_2$, 5.5mM Na_2ATP, 1.3mM EGTA, 188mM KCl, 375mM Tris and a range of calcium concentrations was added. Cell lysis was greater than 95% as determined by lactate dehydrogenase (LDH) activity assays (taking the activity after Triton X-100 lysis to be 100%), and was unaffected by addition of KCl and Tris at 2 mins (Y. Zilberman, personal communication). Calcium chloride was added to give a range of free calcium concentrations from 0.04 to 50µM, as calculated using a computer programme developed by Dr. A.N. Corps from one described by Fabiato and Fabiato (1979).

All reactions were terminated by addition of 3.75 volumes of chloroform/methanol (1:2).

2.7.3 Inositol phosphate extraction

After reaction termination, phases were separated by addition of 0.62ml each of chloroform and water (0.8ml to intact cell HPLC samples). After removal of the upper, aqueous phase to a fresh vial, the organic phase was washed twice with 200µl of a CDTA-containing upper phase (250µl to intact cell HPLC samples) prepared by shaking together 100mM Na_2CDTA, methanol and chloroform (5 : 8 :16) and collecting the upper phase, as described by Berridge et al. (1983). The washings were combined with the upper phase. Samples for HPLC analysis were freeze-dried after addition of 100µl of 50mM mannitol (to aid inositol phosphate recovery), and removal of methanol by evaporation under a nitrogen stream, then stored at -20°C. Samples for non-HPLC analysis were processed as described below (Section 2.7.4).

The lower phase was evaporated to dryness and counted in 5ml of scintillant to determine [^{3}H]inositol-labelled phospholipids.

2.7.4 Inositol phosphate separations on AG1-X8 resin

The combined aqueous phases were loaded onto anion exchange columns containing AG1-X8 resin. The columns were washed with 10ml of water and 1.2ml of 5mM disodium tetraborate/0.2M ammonium formate; then inositol phosphates were eluted sequentially using 9ml of each of the following buffers: 0.2M ammonium formate/0.1M formic acid (InsP); 0.4M ammonium formate/0.1M formic acid ($InsP_2$); 0.7M ammonium formate/0.1M formic acid ($InsP_3$); 1.2M ammonium formate/0.1M formic acid ($InsP_4$). Eluted fractions were oven-dried (80°C), then resuspended in 15ml of scintillant and counted for ^{3}H (Section 2.8).

This elution regime is a modified version of that used by Downes et al. (1986), but with a reduced formate concentration of 0.7M, rather than 0.8M, for $InsP_3$ elution, as 0.8M formate partially eluted $InsP_4$. Recoveries of $InsP_3$ and $InsP_4$ from AG1-X8 columns were 93% and 87% respectively (Table 2.3)

Addition to column	dpm eluted by formate concentration				
	0.2M	0.4M	0.7M	1.2M	Total
[^{3}H]Ins(1,4,5)P_3	10	27	2,054	18	2,109
[^{3}H]$InsP_4$	20	5	103	847	975

TABLE 2.3 $InsP_3$ and $InsP_4$ recovery from AG1-X8 columns
Duplicate aliquots (100µl) of [^{3}H]Ins(1,4,5)P_3 (2,256 dpm) or [^{3}H]$InsP_4$ (1,118 dpm) were mixed with 400µl of salts solution and 2µl of phytic acid hydrolysate, then loaded onto AG1-X8 columns. Columns were eluted as described above. Fractions were collected, dried, and counted for ^{3}H as described below (Section 2.8).

97% of $InsP_3$ counts recovered eluted in the 0.7M formate fraction, and 87% of $InsP_4$ counts recovered eluted in the 1.2M formate fraction.

2.7.5 HPLC analysis of inositol phosphates

Freeze dried samples were dissolved in 0.4ml of water spiked with approx. 200μM AMP, ADP, and ATP, filtered through cellulose acetate filters, and loaded onto a Partisil 10 SAX column (250 x 4.6mm). This was protected by a guard column (50 x 4.6mm) of pellicular SAX, changed after every 10 runs. A flow rate of 1ml/min was used, and inositol phosphates were eluted by increasing concentrations of ammonium formate (buffered to pH 3.7 with orthophosphoric acid). Two protocols were used; one developed from Batty et al. (1985) to separate the two major inositol trisphosphate isomers, Ins(1,3,4)P_3 and Ins(1,4,5)P_3, and inositol tetrakisphosphate, and the other an adaptation of the method described by Heslop et al. (1985) to collect inositol penta- and hexakisphosphates in addition to the $InsP_3$ isomers and $InsP_4$.

2.7.5a $InsP_3$ and $InsP_4$ assay

Inositol, InsP and $InsP_2$ were eluted over 9 mins by increasing formate concentration from 2mM to 750mM in a non-linear gradient (convex no. 0.3 on a Perkin-Elmer gradient former). Ins(1,3,4)P_3 and Ins(1,4,5)P_3 were then eluted by a linear increase over 8 mins to 1.0M ammonium formate, and the buffer was held at this concentration for a further 6 mins. $InsP_4$ was eluted by a linear concentration increase to 1.7M over 10 mins and subsequent maintenance for 20 mins. The column was washed for 5 mins with a 5ml/min 1.7M formate purge before returning to 2mM ammonium formate over 10 mins. The eluent was routinely collected as 0.2ml fractions between 24 and 34 mins and from 40 to 48 mins, and counted for ^3H in 5 ml scintillation fluid.

Fig 2.2 shows a typical elution profile obtained using a standard sample generated by stimulating intact thymocytes with 10μg/ml Con A for 2 minutes. Fractions were continuously collected throughout the run. The first four peaks have not been identified relative to standards, but are likely to be inositol, glycerophosphoinositol, inositol monophosphate and inositol bisphosphate by comparison with data presented in Heslop et al. (1985). The split nature of the putative inositol bisphosphate peak may represent the incomplete separation of two inositol bisphosphate isomers, Ins(1,4)P_2 and Ins(3,4)P_2 formed by degradation of Ins(1,4,5)P_3 and Ins(1,3,4)P_3 respectively.

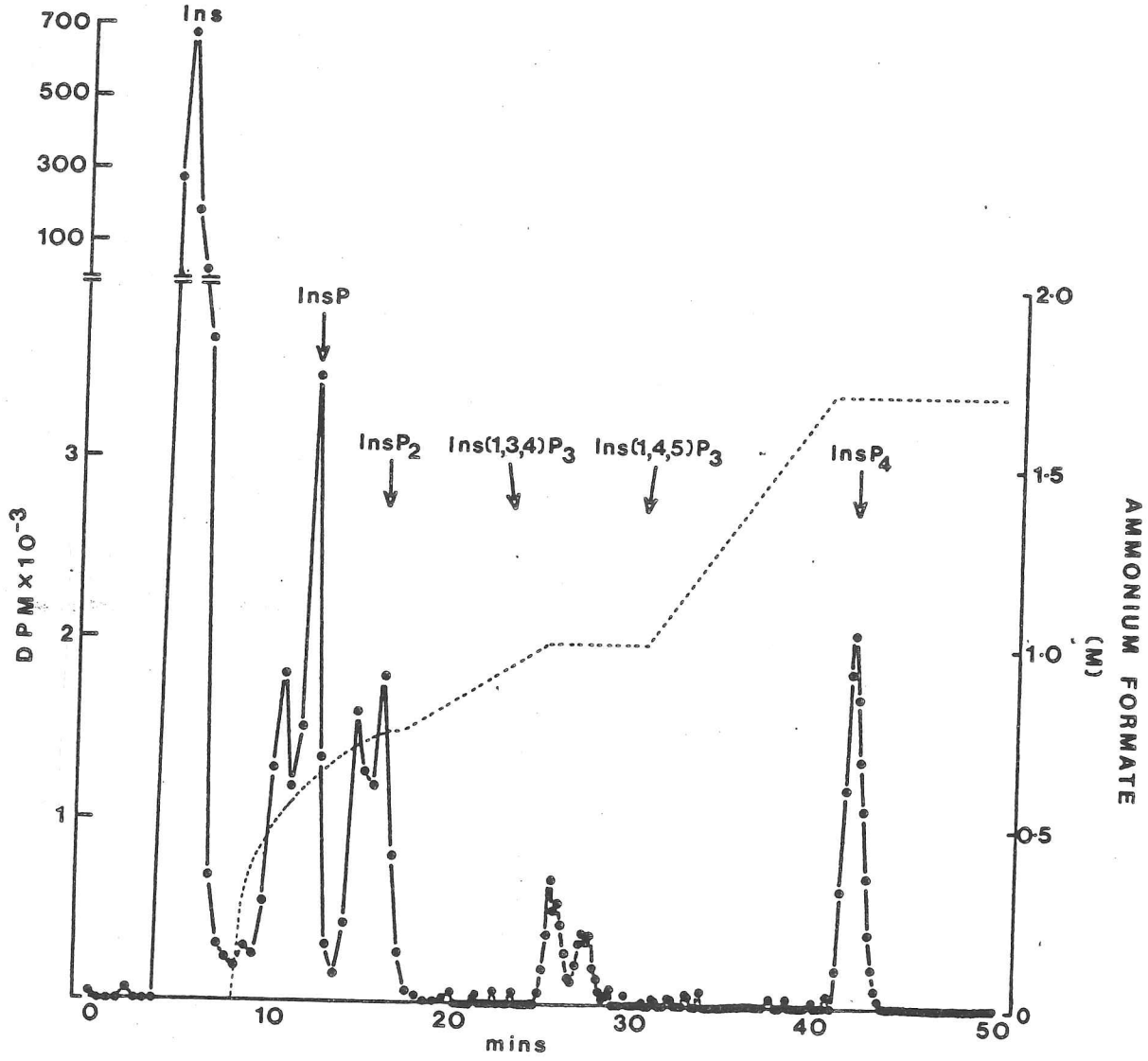


FIGURE 2.2 Elution profile of inositol phosphates extracted from Con A-stimulated thymocytes.

[^{3}H]inositol-labelled BALB/c thymocytes were stimulated for 2 mins with 10μg/ml Con A (Section 2.7.2), then reaction quenched and inositol phosphates extracted as described in Section 2.7.3. Inositol phosphates were separated on a Partisil 10-SAX HPLC column by a modified version of the method of Batty et al. (1985) described in Section 2.7.5a, and the eluate collected as 0.5ml fractions between 0 and 20 mins and as 0.2ml fractions from 20 to 50 mins. Fractions were counted in 4.5ml of scintillation fluid (Section 2.8). Peak identities were assigned as described in the text. The dashed line shows the ammonium formate concentration.

Three further peaks eluted between 20 and 30 mins. Of the two major peaks, the earlier co-eluted with ATP and is therefore assumed to be Ins(1,3,4)P_3 (Irvine et al., 1985), while the later co-eluted with myo[2-^{3}H]inositol 1,4,5 trisphosphate. The minor peak eluting after Ins(1,4,5)P_3 was too small for accurate quantitation, but may be inositol 2,4,5-trisphosphate. A minor peak eluting just before Ins(1,3,4)P_3 was sometimes detected, which may be cyclic inositol 1,4,5 trisphosphate, as described by Connolly et al. (1986a), but was too small to quantitate. The peak at 42 mins co-eluted with [^{3}H]inositol 1,3,4,5 tetrakisphosphate, and thus is assumed to be $InsP_4$.

2.7.5b $InsP_3$, $InsP_4$, $InsP_5$ and $InsP_6$ assay

To elute $InsP_5$ and $InsP_6$, the ammonium formate concentration was increased to 3.4M. Gradient details were as follows: 15 mins at 2mM formate to elute inositol, linear increase over 60 mins to 750mM to elute InsP and $InsP_2$, linear increase over 30 mins to 3.4M formate and subsequent maintenance at this concentration for 15 mins to elute $InsP_3$ isomers, $InsP_4$, $InsP_5$ and $InsP_6$. The concentration was then returned to 2mM formate over 15 mins. The eluent was collected as 0.5ml fractions between 72 and 86 mins, and as 1ml fractions between 90 and 116 mins. 0.2ml aliquots were removed from the fractions and counted in 15ml of scintillant.

This protocol resulted in similar count recovery of $InsP_3$ and $InsP_4$ from a standard sample as was obtained using the previous assay (2.7.5.a). An additional, more strongly retained peak eluted at approx. 2.1M ammonium formate. The $InsP_5$ peak described by Heslop et al. (1985) eluted at a similar formate concentration (Table 2.4) suggesting that the peak eluted by 2.1M formate is $InsP_5$. No more polar peak analogous to $InsP_6$ was observed.

Interestingly, the ratio of $InsP_4$ to Ins(1,4,5)P_3 appeared to be far greater in Con A-stimulated thymocytes than in 5-hydroxytryptamine-stimulated blowfly salivary gland (Table 2.5 and Heslop et al., 1985), carbachol-stimulated brain slices (Batty et al., 1985) or Jurkat cells stimulated with anti-T3 antibody (Stewart et al. 1986).

Inositol phosphate	Eluting formate concentration (M)	
	a) Heslop et al. (1985)	b) This work
$Ins(1,3,4)P_3$	0.63	0.69
$Ins(1,4,5)P_3$	0.68	0.71
$InsP_4$	1.39	1.53
$InsP_5$	2.02	2.12
$InsP_6$	2.66	Not found

TABLE 2.4 Comparison of formate concentrations required for inositol phosphate elution Eluting concentrations were calculated by comparison of elution time and elution regime.

Inositol phosphate	dpm in standard sample	Ratio to dpm in $Ins(1,4,5)P_3$	Ratio from Heslop et al.
$Ins(1,3,4)P_3$	4,452	2.1	0.20
$Ins(1,4,5)P_3$	2,120	1.0	1.0
$InsP_4$	18,325	8.6	0.26
$InsP_5$	3,810	1.8	0.02
$InsP_6$	Not found	-	0.06

TABLE 2.5 Relative radioactivity in $Ins(1,3,4)P_3$, $Ins(1,4,5)P_3$, $InsP_4$ and $InsP_5$ The dpm are shown for inositol polyphosphates in a 2 min standard sample, separated and quantitated as described in Section 2.7.5.b. Ratios are calculated relative to dpm in $Ins(1,4,5)P_3$, and compared with ratios for 5-hydroxytryptamine-stimulated blowfly salivary gland calculated from Heslop et al. (1985).

This difference may be attributable, in part, to the method of extraction employed by the groups cited which involves the use of trichloroacetic acid. Trichloroacetic acid extraction of inositol phosphates (as described below, Section 2.7.6) from thymocytes stimulated for 2 mins with Con A resulted in an $InsP_4$: $Ins(1,4,5)P_3$ ratio 16-fold smaller than that seen when inositol phosphates were extracted using chloroform/methanol (as described in section 2.7.3). To investigate this change in ratio, a comparison of $[^3H]Ins(1,4,5)P_3$ and $[^3H]InsP_4$ recoveries using trichloroacetic acid and chloroform/methanol protocols for reaction termination and inositol phosphate extraction was effected. $[^3H]Ins(1,4,5)P_3$ and $[^3H]InsP_4$ were extracted from media with or without cells using both protocols and the recoveries examined by HPLC separation and quantitation as described (Section 2.7.5a). Trichloroacetic acid had little effect on the

recovery of Ins(1,4,5)P_3 either from salts solution or from a cell suspension (Table 2.6.a), but InsP_4 recovery from trichloroacetic acid-treated samples was only 43% from salts solution, and 5% in the presence of cells, of that from chloroform/methanol-extracted samples (Table 2.6.b). In the absence of cells, low recovery was due to adsorption onto the cellulose acetate filters. However, in the presence of cells only 50% of the unrecovered counts were filter associated. Residual unrecovered counts could not be accounted for by precipitation with protein or dephosphorylation to give lower inositol phosphates, since no counts were found to be associated with the protein precipitate, and no counts eluted earlier than InsP_4 from the HPLC column.

i)

Sample	dpm in Ins(1,4,5)P_3
Control	12,424 +/- 438
a	11,171 +/- 508
b	11,090 +/- 1,427
c	12,018 +/- 16
d	10,910 +/- 230

ii)

Sample	dpm in InsP_4	dpm on filter	Total
Control	1,328 +/- 58	104 +/- 46	1,432
a	1,163 +/- 42	244 +/- 9	1,407
b	1,083 +/- 9	33 +/- 12	1,116
c	498 +/- 35	847 +/- 33	1,345
d	58 +/- 20	691 +/- 153	749

TABLE 2.6 Effect of extraction method on [^{3}H]Ins(1,4,5)P_3 and [^{3}H]InsP_4 recovery 100µl aliquots of a [^{3}H]Ins(1,4,5)P_3 solution ($1.2x10^4$ dpm / 100µl; i) or a [^{3}H]InsP_4 solution ($1.3x10^3$ dpm / 100µl; ii) were added to 0.5ml of salts solution (Control, a, c) or thymocyte suspension (b, d). The control sample was freeze-dried, and the other samples were extracted using a neutral chloroform / methanol method (a, b) or a trichloroacetic acid protocol (c, d). Duplicate samples were assayed for InsP_3 and InsP_4 by HPLC, as described in Section 2.7.5.a. InsP and InsP_2 fractions were also collected but contained no radioactivity. (ii) Cellulose acetate filters were also counted, after drying, in 5ml of scintillant.

InsP_4 loss could be substantially reduced by spiking samples with 2µl phytic acid hydrolysate (13mg phosphate/ml, Wregget et al., 1987). However, chloroform /

methanol extraction was the preferred procedure, since it was a quicker procedure, and was reliable without the need for phytic acid supplements.

2.7.6 Trichloroacetic acid extraction of inositol phosphates

Trichloroacetic acid extraction was according to the method of Batty et al., (1985). Reactions were quenched by the addition of an equal volume of ice-cold 15% w/v trichloroacetic. Tubes were vortexed and incubated at 0°C for 10-15 mins. Precipitated protein was sedimented by centrifugation for 5 mins, and the supernatant removed to a fresh tube and washed five times with 2 volumes of water-saturated diethyl ether to remove trichloroacetic acid. Sample pH was adjusted to 7-8 with 1M $NaHCO_3$, and samples were stored at -20°C. For HPLC analysis, samples were spiked with adenine nucleotides (200μM), filtered, and assayed for $InsP_3$ and $InsP_4$ as described in Section 2.7.5.a.

2.8 Liquid Scintillation counting

Tritium was counted using a Packard Tri-Carb 460C scintillation counter. Filters bearing ^{3}H-DNA (Section 2.4) and fractions from the HPLC (Section 2.7.5) were counted in 5ml plastic scintillation vial inserts containing 4.5ml of scintillation fluid. Inositol phosphate fractions separated on AG1-X8 resin (Section 2.7.4) were eluted into 15ml plastic scintillation vials, and oven dried at 80°C. Inositol phosphate fractions were resuspended by vigorously vortexing for 60s in 15ml scintillant 2-3 times, and counted in these vials.

Two types of scintillant were used during the course of this work. The first, a cocktail consisting of; toluene : Triton X-100 : water (8 : 4 : 1) containing 2.5% w/v PPO (scintillant a) was used to count all HPLC-generated samples and some of the proliferation assays and AG1-X8-generated inositol phosphate fractions. A switch was made to the second, commercially-produced scintillant, Safe Fluor S (scintillant b), to comply with regulations introduced in 1988 concerning the biodegradability of scintillation fluids. This particular biodegradable scintillant was selected for its ability to

solubilise inositol phosphate fractions. It was however necessary to add 6.6% (v/v) water to Safe Fluor S before it was effective at solubilising inositol phosphates.

Counting efficiency was determined using the internal standard of the counter, calibrated by comparison with standard samples of known quenching provided by the manufacturer. The counting efficiency within most experiments only varied approx. 3-5%, and results are therefore expressed as cpm. However the efficiency of counting of HPLC fractions was found to significantly decrease as the formate concentration of the eluent increased. Therefore data for HPLC fractions were converted to dpm and expressed as a percentage of dpm in ^{3}H-labelled lipids to allow comparison between experiments. Counting efficiency was greater with Safe Fluor S (approx. 120% of scintillant a), but the ratio

cpm in inositol phosphate fraction / cpm in lipids (i)

was very similar in both scintillants for all four inositol phosphate fractions (Table 2.3); and the scintillants had very similar counting efficiency calibrations.

Scintillant		InsP	$InsP_2$	$InsP_3$	$InsP_4$
a	cpm	1,866+/-27	358+/-66	296+/-49	378+/-7
	ratio (i)	2.42	0.46	0.38	0.49
b	cpm	2,246+/-15	505+/-63	346+/-38	505+/-43
	ratio (i)	2.43	0.55	0.37	0.55

TABLE 2.7 Comparison of inositol phosphates counts visualised using two scintillation fluids

[^{3}H]inositol-labelled thymocytes were challenged with 10µg/ml Con A for 10 mins, as described in Section 2.7.1. The reaction was terminated, and inositol phosphates extracted, processed and separated as described (Section 2.7.2-4). Fractions were oven dried, resuspended in 15 ml of scintillant, and repeatedly vortexed and counted until counts were stable. Data shown as 'cpm' are the mean cpm +/- s.d. of duplicate determinations. Ratio (i) was calculated using the following values for cpm in lipids: 77,078+/-4,523 (scintillant a) and 92,523+/-800 (scintillant b).

Samples were always counted for sufficient time to ensure that the counting error was considerably less than errors likely to arise elsewhere in the experiment.

2.9 cAMP assay

Clone 4 cells were washed once into salts solution and incubated as 1.0ml aliquots ($0.9\text{-}2x10^7$ cells/ml) at 37°C for 20-30 mins. The reaction was started by reagent addition and quenched by a 3s microfuge spin (13,000xg), rapid removal of the supernatant, cell pellet lysis with 100µl Milli Q water, and boiling for 5 mins. Samples were then frozen at -20°C.

The samples were assayed for cAMP by Dr. S.R.Pennington (Liverpool University) using a competitive binding assay, as described by Brown et al. (1971). 50µl of sample or cAMP standards (containing 0.031-16 pmol cAMP / 50µl in 50mM Tris-HCl, 4mM EDTA, pH7.5) were incubated for 2 hours at 0°C with 50µl of [^{3}H]cAMP (34nCi) and 100µl of cAMP binding protein (prepared by Dr. R. Farndale from bovine adrenal glands). A blank was created by incubating 150µl of buffer (50mM Tris-HCl, 4mM EDTA, pH 7.5) with 50µl of [^{3}H]cAMP. 100µl of 40mg/ml charcoal/0.5mg/ml BSA suspension (in buffer) was added to each sample to absorb unbound cAMP, then samples were centrifuged (2 mins, 13,000xg, 4°C) to pellet the charcoal, and 200µl aliquots of supernatant were counted for ^{3}H. A standard curve was constructed by plotting:

$$\frac{\text{cpm in standard tube - cpm in blank}}{\text{total cpm (34nCi)}}$$

versus [cAMP], and the cAMP content of samples was then calculated using the standard curve.

2.10 c-myc mRNA assay

Thymocytes were prepared in RPMI 1640 as described above, and incubated at $1x10^7$ cells/ml for 5h. Clone 4 cells were washed into RPMI 1640 containing HEPES, $NaHCO_3$, and penicillin/streptomycin, and incubated at 2-5 $x10^6$ cells/ml for 1h. Reaction was started by ligand addition and terminated by rapid centrifugation (30s, 1000g), and resuspension in 1ml of guanidinium isothiocyanate solution (4M GITC, 25mM sodium citrate, 100mM 2-mercaptoethanol, 0.5% N-lauroyl sarcosine, 0.1%

antifoam, pH 7.0). RNA was prepared and analysed as previously described (Moore et al., 1988).

2.10.1 RNA isolation

RNA was isolated by centrifugation through CsCl using a method based on that of Chirgwin et al. (1979). Samples were mixed with 400mg CsCl and loaded onto a cushion of 1.2ml of 5.7M CsCl, 100mM EDTA, pH 7.0 in polyallomer centrifuge tubes. RNA was pelletted by centrifugation (35,000xg, 12-16h), and the pellet resuspended in 200µl of 5mM Na_2EDTA, 1% lithium dodecyl sulphate (LiDS), 10mM HEPES, pH 7.0. Fats and proteins were extracted into an organic phase generated by addition of 100µl of chloroform/butan-1-ol (4 : 1) and centrifugation (2 mins, 14,000xg). The upper phase was removed to a fresh tube, the lower phase washed with 100µl of EDTA / LiDS / HEPES solution, and washing and upper phase combined.

RNA was precipitated by incubation at -70°C for 30 mins with $^1/_{10}$th volume of 3M sodium acetate, pH 5.6, and 2.5 volumes of absolute alcohol, as described by Maniatis et al. (1982). RNA was pelleted by centrifugation (10 mins, 15,000xg, -10°C), vacuum dried, resuspended in 20 µl of sterile water, and quantitated by measuring A_{260}. 10^7 thymocytes typically yielded 4-5µg total RNA. 10^6 clone 4 cells typically yielded 2-3µg total RNA.

2.10.2 RNA fractionation

RNA was fractionated by gel electrophoresis as described by Rave et al. (1979). Total cellular RNA was denatured by incubation for 15 mins at 58°C in electrophoresis buffer (20mM MOPS, 5mM sodium acetate, 1mM Na_2EDTA, pH 6.8) supplemented with 6.6% formaldehyde and 50% de-ionised formamide, and fractionated on a 0.8% agarose / 7% formaldehyde gel. The loading of equal amounts of total RNA in each lane was confirmed by ethidium bromide staining, and gels photographed on an ultraviolet transilluminator (254nm) after destaining in sterile water.

2.10.3 Transfer of RNA to nitrocellulose membrane

RNA was transferred from the gel to nitrocellulose membrane using a modified version of the protocol described by Thomas (1980). The gel was sandwiched between

buffer-saturated filter paper (extending into a buffer reservoir) and a sheet of nitrocellulose, covered by 30-40 sheets of filter paper and a 1 kilogram weight. Transfer was complete after 4h. The buffer used was 1.5M sodium chloride, 0.15M tri-sodium citrate, pH 7.0 (10xSSC). RNA was fixed by baking in vacuo for 3h at 80°C. The location of 18S and 23S rRNA in the filters was determined by staining selected lanes with methylene blue dye (0.04% in 500mM sodium acetate, pH 6.0), at regular intervals to confirm band assignations.

2.10.4 Hybridisation with c-myc cDNA probe

Purified c-myc cDNA (complementary to exons 2 and 3 of the mouse c-myc gene; Neuberger and Calabi, 1983) was labelled with [α-^{32}P]dATP by nick translation (Rigby et al., 1977), and isolated from the reaction mixture by alcohol precipitation and resuspension in sterile water.

[^{32}P]-labelled c-myc probe was hybridised to nitrocellulose membranes using a protocol based on that of Wahl et al. (1979). Nitrocellulose membranes were soaked for 2-3 mins in hybridisation solution (50% formamide, 10% dextran sulphate, 0.6M sodium chloride, 0.06M trisodium citrate, 20μg/ml sonicated herring sperm DNA and 1x Denhardts reagent), then incubated at 42°C for 16-24h with denatured ^{32}P-labelled c-myc probe (0.4-1.0x10^7 dpm) in 10ml of hybridisation solution in a sealed plastic bag.

Nitrocellulose membranes were washed twice for 30 mins in 500ml of 2x SSC, 0.1% SDS at room temperature, twice for 30 mins in 0.1xSSC, 0.05% SDS at 55°C (Shank et al., 1978), then exposed to preflashed Kodak X-omat x-ray film for 1-14 days at -70°C with a single intensifying screen. The intensities of the major bands were determined by scanning densitometry.

CHAPTER 3

MITOGENIC STIMULATION OF T LYMPHOCYTES

CHAPTER 3

MITOGENIC STIMULATION OF T LYMPHOCYTES

3.1 Mitogenic stimulation of clone 4

3.1.1 Stimulation of clone 4 by interleukin 2

Clone 4, a helper T cell clone, expresses antigen receptors directed against E^k antigen, and cultures were therefore stimulated with a combination of E^k-bearing cells and IL2 (Tomonari, 1985; and see Fig. 2.1). Stable expression of IL2r enables clone 4 also to respond to IL2 alone (Fig. 3.1a). IL2 was mitogenic at concentrations greater than 2 U/ml with maximal [^{3}H]thymidine incorporation at 50 U/ml IL2, and supobtimal responses at concentrations above 50 U/ml (e.g. 80% with 200 U/ml). IL2-stimulated clone 4 mitogenesis was inhibited by the anti-IL2 receptor (IL2r) antibody PC61-53. PC61-53 alone did not stimulate DNA synthesis, but was a potent antagonist of stimulation by 50 U/ml IL2 (Fig 3.1b). K_i was approximately equal to 0.35μg/ml, and 12.5μg/ml PC61-53 caused greater than 98% inhibition. The ability of PC61-53 to antagonise IL2-stimulated mitogenesis despite its failure to stimulate mitogenesis suggests that PC61-53 does not recognise an activating epitope of the IL2 receptor, but either evokes responses antagonistic to those evoked by IL2, or more probably impairs binding of IL2 to IL2r, possibly by steric hindrance due to recognition of an overlapping epitope. The presumed ability of PC61-53 to inhibit productive interaction of IL2 with IL2r makes it a useful tool for establishing which mitogens operate via an IL2-IL2r interaction. The involvement of such an interaction in mitogenic stimulation through Thy 1 was examined, using the anti-Thy 1 antibody KT16.

3.1.2 Stimulation of clone 4 by anti-Thy 1 antibody

KT16 stimulated DNA synthesis maximally in clone 4 over a narrow concentration range between 100 and 200 ng/ml (Fig. 3.2a). Maximal KT16 responses were 10-20% of those with 50 U/ml IL2.

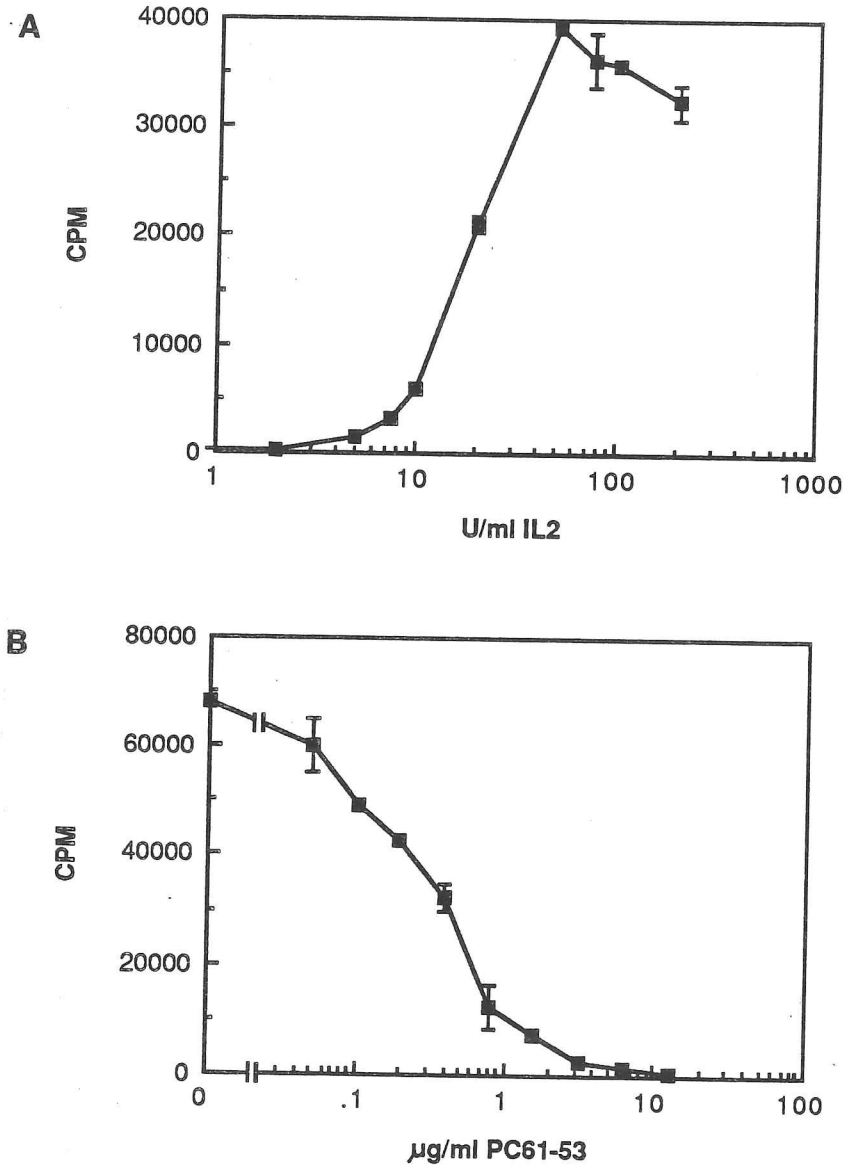


FIGURE 3.1 A. Mitogenic stimulation of clone 4 by IL2. Clone 4 cells were cultured with the indicated IL2 concentrations for 96h, and DNA synthesis assayed by [^{3}H]thymidine incorporation as described in Section 2.4.
B. Effect of anti-IL2r antibody on IL2-stimulated clone 4 proliferation. Clone 4 cells were cultured with 50U/ml and the indicated concentrations of anti-IL2r antibody PC61-53 for 96h and DNA synthesis measured as in a. The ordinate in this and all subsequent figures depicting DNA synthesis data shows cpm of [^{3}H]thymidine incorporated. Each point is the mean (+/- standard deviation) of duplicate determinations.

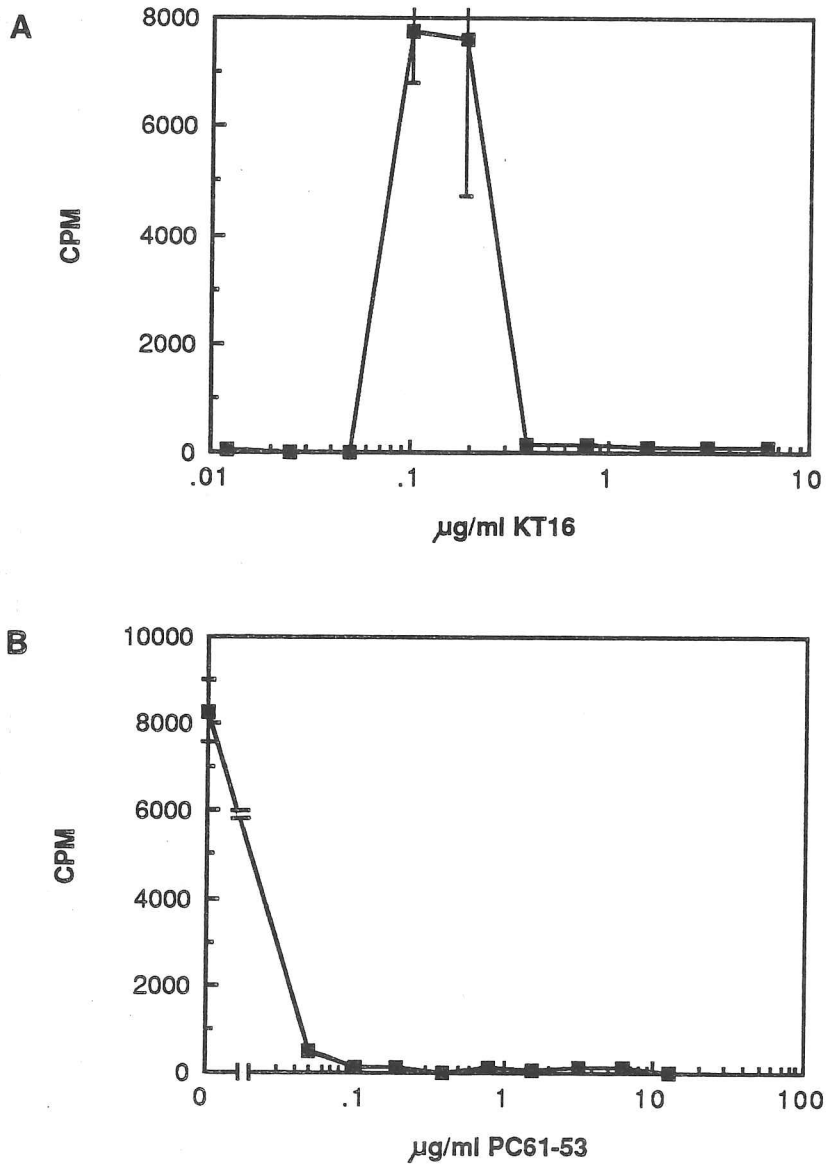


FIGURE 3.2 A. Mitogenic stimulation of clone 4 by anti-Thy 1 antibody.
Clone 4 cells were cultured with the indicated concentrations of anti-Thy 1 antibody KT16 for 96h and DNA synthesis assayed as descibed in the legend to Fig. 3.1.
B. Effect of anti IL2r antibody on KT16-stimulated clone 4 proliferation. Clone 4 cells were cultured with 125ng/ml KT16 and the indicated concentrations of anti-IL2r antibody PC61-53 for 96h and DNA synthesis assayed. Each point is the mean (+/- standard deviation) of duplicate determinations.

Mitogenic stimulation of clone 4 by 150ng/ml KT16 was inhibited by PC61-53 (Fig. 3.2b), suggesting IL2-IL2r interaction to be essential for mitogenic stimulation through Thy 1. KT16-mediated mitogenesis was inhibited by greater than 98% by 0.1μg/ml PC61-53, compared with a K_i of 0.35μg/ml for inhibition of IL2-stimulated mitogenesis, possibly indicating that significantly less than 50U/ml IL2 was secreted by clone 4 in response to KT16 doses optimal for mitogenesis. In this context, it is interesting to note that KT16-stimulated clone 4 proliferation was inhibited by 50U/ml IL2 (see below).

3.1.3 Responses to mitogen combinations

KT16 and IL2 both activated DNA synthesis individually, but when combined were mutually antagonistic. Titration of KT16 against an optimal mitogenic IL2 concentration showed concentration dependent inhibition of IL2-stimulated mitogenesis by KT16 (Fig. 3.3a). Combination of optimal concentrations of KT16 and IL2 stimulated virtually no DNA synthesis. Thus although IL2-IL2r interaction was essential for KT16 to stimulate proliferation, exogenous IL2 was antagonistic to KT16. This apparent paradox may be resolved by postulating that optimal KT16 concentrations stimulate secretion of less than 50U/ml IL2 (as suggested above). Furthermore, since supraoptimal KT16 concentrations presumably stimulate sufficient IL2 production to antagonise stimulation through Thy 1, IL2-KT16 antagonism may explain the narrow concentration range over which KT16 is mitogenic.

IL2-activated DNA synthesis in clone 4 was also antagonised by the polyvalent lectin Con A (Fig. 3.3b), although Con A was not mitogenic alone. The failure of Con A to stimulate clone 4 proliferation may reflect its inability to elicit IL2 secretion from clone 4, or conversely may indicate that Con A induces secretion of antagonistic concentrations of IL2.

The anti-T3 antibody 145-2C11 was also ineffective as a mitogen for clone 4. This result is surprising because 145-2C11 is a potent mitogen for purified T3-T_i^+ T cells (Leo et al., 1987b) and clone 4 expresses T3-T_i.

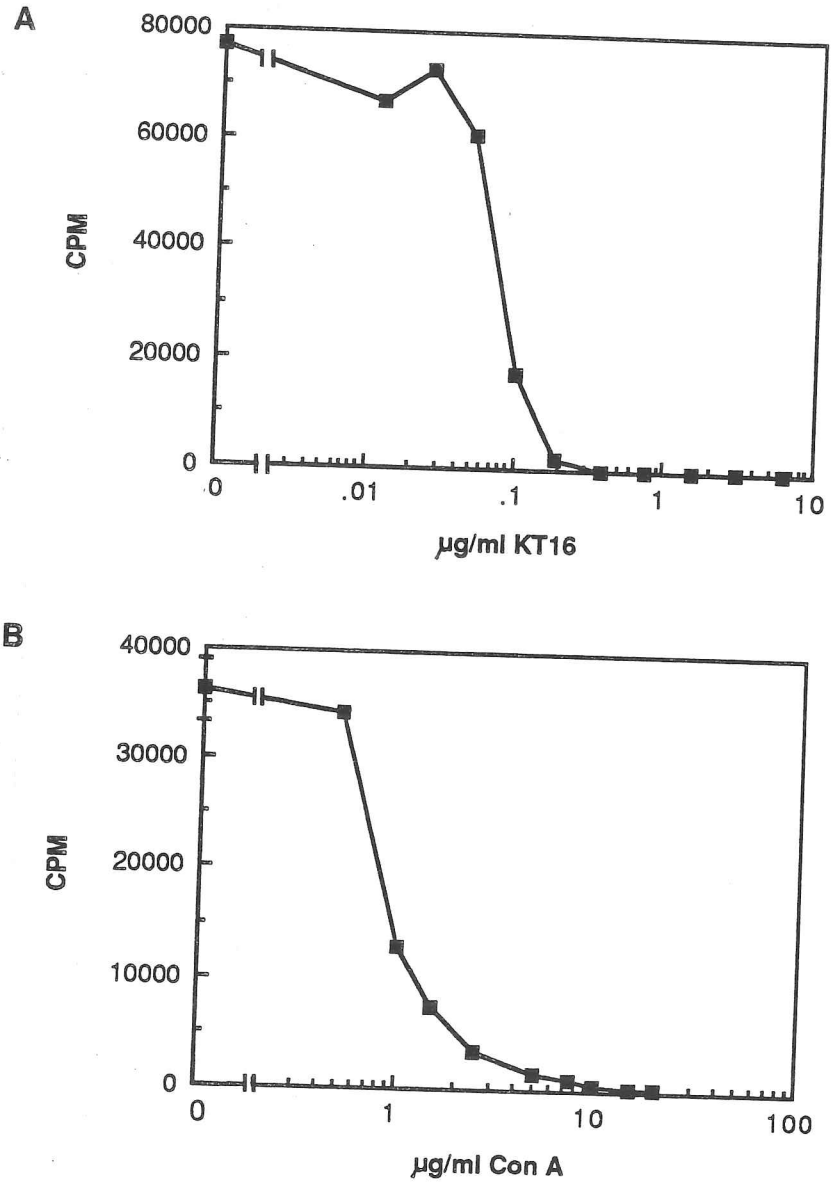


FIGURE 3.3 Inhibition of IL2-stimulated clone 4 proliferation by KT16 and Con A. Clone 4 cells were cultured with 50U/ml IL2 and the indicated concentrations of KT16 (A) or Con A (B) for 96h. DNA synthesis was assayed as described in the legend to Fig. 3.1. Each point is the mean (+/- standard deviation) of duplicate determinations.

However, the failure of 145-2C11 as a mitogen for clone 4 may reflect the absence of agents capable of cross-linking 145-2C11 on the surface of clone 4, since ligand cross-linking appears to be an important component of the T3-T_i-mediated mitogenic signal (see Section 1.2.3). Cross-linking could be achieved by interaction of the F_c portion of 145-2C11 with F_c receptors on the surface of accessory cells (as used by Leo et al., 1987b), or on the T cell surface. Therefore these results suggest that clone 4 lacks an F_c receptor specific for hamster IgM. Alternatively, 145-2C11 may simply stimulate secretion of either insufficient or antagonistic amounts of IL2 from clone 4.

3.2 Mitogenic stimulation of BALB/c thymocytes

3.2.1 Stimulation of thymocytes by Con A

In contrast to clone 4, only a very small proportion of thymocytes derived from BALB/c mice can respond to a single antigen since each individual thymocyte expresses a unique T_i. However, BALB/c thymocytes were mitogenically stimulated by Con A (Fig 3.4a), presumably due to interaction with T3, or non-polymorphic determinants of T_i (see Section 1.2.2). Maximal incorporation was stimulated by 0.75-1.0μg/ml Con A. Supraoptimal concentrations evoked diminished responses, and no incorporation was stimulated by concentrations greater than 2.5μg/ml. IL2 synergised with 0.8μg/ml Con A, with maximal stimulation at 20U/ml IL2 (Fig. 3.4b), but was ineffective alone, presumably due to the virtual absence of IL2r from resting thymocytes. IL2 increased maximum Con A-induced [^{3}H]thymidine incorporation 2-3 fold.

The phorbol ester 12-O-tetradecanoyl phorbol 13-acetate (TPA), like IL2, synergised with Con A, but was ineffective alone. 10nM TPA increased maximum incorporation 4-fold, and shifted the optimum Con A concentration to 0.5μg/ml (Fig. 3.4a). Although both TPA and IL2 singly were ineffective, together they stimulated [^{3}H]thymidine incorporation into BALB/c thymocytes (data not shown). This suggests that protein kinase C activation may be sufficient to stimulate IL2r expression, as TPA activates protein kinase C (Castagna et al., 1982).

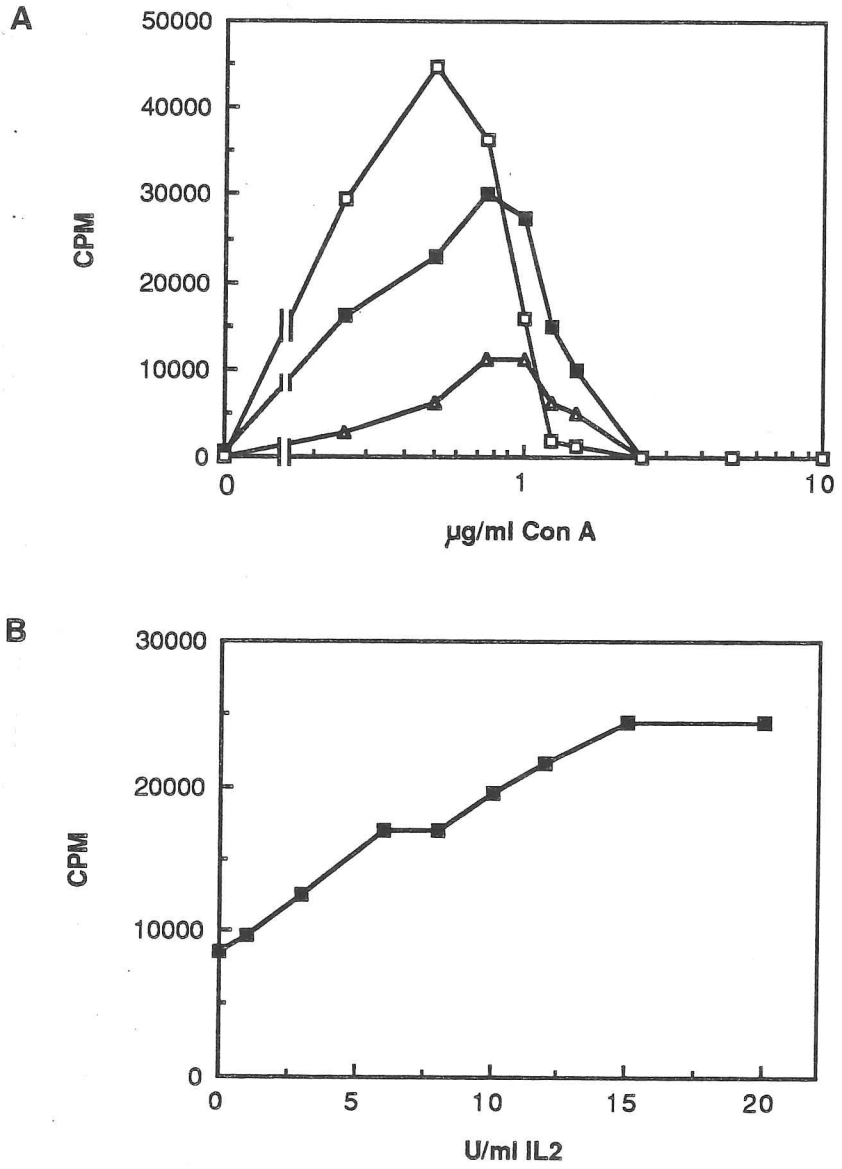


FIGURE 3.4 Mitogenic stimulation of BALB/c thymocytes by Con A.
A. Thymocytes were cultured with the indicated Con A concentrations plus medium (△), 20U/ml IL2 (■) or 10nM TPA (□) for 48h, and DNA synthesis assayed as described in Section 2.4. Each point is the mean of duplicate determinations. Standard deviations are omitted for the sake of clarity.

B. Thymocytes were cultured with 0.8µg/ml Con A plus the indicated IL2 concentrations for 48h, and DNA synthesis assayed. Each point is a single determination.

However, as resting thymocytes may express a low level of IL2r (Jenkinson et al., 1987), IL2-IL2r interaction may also have positive feedback effects on IL2r expression.

3.2.2 Stimulation of thymocytes by anti-Thy 1 and anti-T3 antibodies

Thymocyte DNA synthesis was also stimulated by anti-Thy 1 antibody, KT16, and anti-T3 antibody, 145-2C11 (Fig. 3.5a&b respectively). Both antibodies were only weakly mitogenic alone. KT16 and 145-2C11 stimulated 29% and 23% respectively of the incorporation stimulated by 0.8μg/ml Con A. However the potencies of both KT16 and 145-2C11 were enhanced 3-4 fold by IL2 (20U/ml) suggesting that the low potencies of the antibodies alone relative to Con A were due to their inability to stimulate significant IL2 production. Since, TPA also enhanced Thy 1- and T3-induced thymocyte proliferation (TPA increased KT16 and 145-2C11 stimulation 7-8 fold and 12 fold respectively), these data reinforce the idea that activation of protein kinase C is required for stimulated IL2 secretion, and suggest that neither KT16 nor 145-2C11 stimulate appreciable protein kinase C activation in BALB/c thymocytes (however see Section 3.2.3).

The relationship between [^{3}H]thymidine incorporation (as a percentage of maximal stimulation) and KT16 concentration was similar in cells co-stimulated with KT16 plus either TPA or IL2. Proliferation increased sharply between 3 and 6 μg/ml KT16, was maximal by 12μg/ml, and did not significantly decrease at concentrations up to 200μg/ml (Fig. 3.5a; and additional experiments). This concentration dependence profile differed from that seen with Con A since [^{3}H]thymidine incorporation declined at supraoptimal Con A concentrations. The profile of 145-2C11 concentration dependence was more similar to that seen with Con A. Maximum [^{3}H]thymidine incorporation was stimulated by 145-2C11 concentrations between 1 and 25 μg/ml. Concentrations below 0.2μg/ml and above 100μg/ml stimulated less than 8% of maximal incorporation.

The profiles exhibited by Con A and 145-2C11 demonstrate inhibition of mitogenesis by supramitogenic concentrations of these ligands. High ligand concentrations may cause increased receptor modulation (e.g. capping), resulting in signals of insufficient duration to activate DNA synthesis.

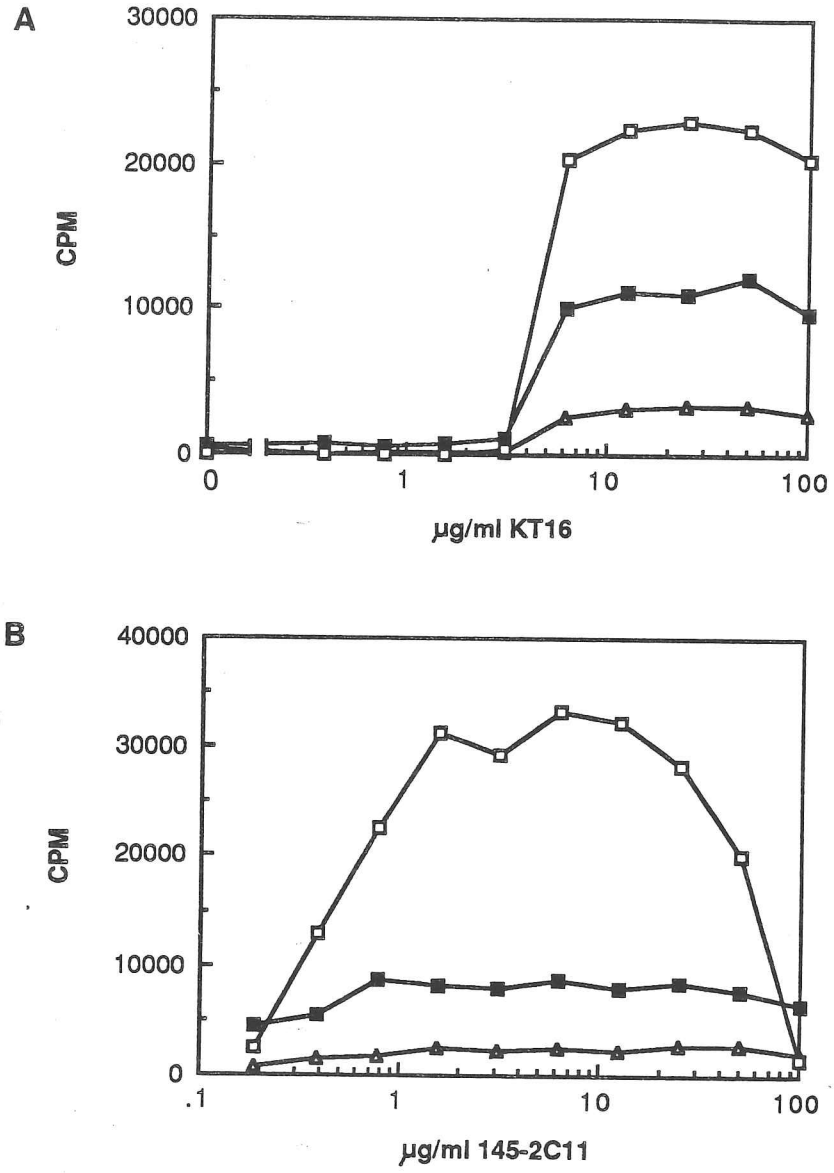


FIGURE 3.5 Mitogenic stimulation of BALB/c thymocytes by KT16 and 145-2C11. Thymocytes were stimulated with the indicated concentrations of KT16 (A) or 145-2C11 (B) plus medium (△), 20U/ml IL2 (■) or 10nM TPA (□), and DNA synthesis assayed as described in Section 4.2. Each point is a single determination. Similar results were obtained in several experiments.

In support of this, Pozzan et al. (1981) demonstrated that addition of anti-Con A antibody to Con A-challenged cells accelerated the rate of Con A capping and reduced mitogenic stimulation. The failure of high KT16 concentrations to inhibit thymocyte DNA synthesis correlates with a slow capping rate of Thy 1 relative to Con A receptor(s) (Corps et al., 1982), although it should be noted that the capping studies were performed with a different anti-Thy 1 antibody, and may therefore not be comparable to the data described herein. However, lack of inhibition of proliferation by supramitogenic KT16 concentrations could be a function of slow Thy 1 capping.

The results for thymocytes contrast strongly with the situation in clone 4 where mitogenesis was strongly inhibited by supraoptimal KT16 concentrations. However, this difference may be explained on the basis of differing IL2r expression, as resting thymocytes express only very low levels or no IL2r, they are likely to be less susceptible to IL2-mediated inhibition.

3.2.3 IL2-IL2r interactions in thymocyte mitogenesis

The ability of TPA to enhance the mitogenicity of anti-Thy 1 and anti-T3 antibodies may reflect increased expression of IL2r stimulated by TPA (see above; Shackelford and Trowbridge, 1984; McCrady et al., 1988). If so, TPA must also have synergised with the antibodies to produce IL2, since the antibodies alone apparently stimulated little IL2 production (i.e. the antibodies were weak mitogens in the absence of exogenous IL2). Alternatively, TPA in combination with the antibodies might have stimulated mitogenesis via an IL2-IL2r independent pathway. This possibility was examined using the anti-IL2r antibody PC61-53.

Proliferation induced by Con A alone, or by KT16 plus TPA, was inhibited by greater than 80% at PC61-53 concentrations above 0.8μg/ml, but concentrations of PC61-53 up to 200μg/ml caused less than 60% inhibition of the stimulation evoked by Con A and TPA (Fig. 3.6). 0.8μg/ml PC61-53 only inhibited TPA plus 145-2C11 mediated stimulation by approx. 50%, and 90% inhibition only occurred at PC61-53 concentrations above 50μg/ml.

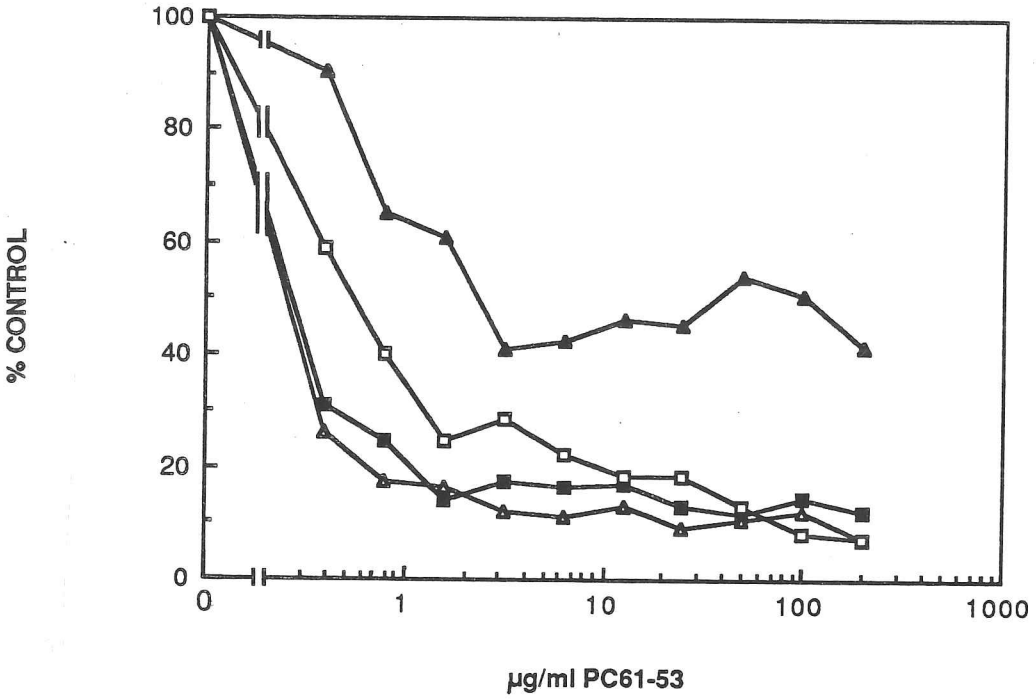


FIGURE 3.6 Inhibition of thymocyte proliferation by anti-IL2r antibody.
Thymocytes were cultured with the indicated concentrations of PC61-53 plus the following stimulatory ligand combinations: 0.8µg/ml Con A (■), 0.8µg/ml Con A + 10nM TPA (▲), 20µg/ml KT16 + 10nM TPA (△) or 10µg/ml 145-2C11 + 10nM TPA (□), then assayed for DNA synthesis as described in Section 2.4. Results are expressed as a percentage of stimulation by these ligands in the absence of PC61-53.

Thus, TPA synergised with Con A or 145-2C11 to stimulate thymocytes by a pathway with reduced sensitivity to PC61-53 compared with the pathway stimulated by Con A alone. These data suggest that TPA in synergism with Con A (or 145-2C11) either stimulated proliferation via an IL2-independent pathway or amplified IL2r expression to a level at which PC61-53 could only partially block IL2-IL2r interaction. If this latter were true, increased amounts of PC61-53 should be effective at antagonising proliferation. Since this was not true for proliferation evoked by Con A plus TPA, it is most likely that this ligand combination stimulated proliferation via an IL2-independent pathway. Consistent with this, Kim et al. (1986) have demonstrated PC61-53-insensitive, TPA-stimulated proliferation of a cytolytic T cell clone. If TPA does stimulate proliferation via a pathway other than IL2-IL2r interaction, then protein kinase C activation may not be sufficient to stimulate IL2 expression. In turn, this suggests that the failure of KT16 and 145-2C11 to stimulate IL2 secretion from thymocytes might not be due to an inability to activate protein kinase C, but rather a failure to induce some other required response, possibly activation of the lymphocyte-specific tyrosine kinase $pp56^{lck}$ (see below).

3.2.4 Identity of the Con A receptor(s)

Con A has been demonstrated to interact with a variety of cell surface glycoproteins including the T3 complex (see Section 1.2.2). Consistent with its ability to bind to T3, several similarities were observed between the effects of 145-2C11 (anti-T3 antibody) and Con A, suggesting that at least some of the responses to Con A might be mediated via its interaction with T3 (although see Section 4.3.2). Co-incidence of the effects of Con A and 145-2C11 was highlighted by comparison with the effects of the anti-Thy 1 antibody KT16. Contrasting effects were as follows:

i Supraoptimal concentrations of Con A and 145-2C11, but not KT16, inhibited mitogenesis, implying that supraoptimal Con A and 145-2C11 concentrations caused rapid modulation of a surface receptor (e.g. by rapid capping) or generated responses antagonistic to DNA synthesis.

ii Proliferation stimulated by synergistic combinations of either Con A or 145-2C11 (but not KT16) with TPA was less inhibited by PC61-53 than that stimulated by Con A alone.

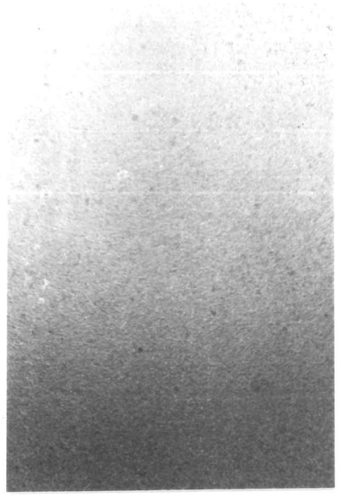
iii While KT16 produced large sausage-like blasts (Fig. 3.7), Con A and 145-2C11 both caused formation of small round blasts, suggesting that both Con A and 145-2C11 recognise receptors of similar cell surface distribution and mobility.

The possibility that T3 mediates the responses to Con A was further examined by assaying proliferation stimulated by ligand combinations. This technique was particularly useful since both KT16 and 145-2C11 were only weakly mitogenic in the absence of IL2 or TPA, and their effect on Con A-stimulated mitogenesis could therefore be evaluated.

KT16 slightly enhanced activation of DNA synthesis by Con A or 145-2C11, but 145-2C11 inhibited Con A-stimulated proliferation (Fig. 3.8). 145-2C11 and Con A were present at roughly equal molar concentrations (11μM and 8μM respectively), resulting in an approximate 2 fold excess of 145-2C11 binding moieties since Con A is tetrameric but IgM molecules are pentameric associations of subunits which are themselves dimers ($\lambda_2\mu_2$). 145-2C11-mediated inhibition of Con A could therefore reflect competition for an identical binding site, if the affinities of the binding site for the two ligands were similar. Alternatively, 145-2C11-mediated cross-linking of T3 might result in more rapid capping of the Con A receptor, and thus cause an abbreviated response similar to that seen with supraoptimal concentrations of either of the ligands individually.

Thus these data are consistent with the suggestion that Con A may interact with T3, but further evidence would be necessary to substantiate this hypothesis. In apparent contradiction, the ability of Con A to stimulate considerable proliferation in the absence of IL2 or TPA argues that Con A cannot be operating via T3, since anti-T3 is a weak mitogen in the absence of IL2 or TPA. The most probable explanation is that Con A is effective due to interactions both with T3 and with additional cell-surface glycoproteins, the latter not necessarily located on T3-bearing cells, but possibly on co-operating accessory cells. These additional interactions may stimulate secretion of potentiating lymphokines, such as interleukin 1 (see Section 1.2.3), from accessory cells.

Control



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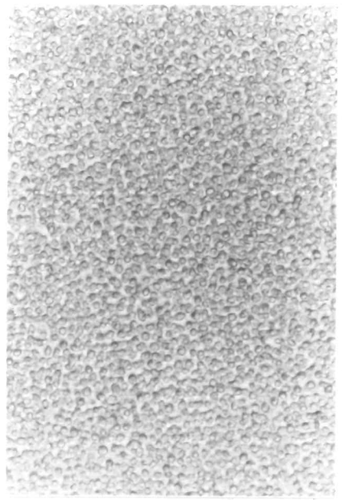

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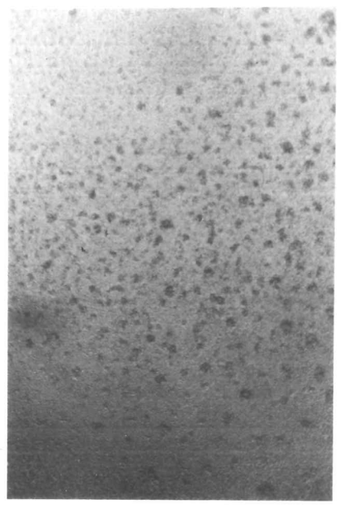
Con A



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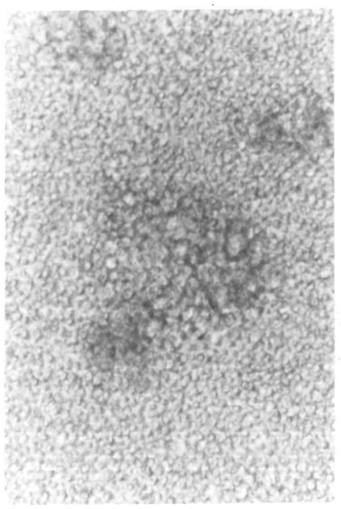

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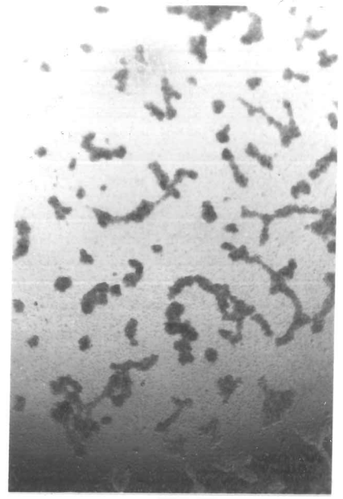
KT16



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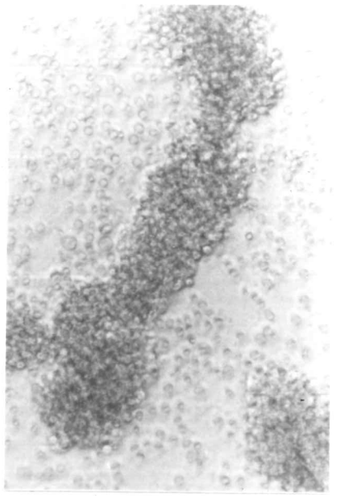

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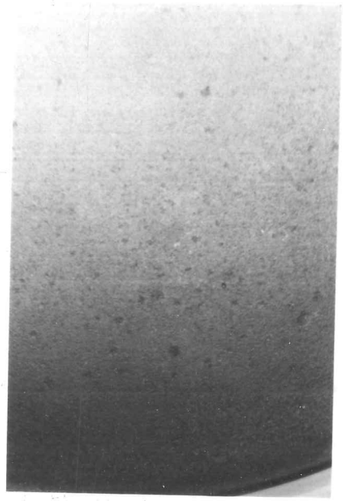
145-2C11



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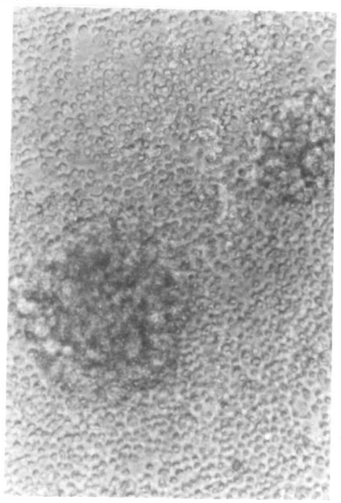

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FIGURE 3.7 Light micrographs of ligand-stimulated BALB/c thymocytes. BALB/c thymocytes were cultured for 48h. with the following ligands: Control (no addition), Con A (0.8µg/ml), KT16 (20µg/ml) and 145-2C11 (10µg/ml) as described in Section 2.4.1: then photographs were taken as described in Section 2.5.

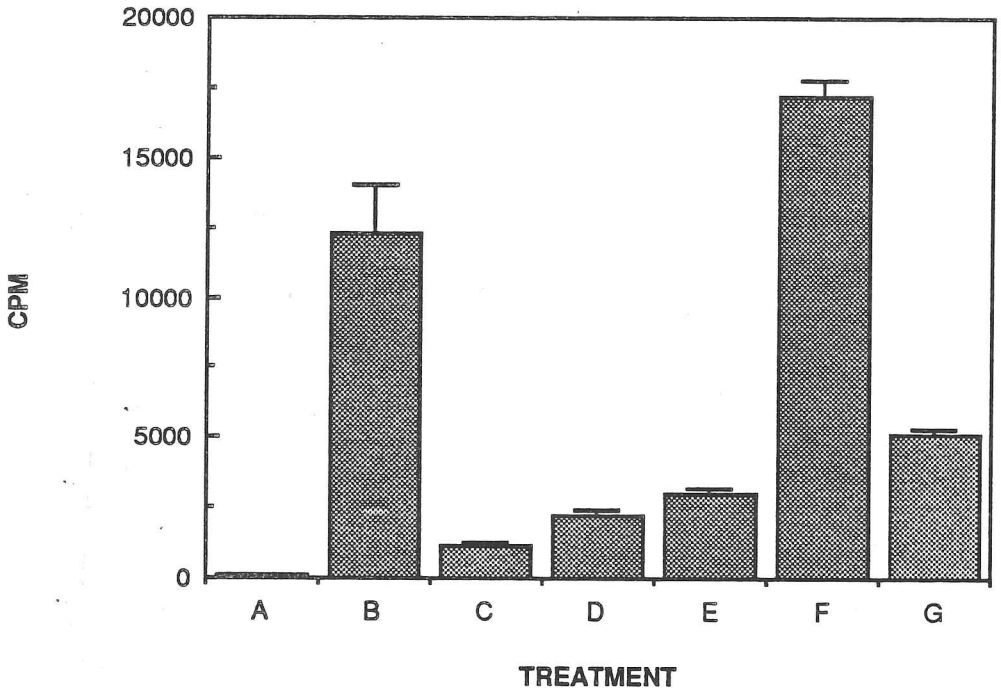


FIGURE 3.8 Ability of ligand combinations to stimulate BALB/c thymocytes. Thymocytes were assayed for DNA synthesis (Section 2.4) after 48h incubation with the following ligands; medium (A), 0.8µg/ml Con A (B), 20µg/ml KT16 (C), 10µg/ml 145-2C11 (D), Con A + 145-2C11 (E), Con A + KT16 (F), 145-2C11 + KT16 (G). Results shown are the means (+/- standard deviation) of triplicate determinations.

3.3 Effect of anti-CD4 antibody on T cell proliferation

The ability of the CD4 molecule to inhibit T cell proliferation was investigated using the anti-CD4 antibody KT6. KT6 alone stimulated no DNA synthesis in BALB/c thymocytes or clone 4, but was a potent antagonist of KT16-stimulated clone 4 proliferation (50% at 6 ng/ml and 95% at concentrations above 20 ng/ml; Fig. 3.9a). In contrast, KT6 concentrations up to 2.5μg/ml had no effect on IL2-stimulated DNA synthesis in clone 4 (Fig. 3.9b), suggesting that IL2 and KT16 activated DNA synthesis in clone 4 by different mechanisms. Consistent with this, IL2 and KT16 evoked different patterns of early responses in clone 4 (see Chapter 4).

The effect of KT6 on thymocyte proliferation was also examined (Fig. 3.10). KT6 concentrations up to 20μg/ml had no effect on thymocyte mitogenesis stimulated by KT16 plus TPA, and modulated Con A stimulation by less than +/-20%. The failure of KT6 to significantly inhibit thymocyte proliferation may have several explanations. Con A and KT16 may stimulate predominantly $CD4^-$ thymocytes, and thus anti-CD4 would be ineffective. However this seems unlikely as 80-90% of thymocytes are $CD4^+$ (Scollay et al., 1984). Alternatively, CD4 may be in a different functional state in immature cells (thymocytes) compared with mature, peripheral cells or T cell clones. This possibility is consistent with the observation that treatment of murine thymocytes, but not mature peripheral T cells, with the phorbol ester TPA results in a rapid disappearance of CD4 molecules from the surface of thymocytes, which persists for at least 72h (Wang et al., 1987). Since $CD4^+$ peripheral cells predominantly fail to express murine CD8, but murine thymocytes are 70-80% $CD4^+CD8^+$ (Scollay et al., 1984), this difference in functional state may depend on the presence/absence of co-expressed CD8. Consistent with this, Blue et al. (1988) have reported potent inhibition of T3-induced $[Ca^{2+}]_i$ increases in a $CD4^+CD8^-$ clone by an anti-CD4 antibody incapable of abrogating similar responses in a $CD4^+CD8^+$ clone.

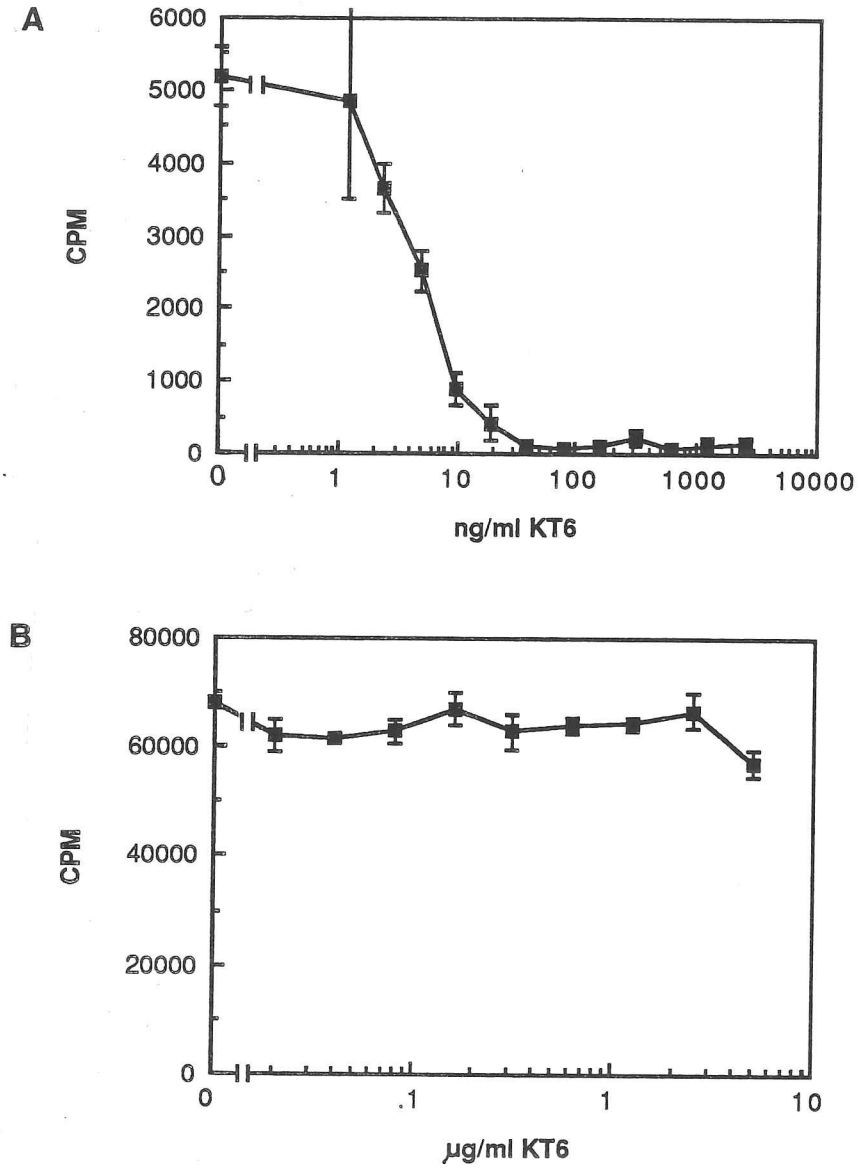


FIGURE 3.9 Effect of anti-CD4 antibody on clone 4 proliferation.
Clone 4 cells were cultured with the indicated concentrations of KT6 plus 50ng/ml KT16 (A) or 50U/ml IL2 (B) for 96h, and DNA synthesis was assayed as described in Section 2.4. Each point is the mean (+/- standard deviation) of triplicate (A) or duplicate (B) determinations. The data in A were generated by Dr. K. Tomonari and are included with his permission.

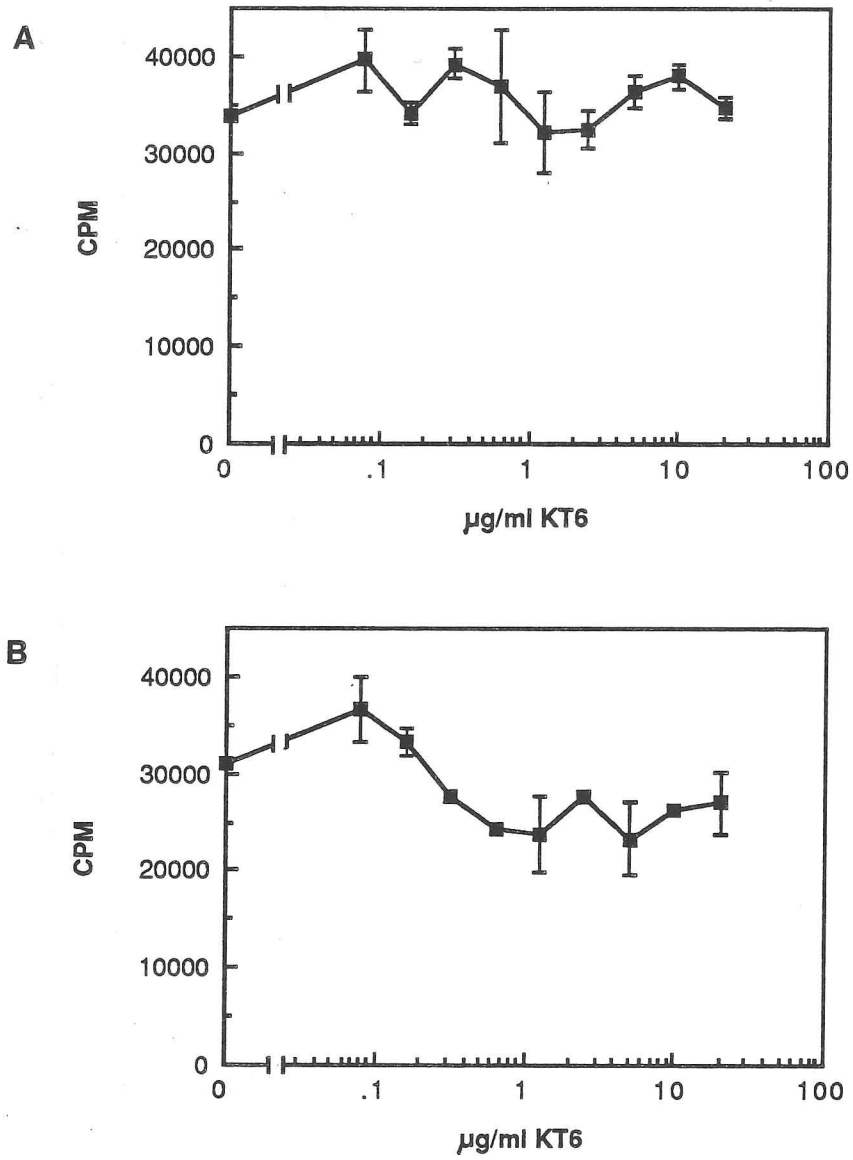


FIGURE 3.10 Effect of anti-CD4 antibody on BALB/c thymocyte proliferation. Thymocytes were cultured with the indicated concentrations of KT6 plus 0.8µg/ml Con A (A) or 20µg/ml KT16 and 10nM TPA (B), and DNA synthesis assayed (Section 2.4). Each point is the mean (+/- standard deviation) of duplicate determinations.

A third highly speculative explanation hinges on the differential expression of class II MHC molecules on immature and partially activated T cells. MHC class II proteins are expressed on activated T cells and most T cell clones (which are considered to be partially activated since they express IL2r), but not on quiescent, immature cells such as primary thymocytes. Class II proteins probably interact with CD4 (Section 1.2.5) and interaction of these molecules on adjacent T cells (or between T cells and B lymphocytes or macrophages) may result in activation of the CD4-associated tyrosine kinase $pp56^{lck}$, which may be involved in T cell mitogenesis (Section 1.5.3). Anti-CD4 antibody may destabilise/prohibit this interaction, thereby abrogating ligand-stimulated proliferation of mature T cells. However since quiescent thymocytes do not express class II molecules, such intercellular interaction cannot occur and thymocytes are therefore not sensitive to inhibition via CD4. Non-specific ligands such as Con A could activate $pp56^{lck}$ by co-cross-linking CD4 with other molecules, and would therefore be free from a requirement for class II-bearing cells, and less sensitive to inhibition by anti-CD4. However, specific ligands such as KT16 would require class II:CD4 interaction, and thus KT16 would be mitogenic for activated but not immature, quiescent cells. This provides an elegant though highly speculative explanation for the failure of KT16 to stimulate appreciable thymocyte proliferation in the absence of exogenous IL2, and further suggests that tyrosine kinase activation may be a necessary precursor of IL2 secretion. This is also suggested by the recent demonstration that anti-Thy 1 antibody (G7) can stimulate IL2 secretion from a mutant T cell hybridoma in the absence of increases in $[Ca^{2+}]_i$ or inositol phosphates (Sussman et al, 1988b).

3.4 Summary

The mitogenic responses to a panel of ligands have been characterised and compared in two murine T cell populations, a mature monoclonal helper T cell line, clone 4, and an immature heterogeneous T cell preparation derived from BALB/c thymi. Both cell types have been challenged with four ligands; Con A, anti-Thy 1 antibody (KT16), anti-T3 antibody (145-2C11), and IL2.

Con A was a potent mitogen for BALB/c thymocytes. In comparison, KT16 and 145-2C11 were weak mitogens but both synergised with IL2, suggesting stimulation of IL2 secretion to be their main deficiency. In turn this suggested that both antibodies require an IL2-IL2r interaction to activate DNA synthesis. This was confirmed by the ability of anti-IL2r antibody PC61-53 to inhibit mitogenesis stimulated by either KT16 or 145-2C11 in conjunction with TPA, or by Con A alone. However IL2 alone was not mitogenic for thymocytes presumably since the majority of resting thymocytes do not express IL2r.

Several pieces of data, notably the inhibition by anti-T3 antibody of Con A-stimulated DNA synthesis, suggested that the effects of Con A might be due to its interaction with the T3-T_i complex. However, since Con A was a potent mitogen in the absence of exogenous IL2 (cf 145-2C11) it must be assumed to also interact with additional receptors, possibly on accessory cells, to augment IL2 production.

Anti-CD4 antibody (KT6) had no effect on thymocyte proliferation stimulated by KT16 plus TPA, and little effect on the response to Con A. Since this antibody did inhibit KT16-induced clone 4 proliferation (see below), the CD4 molecule may exist in different functional states in the two cell populations, or may interact with different proteins on the surface of thymocytes or clone 4.

The mature T cell, clone 4, was mitogenically stimulated by IL2 and KT16. These mitogens apparently worked via different mechanisms since proliferation induced by KT16 but not IL2 was sensitive to inhibition by anti-CD4 antibody. Furthermore optimal mitogenic concentrations of IL2 and KT16 were mutually antagonistic implying that they may generate distinct, mutually antagonistic responses. Despite this, KT16 required an IL2-IL2r interaction to activate DNA synthesis, as adjudged by the sensitivity of KT16-stimulated proliferation to PC61-53. However, these data are not inconsistent since optimal mitogenic KT16 concentrations may generate insufficient IL2 to antagonise DNA synthesis.

IL2-induced DNA synthesis was also inhibited by Con A, but neither Con A nor 145-2C11 were mitogenic for clone 4. The failure of Con A to stimulate clone 4 is consistent

with the idea that Con A stimulates thymocyte mitogenesis via interaction with both T cells and accessory cells since no accessory cells are present in assays of clone 4 proliferation. Equally however the apparent failure of Con A to stimulate clone 4 could be a consequence of IL2 over-production.

CHAPTER 4

MITOGEN-STIMULATED INCREASES IN INOSITOL PHOSPHATES, $[Ca^{2+}]_i$ AND c-myc mRNA IN T LYMPHOCYTES

CHAPTER 4

MITOGEN-STIMULATED INCREASES IN INOSITOL PHOSPHATES, $[Ca^{2+}]_i$ AND c-myc mRNA IN T LYMPHOCYTES

4.1 Con A-stimulated increases in inositol phosphates and $[Ca^{2+}]_i$ in BALB/c thymocytes

4.1.1 Assays of inositol phosphates

Early studies of ligand-stimulated phosphoinositide metabolism characterised changes in lipid labelling rather than increases in inositol phosphates. Hokin and Hokin (1953) observed incorporation of [^{32}P]phosphate into lipid on stimulation of pigeon pancreas slices with acetylcholine (ACh) and subsequently identified phosphatidylinositol as the lipid into which [^{32}P]phosphate was incorporated (1964). This facilitated analysis since phosphoinositides could be specifically labelled with [^{3}H]inositol prior to reaction, and products of phosphodiesteratic cleavage easily quantitated. Durell et al. (1968) demonstrated ACh-stimulated release of [^{3}H]inositol phosphates from [^{3}H]inositol-labelled guinea pig brains. Inositol phosphates thus generated were crudely separated into inositol monophosphate, diphosphate and triphosphate peaks by high voltage paper electrophoresis, and quantitated by scanning. A more convenient separation protocol employing sequential elution from a Dowex anion exchange column was devised by Berridge et al. (1983). This separation protocol resulted in three fractions; inositol mono-, bis-, and tris-phosphate. Using this protocol, Taylor et al. (1984) analysed [^{3}H]inositol phosphates extracted from Con A-stimulated BALB/c thymocytes. Inositol trisphosphate increased rapidly between 0 and 4 mins in response to 1μg/ml Con A, but then remained approx. constant for a further 10 mins. Total inositol phosphates increased rapidly from 0 to 7 mins, more slowly between 7 and 10 mins, and remained constant between 10 and 14 mins.

Subsequent to the characterisation of inositol phosphate production in Con A-stimulated thymocytes, several novel inositol phosphates were identified including a

second $InsP_3$ isomer $Ins(1,3,4)P_3$ (Irvine et al., 1984, 1985), $InsP_4$ (Batty et al., 1985), $InsP_5$ and $InsP_6$ (Heslop et al., 1985) (see Section 1.4.3). All of these novel inositol phosphates were initially separated by anion-exchange HPLC, by elution from a Partisil 10 SAX column with a continuous formate gradient (pH adjusted to 3.7 with orthophosphoric acid). However, Downes et al. (1986) subsequently designed a non-HPLC separation of $InsP_4$ from the $InsP_3$ isomers using Biorad AG1-X8 anion exchange resin. A similar method devised by Wreggett and Irvine (1987) which employed Waters QMA SEP-PAKS is no longer utilisable since manufacture of the resin has ceased.

An analysis of inositol phosphate extracts from BALB/c thymocytes was undertaken using anion-exchange HPLC to establish which of these novel inositol phosphates are present in resting and Con A-stimulated cells. After completion of this work, Stewart et al. (1986, 1987) reported the presence of $InsP_4$ and $InsP_5$ in [^{3}H]inositol-labelled Jurkats, but no detailed time course data for inositol polyphosphate production in T cells comparable to the data presented here has been published, excepting an account of this work (Zilberman et al., 1987).

4.1.2 The effect of Con A on inositol polyphosphates

Thymocytes pre-labelled with [^{3}H]inositol were incubated with 10μg/ml Con A, the optimal concentration for inositol phosphate accumulation (J.P.Moore, personal communication); extracted inositol phosphates were then assayed by HPLC. Quiescent thymocytes contained at least 5 fold more $InsP_5$ than the combined amounts of $InsP_3$ and $InsP_4$, but no $InsP_6$ could be detected (Fig. 4.1, and see Table 2.4). $InsP_5$ did not vary by more than 10% after addition of Con A. These data suggest $InsP_5$ may have no signalling function in thymocyte stimulation by Con A, but do not exclude the possibility of small, transient changes in $InsP_5$ within 30s of Con A addition.

The accumulation of $InsP_3$ isomers and $InsP_4$ is also shown. $InsP_4$ increased rapidly for 2 mins, declined between 2 and 5 mins, then increased slowly up to 20 mins. $Ins(1,3,4)P_3$ rose rapidly between 1 and 2 mins, then accumulated monotonically, reaching ~30% of $InsP_4$ at 20 mins. $Ins(1,4,5)P_3$ accumulation was transient, peaking 1 min after Con A addition and declining to near basal within 5 minutes.

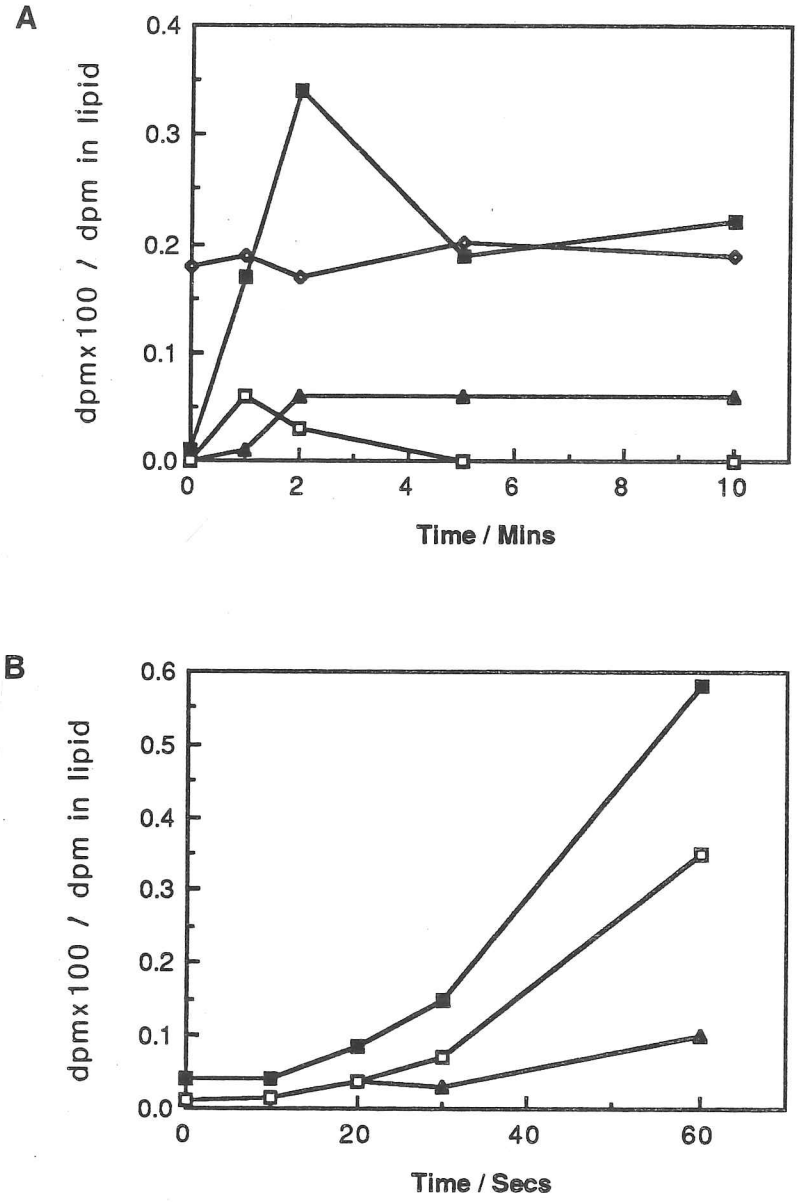


FIGURE 4.1 Time course of inositol polyphosphate production in Con A-stimulated thymocytes.

BALB/c thymocytes were labelled with [^{3}H]inositol (Section 2.7.1) and challenged with 10μg/ml Con A as described in Section 2.7.2. Reactions were quenched at the times indicated, and inositol phosphates extracted (Section 2.7.3), separated by anion-exchange HPLC (Section 2.7.5a), and quantitated by liquid scintillation counting (Section 2.8). A and B show 10min and 1 min time courses respectively. Amounts of inositol phosphates are expressed as a percentage of the total dpm in [^{3}H]inositol-containing lipids in this and subsequent figures showing data from HPLC experiments. Each point is the mean of duplicate determinations in this and all subsequent HPLC experiments, but standard deviations are omitted for clarity. Symbols: □, Ins(1,4,5)P_3; ▲, Ins(1,3,4)P_3; ■, $InsP_4$; ◇, $InsP_5$.

The increases in amounts of these inositol phosphates 1 min after stimulation were in the order $InsP_4$ > $Ins(1,4,5)P_3$ > $Ins(1,3,4)P_3$, but it was not possible to distinguish the times at which the increases could be detected above background. All showed an initial detectable increase at 20s (Fig. 4.1b) consistent with the lag of ~15s in the $[Ca^{2+}]_i$ response to Con A in these cells (Hesketh et al., 1983b,1985), and no increases were detectable within 10s (data not shown). Therefore no substrate-product relationships could be inferred from the initial time courses, but the data were not inconsistent, subject to certain conditions (see below), with the interconversion scheme suggested by Irvine et al. (1986a); $Ins(1,4,5)P_3$ --> $InsP_4$ --> $Ins(1,3,4)P_3$.

Since TPA enhanced DNA synthesis activation by 0.8μg/ml Con A (Section 3.2.1), the effect on Con A-stimulated inositol phosphate accumulation of 10 min pre-incubation with 10nM TPA was determined (Fig. 4.2). The pattern of responses generated by the optimal mitogenic concentration of Con A (0.8μg/ml) was qualitatively similar to that seen with 10μg/ml Con A (Fig. 4.2a), but the $Ins(1,4,5)P_3$ maximum occurred at 2 mins rather than 1 min and the amount of $Ins(1,4,5)P_3$ only declined to ~50% of maximum by 5 mins. However, the approx. 2 fold reduction rendered the responses too small for accurate routine detection by HPLC. Therefore subsequent experiments were performed with 10μg/ml Con A.

TPA pre-treatment slightly inhibited $InsP_4$ production, but neither $InsP_3$ isomer was significantly affected. Since $InsP_4$ was probably generated by $Ins(1,4,5)P_3$ phosphorylation, TPA-mediated $InsP_4$ reduction might have been due to preferential dephosphorylation of $Ins(1,4,5)P_3$ in the presence of TPA. This would be consistent with data from the platelet system suggesting that $Ins(1,4,5)P_3$ 5'-phosphomonoesterase is stimulated by protein kinase C (Connolly et al., 1986a; Molina y Vedia and Lapetina, 1986).

The effect of TPA on Con A-stimulated $InsP_3$ and $InsP_4$ accumulation described here is inconsistent with the data of Taylor et al. (1984), who found that TPA increased counts in the Con A-stimulated "$InsP_3$" fraction eluted from Dowex resin which consists of both $InsP_3$ isomers and a substantial proportion of the $InsP_4$.

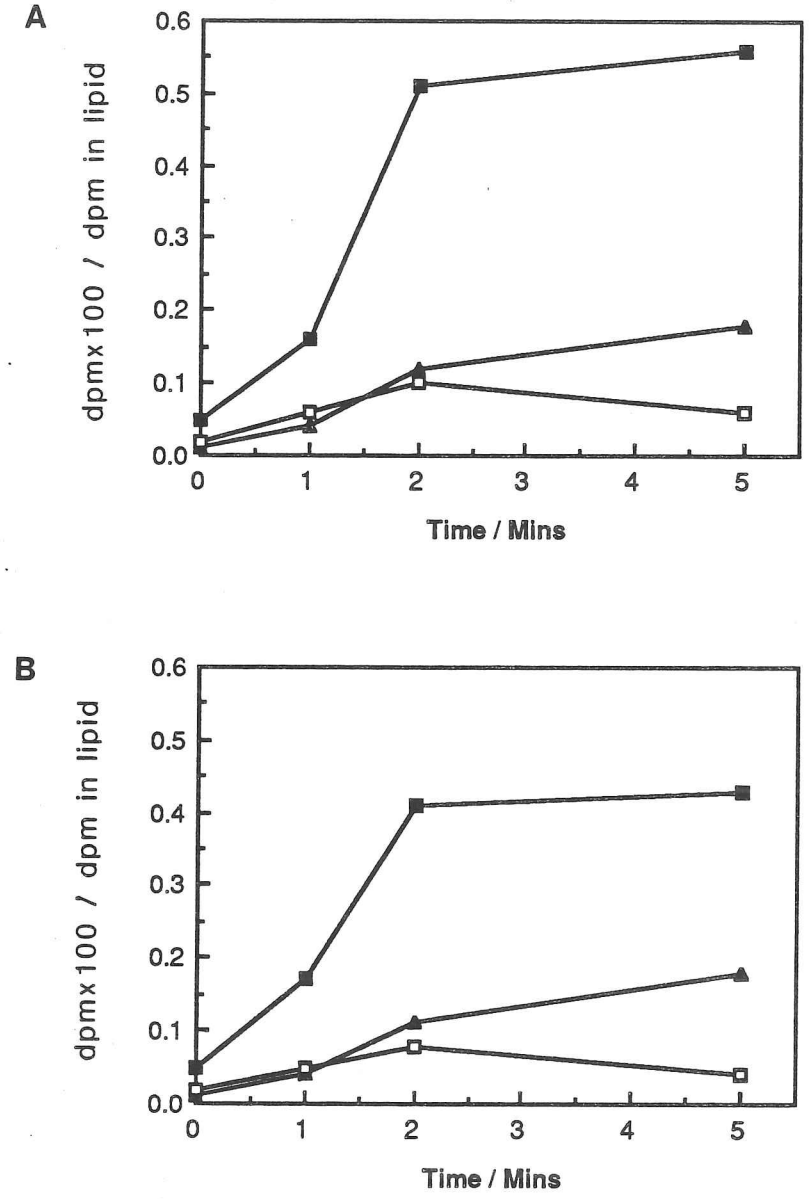


FIGURE 4.2 Effect of TPA on Con A-stimulated inositol phosphate accumulation. [^{3}H]inositol-labelled thymocytes were challenged with 0.8μg/ml Con A after 10 mins pre-incubation with medium (A) or 10nM TPA (B). Inositol phosphates were assayed as described in the legend to Fig. 4.1. Symbols: □, $Ins(1,4,5)P_3$; ▲, $Ins(1,3,4)P_3$; ■, ` $InsP_4$.

The experiments are not directly comparable since Taylor et al. pre-incubated cells with 5mM Li^+ to inhibit inositol-1-phosphate phosphomonoesterase and thereby maximise inositol phosphate accumulation (Berridge et al., 1982), but Li^+ was omitted from the experiments described here to avoid peturbations of inositol phosphate interconversions. In addition, there may be disparities due to the different separation methods employed. Therefore cells were pre-incubated with or without Li^+, challenged with Con A plus or minus TPA pre-treatment, and inositol phosphates separated on Dowex resin. No significant effect of TPA pre-treatment on any inositol phosphate fraction was observed, irrespective of whether cells were pre-incubated with Li^+ (data not shown). Thus the reason for the inconsistency between the data presented here and those of Taylor et al. is unclear. However, an inhibitory effect of TPA on ligand-stimulated inositol phosphate accumulation has been observed in a variety of systems other than T cells, including platelets (Tohmatsu et al., 1986). B cells (Bijsterbosch and Klaus, 1987; Harnett and Klaus, 1988), fibroblasts (Jamieson Jr. and Villereal, 1985; Brown et al., 1987) and vascular smooth muscle cells (Brock et al., 1985; Takata et al., 1988), implying that activated protein kinase C may have a role in negative feedback.

4.1.3. The effect of $[Ca^{2+}]_i$ on $InsP_3$ kinase

The time course data described above were not incompatible with the proposed interconversion scheme; $Ins(1,4,5)P_3$ --> $InsP_4$ --> $Ins(1,3,4)P_3$, provided that the initial rate of $Ins(1,4,5)P_3$ phosphorylation to $InsP_4$ was greater than the rate of $Ins(1,4,5)P_3$ generation (necessary to explain the more rapid accumulation of $InsP_4$ relative to $Ins(1,4,5)P_3$). Further, the observed transience of $Ins(1,4,5)P_3$ accumulation suggested that $Ins(1,4,5)P_3$ conversion to $InsP_4$ (or possibly to $InsP_2$) might be accelerated by a component of the Con A response. An alternative explanation for the observed $Ins(1,4,5)P_3$ transience might be that $PtdInsP_2$ hydrolysis was transient. However, this seemed unlikely since displacement of Con A from the cell surface with α-methyl mannoside resulted in rapid decreases in the $InsP_3$ + $InsP_4$ pool at a rate similar to the initial rate of $InsP_3$ + $InsP_4$ production, implying that Con A:receptor interaction generated continuous $Ins(1,4,5)P_3$ production (Zilberman et al., 1987). Therefore it

seemed more likely that Ins(1,4,5)P_3 metabolism was accelerated by some Con A-evoked event. Con A-induced $[Ca^{2+}]_i$ elevation appeared to be a prime candidate, as $[Ca^{2+}]_i$ was significantly elevated before onset of the decline in Ins(1,4,5)P_3 (Fig. 4.3).

The maximum rate of increase of $[Ca^{2+}]_i$ stimulated by 10μg/ml Con A occurred between 1 and 2 mins after Con A addition, thus coinciding with the onset of Ins(1,4,5)P_3 decline. Correspondingly, the later Ins(1,4,5)P_3 peak in thymocytes stimulated with 0.8μg/ml Con A correlated with a slower rate of increase of $[Ca^{2+}]_i$ (Fig. 4.3b).

These correlations suggested that changes in $[Ca^{2+}]_i$ might regulate conversion of Ins(1,4,5)P_3 to InsP_4. This possibility was examined by manipulating $[Ca^{2+}]_i$ by modulation of $[Ca^{2+}]_o$. When $[Ca^{2+}]_o$ was reduced to 0.1μM by addition of 1mM EGTA, $[Ca^{2+}]_i$ increased only transiently in response to Con A and returned to near-basal level within 5 minutes (Fig. 4.3a). Thus extracellular EGTA reduced the absolute magnitude and abolished the persistent component of the $[Ca^{2+}]_i$ response. Con A inositol phosphate responses were assayed in cells from the same [^{3}H]inositol-labelled cell preparation in normal and low free Ca^{2+} (i.e. EGTA-containing) medium to assess the effect of $[Ca^{2+}]_i$ on the Ins(1,4,5)P_3 response (Fig. 4.4). In the presence of extracellular EGTA, Ins(1,4,5)P_3 remained elevated up to 5 mins, instead of declining back to basal as in control cells, and the profile of InsP_4 accumulation was very similar to that of Ins(1,4,5)P_3 accumulation. Ins(1,3,4)P_3 accumulation at 5 mins was reduced by greater than 50%. The total amount of InsP_3 + InsP_4 was reduced by ~40% compared with control cells at 5 mins, suggesting that receptor coupling to Ins(1,4,5)P_3 formation might be modulated by extracellular calcium. (Fig. 4.4c) However, such modulation was only minor; total accumulation of inositol phosphates (which approximates to total PtdInsP_2 hydrolysis in the presence of Li^+) was reduced only 15-20% by EGTA pretreatment.

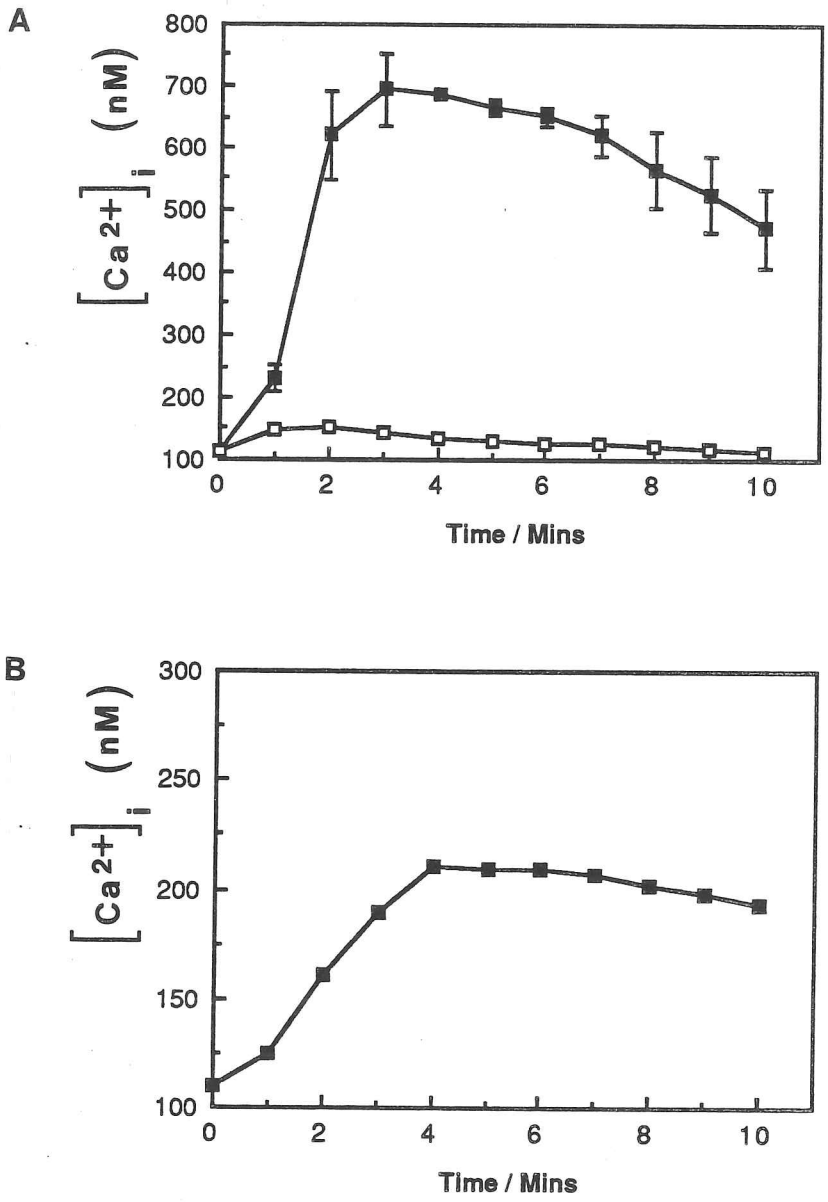


FIGURE 4.3 Effect of Con A on $[Ca^{2+}]_i$ in thymocytes
Quin 2-loaded thymocytes prepared as described in Section 2.6.1 were challenged with 10μg/ml Con A (A) or 0.8μg/ml Con A (B) in medium containing 0.43mM free Ca^{2+} (■) or 0.1μM free Ca^{2+} (□). Con A was added at time 0. Fluorescence intensity (FI) was measured continuously and FI values at minute intervals converted to $[Ca^{2+}]_i$ as described in Section 2.6.3. Data for all $[Ca^{2+}]_i$ measurement experiments are means of duplicate determinations unless otherwise stated. Standard deviations are not shown, but were usually less than 10% of the mean.

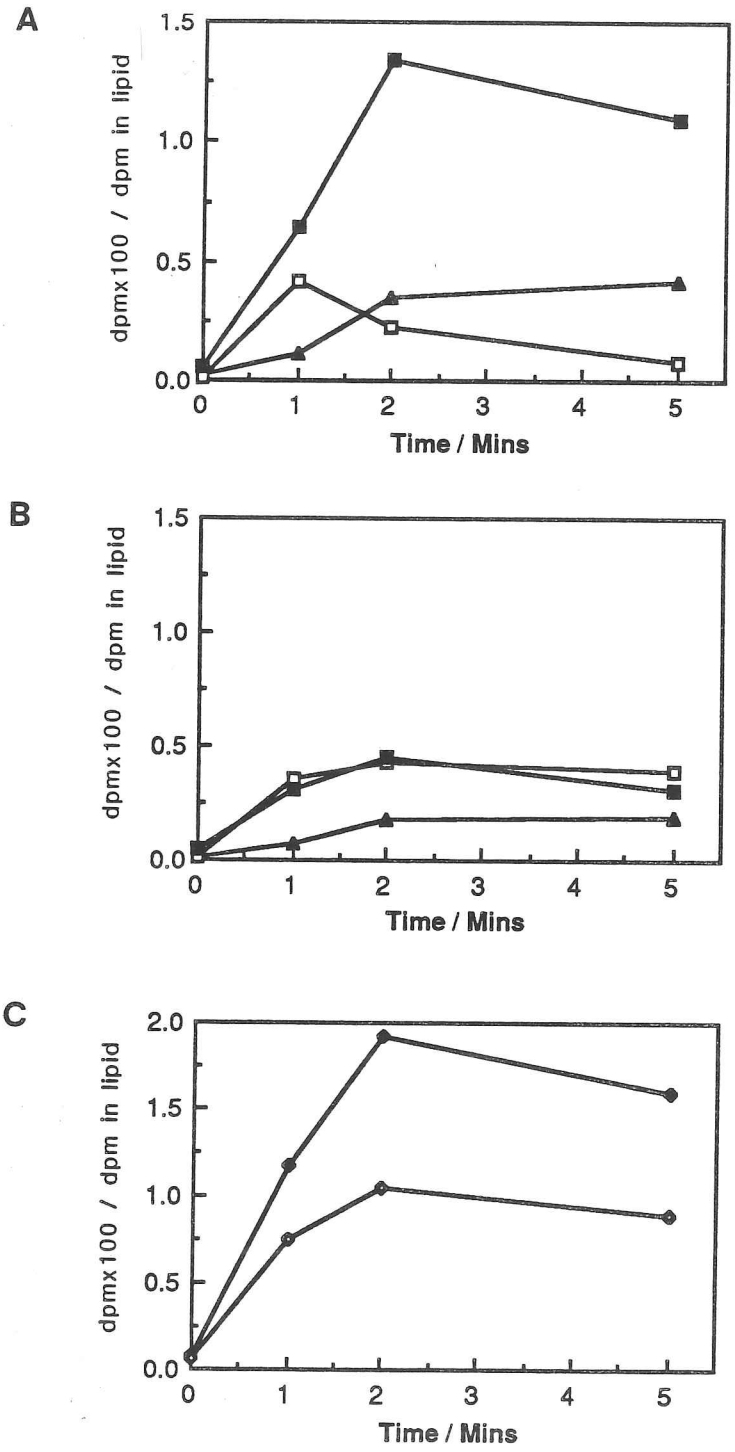


FIGURE 4.4 Effect of extracellular Ca^{2+} concentration on Con A-stimulated inositol phosphate accumulation

[^{3}H]inositol-labelled thymocytes were challenged with 10μg/ml Con A in medium containing 0.43mM free Ca^{2+} (A) or 0.1μM free Ca^{2+} (B), reactions quenched at the times indicated, and inositol phosphates assayed as described in the legend to Fig. 4.1. Symbols: □, $Ins(1,4,5)P_3$; ▲, $Ins(1,3,4)P_3$; ■, $InsP_4$.

C. Total $InsP_3$+$InsP_4$ accumulation in medium containing 0.43mM free Ca^{2+} (◆) or 0.1μM free Ca^{2+} (◇). Subsequent experiments were performed in medium containing 0.43mM free Ca^{2+} unless otherwise stated.

The increase in Ins(1,4,5)P_3 accumulation and corresponding decrease in $InsP_4$ accumulation observed in EGTA-treated compared with control cells strongly suggested the conversion of Ins(1,4,5)P_3 to $InsP_4$ to be Ca^{2+}-sensitive. To examine this further, the interconversion of [^{3}H]Ins(1,4,5)P_3 and $InsP_4$ was assayed in a lysed cell preparation. The data in Figure 4.5 were generated in collaboration with Dr. Y. Zilberman and are included here with her permission.

Fig. 4.5a shows the time course of Ins(1,4,5)P_3 metabolism by lysed cells. Six minutes after cell lysis into medium containing [^{3}H]Ins(1,4,5)P_3, greater than 50% of the [^{3}H] was present as [^{3}H]$InsP_4$ and by 20 mins this had increased to 70%. The amount of [^{3}H]Ins(1,3,4)P_3 started rising slowly at 6 mins, and was ~30% of the amount of [^{3}H]$InsP_4$ at 42 mins. A gradual decline in the total amount of $InsP_3$ + $InsP_4$ was attributable to slow conversion to InsP and $InsP_2$.

The ability of the lysed cell preparation to phosphorylate Ins(1,4,5)P_3 in the presence of ATP was employed to examine the effect of free calcium concentration on the Ins(1,4,5)P_3 -> $InsP_4$ conversion (Fig. 4.5b). Cells were lysed into medium containing [^{3}H]Ins(1,4,5)P_3 plus a range of free calcium concentrations, and reactions were quenched at 12 mins. The rate of Ins(1,4,5)P_3 phosphorylation as judged by the ratio of $InsP_4$ to Ins(1,4,5)P_3 increased substantially between 0.1 and 16μM free Ca^{2+}, but little Ins(1,3,4)P_3 accumulated at any calcium concentration, implying that $InsP_4$ dephosphorylation was independent of [Ca^{2+}]. The [Ca^{2+}] sensitivity of $InsP_3$ kinase correlated well with the physiological range over which $[Ca^{2+}]_i$ changed in Con A-stimulated cells.

Taken together these data suggest that Ins(1,4,5)P_3 conversion to $InsP_4$ is stimulated by Ins(1,4,5)P_3-mediated release of intracellular calcium stores, but give little clue as to the role of $InsP_4$ in $[Ca^{2+}]_i$ homeostasis. If $InsP_4$ modulates extracellular Ca^{2+} entry, as has been suggested (Irvine and Moor, 1986; Morris, A.J. et al., 1987), such a mechanism would facilitate switching from $[Ca^{2+}]_i$ elevation by intracellular Ca^{2+} store release to maintenance of elevated $[Ca^{2+}]_i$ by extracellular Ca^{2+} entry.

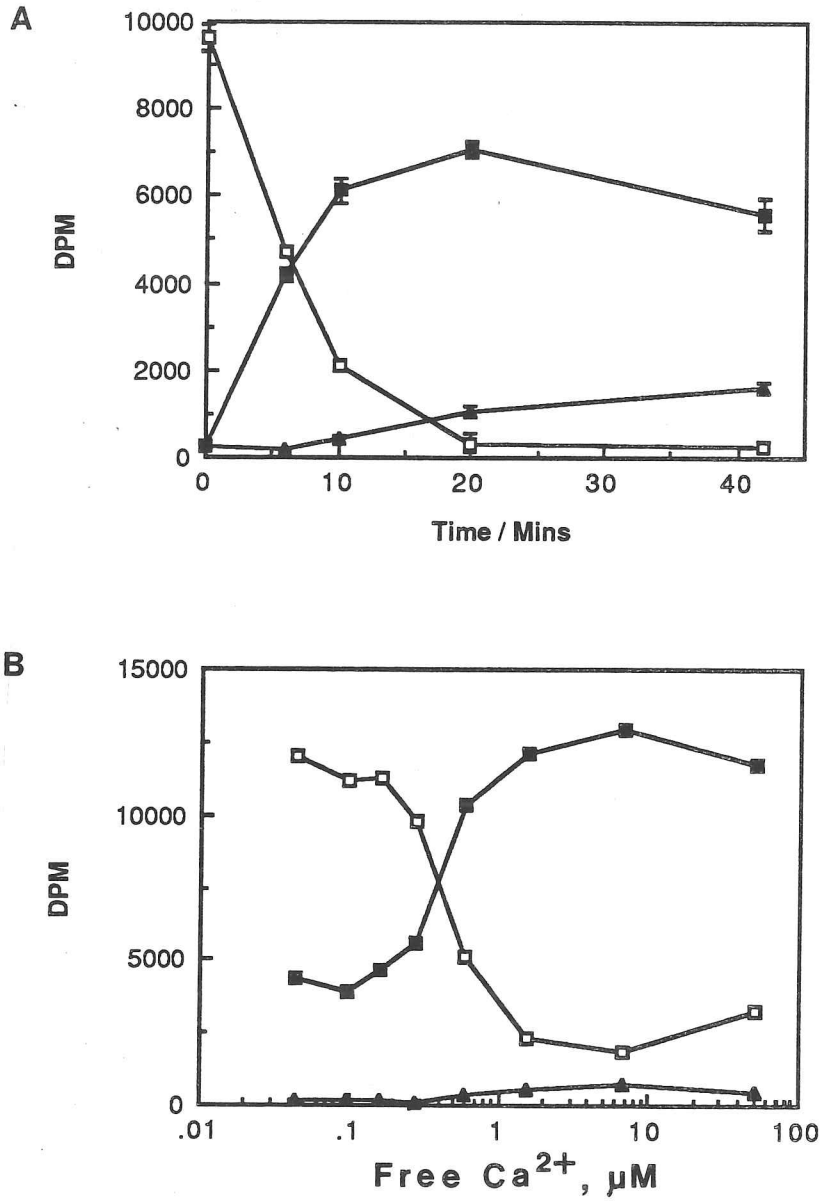


FIGURE 4.5 Metabolism of [^{3}H]Ins(1,4,5)P_3 by lysed thymocytes.
A. Time course of [^{3}H]Ins(1,4,5)P_3 metabolism. Thymocytes were lysed by dilution into hypo-osmotic medium containing 12.5nM [^{3}H]Ins(1,4,5)P_3, and Ca^{2+}/EGTA added with Tris/KCl buffer at two minutes to adjust the free [Ca^{2+}] to 50µM (Section 2.7.2). Reactions were terminated at the times indicated and inositol phosphates assayed as described in the legend to Fig. 4.1.
B. [Ca^{2+}] dependence of Ins(1,4,5)P_3 phosphorylation. Thymocytes were lysed as above, and Ca^{2+}/EGTA added with Tris/KCl buffer at two minutes to give free Ca^{2+} concentrations as indicated. Reactions were terminated at 12 mins and inositol phosphates assayed as above.
Symbols: □, Ins(1,4,5)P_3; ▲, Ins(1,3,4)P_3; ■, InsP_4.

However, the demonstration of an Ins(1,4,5)P_3-sensitive plasma membrane Ca^{2+} channel in Jurkats (Kuno and Gardner, 1987) and the observation that a PtdInsP_2-hydrolysing ligand (substance P) can stimulate $Ca^{2+}{}_o$ entry into a pancreatoma cell line in the absence of Ins(1,3,4,5)P_4 accumulation, bring into question the involvement of InsP_4 in $Ca^{2+}{}_o$ influx. Furthermore, it is clear that some ligands can stimulate $Ca^{2+}{}_o$ entry without affecting inositol phosphate levels (e.g. EGF on 3T3 fibroblasts; Hesketh et al., 1988). Therefore the mechanism(s) by which ligands mediate $Ca^{2+}{}_o$ influx is (are) currently unclear.

4.1.4 The effect of azide on Con A-stimulated inositol phosphate and $[Ca^{2+}]_i$ increases

The effect of azide on inositol phosphate accumulation was examined as azide antagonised ligand-induced increases in $[Ca^{2+}]_i$. 10mM sodium azide reduced Con A-stimulated $[Ca^{2+}]_i$ increases, but itself caused a small $[Ca^{2+}]_i$ elevation (Fig. 4.6). The azide-induced increase was due to intracellular store release, possibly mitochondrial, since it occurred in the presence of extracellular EGTA (data not shown), but was not Ins(1,4,5)P_3-mediated (i.e. InsP_3 and InsP_4 were unchanged by 10 min incubation of thymocytes with 10mM azide). Pre-incubation with azide reduced the maximal $[Ca^{2+}]_i$ induced by Con A from 700nM to ~150nM, resulting in a signal comparable to that observed in the presence of 1mM EGTA. The pattern of Con A-stimulated inositol phosphate production after azide pre-treatment was also similar to that generated in the presence of 1mM extracellular EGTA. Ins(1,4,5)P_3 amounts at 2 and 5 mins were 2-fold those in control cells, and Ins(1,3,4)P_3 and InsP_4 accumulation were reduced by greater than 50% at 5 mins (Fig. 4.7a versus b).

Azide-induced ATP depletion might be expected to affect a number of activities including ATP-dependent ion channels, ATP-requiring phosphorylations, and, via reduction of ATP-GTP exchange, GTP-dependent processes such as G-protein-stimulated reactions.

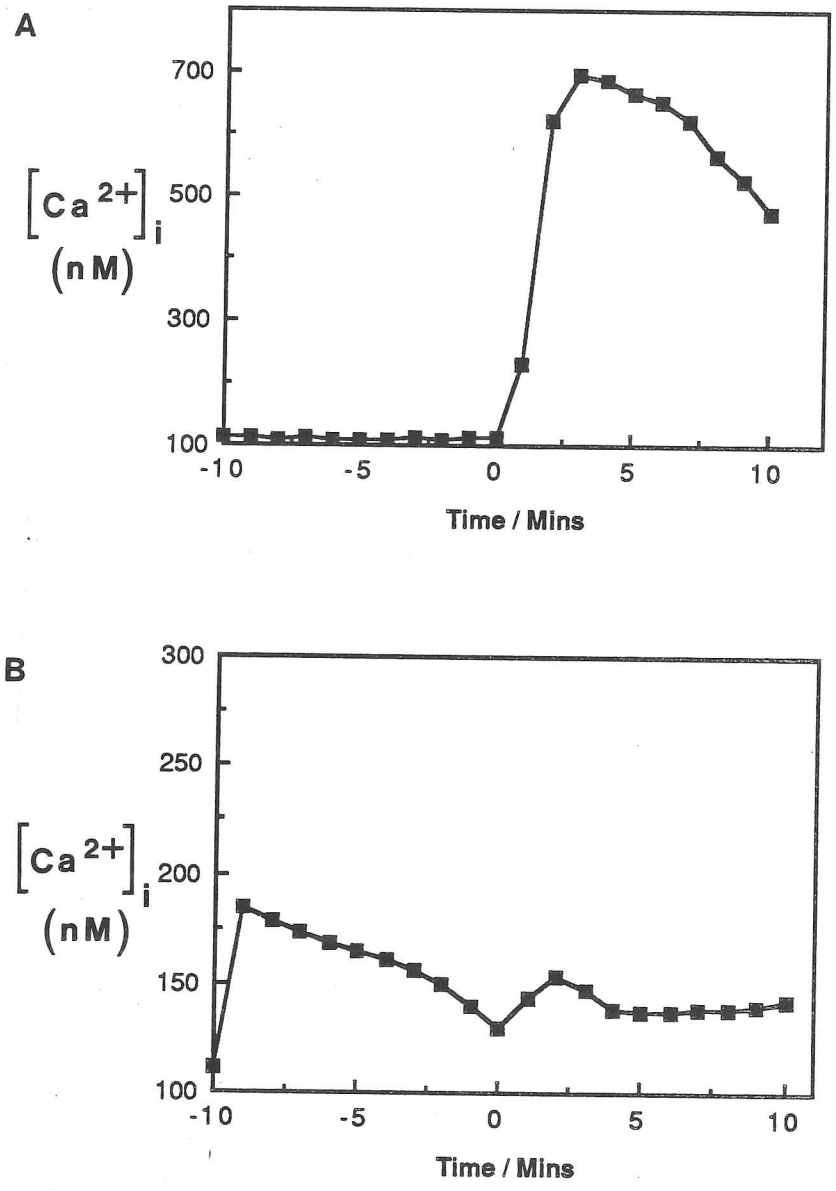


FIGURE 4.6 Effect of sodium azide on Con A-stimulated $[Ca^{2+}]_i$ increase.
Quin 2-loaded thymocytes prepared as described in Section 2.6.1 were challenged with 10μg/ml Con A (added at time 0) after 10 mins pre-incubation with medium (A) or 10mM sodium azide (B). $[Ca^{2+}]_i$ was measured as described in the legend to Fig. 4.3. this and subsequent experiments were performed in medium containing 0.43mM free Ca^{2+} unless otherwise stated.

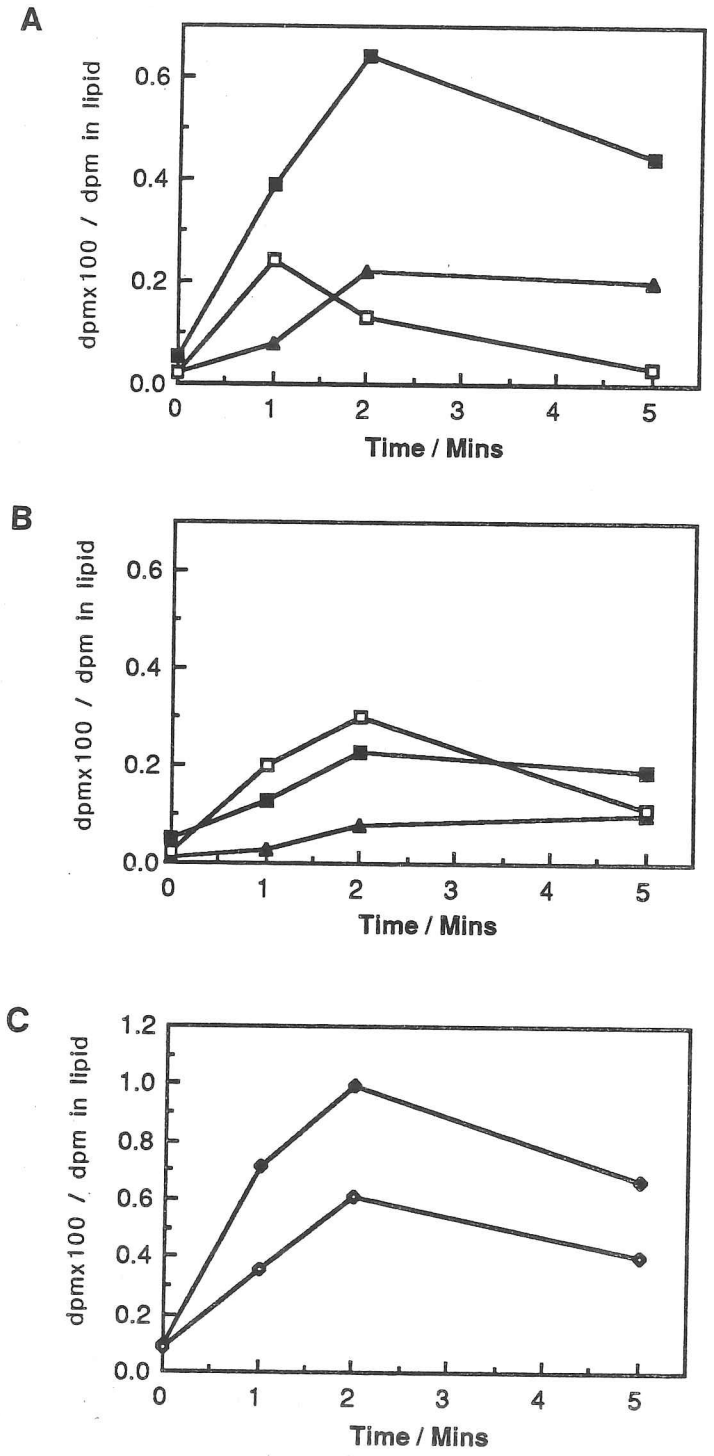


FIGURE 4.7 Effect of sodium azide on Con A-stimulated inositol phosphate accumulation.

[^{3}H]inositol-labelled thymocytes were challenged with 10μg/ml Con A (added at time 0) after 10 min pre-incubation with medium (A) or 10mM sodium azide (B). Reactions were terminated at the times indicated and inositol phosphates assayed as described in the legend to Fig. 4.1. Symbols: □, Ins(1,4,5)P_3; ▲, Ins(1,3,4)P_3; ■, InsP_4.
C. Total InsP_3+InsP_4 accumulation in cells pre-treated with medium (◆) or sodium azide (◇).

However, the marked similarity between azide and EGTA effects on Con A-stimulated $InsP_3$ + $InsP_4$ accumulation (compare Figs. 4.4c and 4.7c), suggests that azide-mediated inhibition could be mainly due to an effect on a plasma membrane Ca^{2+} channel (although the similarities could be purely coincidental). In turn, reduced Ca^{2+}_o influx might prevent full activation of Ins(1,4,5)P_3 kinase resulting in a higher ratio of Ins(1,4,5)P_3 to $InsP_4$. However, the azide-mediated reduction of both basal and stimulated amounts of PtdInsP and $PtdInsP_2$ (15-20%; Taylor et al., 1984) may also contribute to the observed reduction in total $InsP_3$ + $InsP_4$ accumulation.

4.1.5 The effect of CGS9343B on Con A-stimulated inositol phosphate and $[Ca^{2+}]_i$ increases

Ca^{2+}-sensitive Ins(1,4,5)P_3 kinase activity has now been demonstrated in a variety of cells other T cells including; turkey erythrocytes (Morris, A.J. et al., 1987), aortic smooth muscle cells (Yamaguchi et al., 1987; Rossier et al., 1987), platelets (Daniel et al., 1988), and rat hepatocytes (Biden et al., 1988). Calmodulin confers Ca^{2+}-sensitivity to partially purified $InsP_3$ kinase (Biden et al., 1987; Yamaguchi et al., 1988; Johanson et al., 1988), suggesting that the enzyme is activated by a Ca^{2+}-calmodulin complex. Therefore it was of interest to determine the effect of the calmodulin antagonist CGS9343B (Norman et al., 1987) on Con A-stimulated inositol phosphate accumulation.

CGS9343B pre-treatment (5 mins) caused a dose-dependent increase in the ratio of Ins(1,4,5)P_3 to $InsP_4$ in intact thymocytes stimulated with Con A for 2 mins (Fig. 4.8). A corresponding decrease in Ins(1,3,4)P_3 over the same concentration range was presumed to reflect the reduced availability of $InsP_4$ for 5' dephosphorylation. The ability of CGS9343B to reduce Ins(1,4,5)P_3 phosphorylation was consistent with the reported sensitivity of $InsP_3$ kinase to calmodulin. However, the effect of CGS9343B may be partially due to antagonistic effects on Ins(1,4,5)P_3-mediated Ca^{2+} release, since Hill et al. (1988a) have demonstrated CGS9343B-mediated inhibition of the latter in electropermeabilised rat liver epithelial cells.

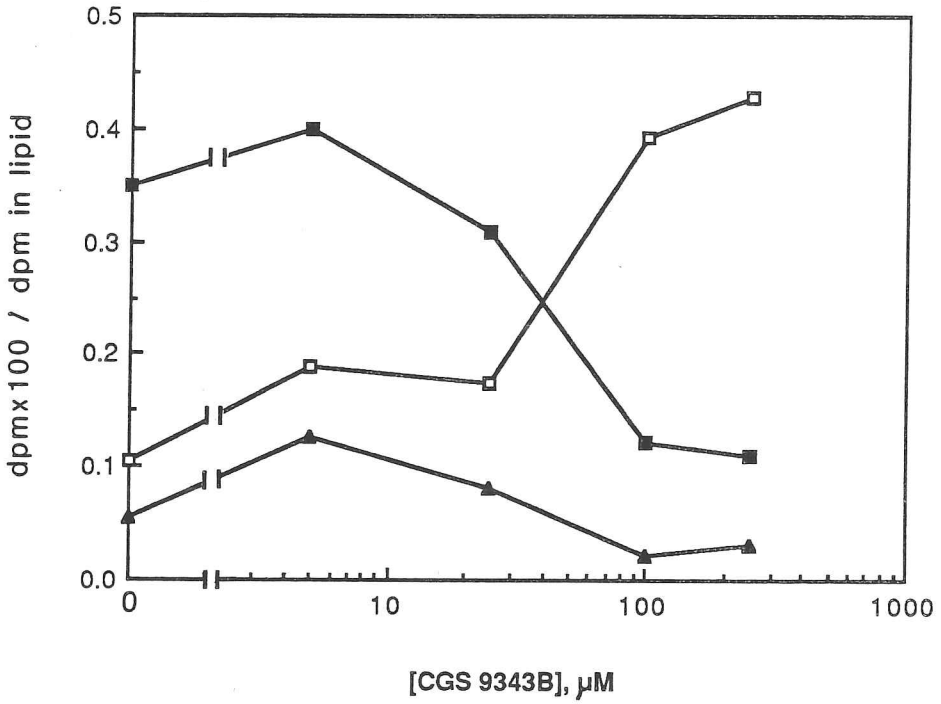


FIGURE 4.8 Effect of CGS9343B on Con A-stimulated inositol phosphate accumulation.

[^{3}H]inositol-labelled thymocytes were challenged with 10µg/ml Con A for 2 mins after 5 min pre-incubation with the indicated CGS9343B concentrations. After reaction termination, inositol phosphates were assayed as described in the legend to Fig. 4.1. Symbols: □, Ins(1,4,5)P_3; ▲, Ins(1,3,4)P_3; ■, $InsP_4$.

To test this possibility, attempts were made to measure the effect of CGS9343B on Con A-induced $[Ca^{2+}]_i$ elevation, but the assay was complicated by the fact that addition of small aliquots of 2mM stock solutions to salts solution resulted in light scattering due to CGS9343B precipitation. Resuspension of CGS9343B occurred gradually, resulting in an apparent $[Ca^{2+}]_i$ increase. Figure 4.9 shows traces illustrating this phenomenon, and also attempted assays of CGS9343B effect on ligand-induced $[Ca^{2+}]_i$ responses. CGS9343B may cause a reduction in stimulated $[Ca^{2+}]_i$, but this was impossible to quantitate. Therefore it was not possible to distinguish between calmodulin-stimulated Ca^{2+} store release and $InsP_3$ kinase activity as the sites for CGS9343B action.

4.2 The effect of anti-Thy 1 and anti-T3 antibodies on inositol phosphates and $[Ca^{2+}]_i$ in BALB/c thymocytes

4.2.1 Inositol phosphate responses

Accurate analysis of inositol polyphosphate interconversions depended on the ability to resolve the various inositol phosphate isomers by HPLC. However, for evaluation of the $PtdInsP_2$-hydrolysing ability of ligands, a less sophisticated, less time-consuming assay was preferable. Therefore inositol phosphate extracts from thymocytes stimulated with anti-Thy 1 and anti-T3 antibodies were assayed using AG1-X8 anion exchange resin, as described by Downes et al. (1986).

Both KT16 and 145-2C11 stimulated inositol phosphate accumulation in BALB/c thymocytes. KT16 stimulated increases in all inositol phosphates (measured 10 mins after ligand addition), although only small increases in $InsP_4$ were observed (Fig. 4.10). The index of stimulation increased over a similar concentration range as KT16-stimulated DNA synthesis (5-50μg/ml; Fig. 3.5a), suggesting a causal link. 145-2C11 induced much larger responses (expressed as a percentage of unstimulated levels), with maximal accumulation occurring at concentrations greater than those required to evoke optimal DNA synthesis (Fig. 4.11). Above 12.5μg/ml 145-2C11 both inositol phosphate responses and DNA synthesis were sub-maximal.

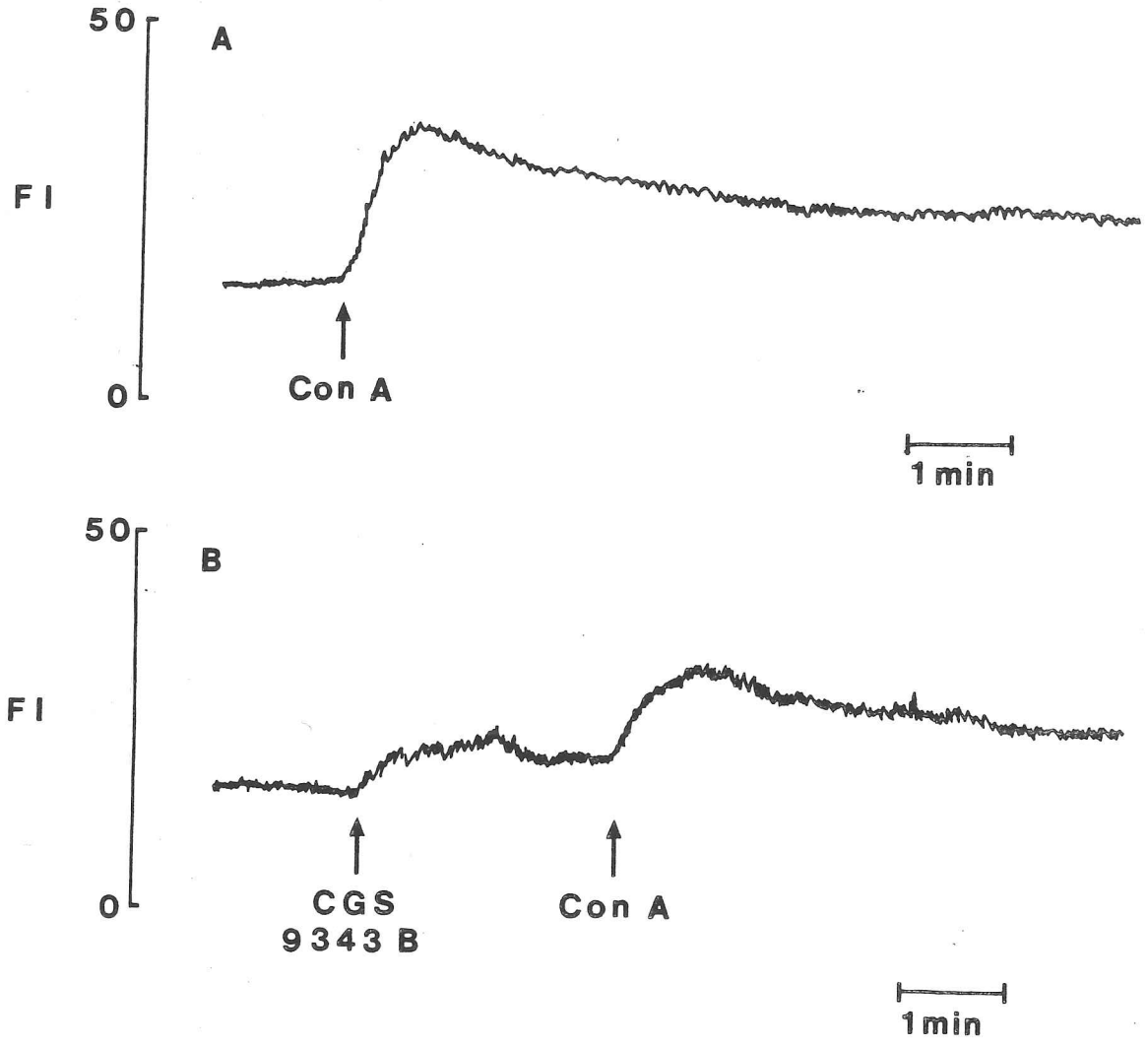


FIGURE 4.9 Effect of CGS9343B on the $[Ca^{2+}]_i$ response to Con A.
Quin 2-loaded thymocytes were challenged with 10μg/ml Con A after 3.5 min pre-incubation with medium (A) or 25μM CGS9343B (B). A continuous recording of fluorescence intensity (arbitrary units) is shown. CGS9343B caused a similar increase in FI when added to medium in the absence of cells, suggesting the increases to be a light-scattering effect due to precipitation of CGS9343B from the medium (not shown).

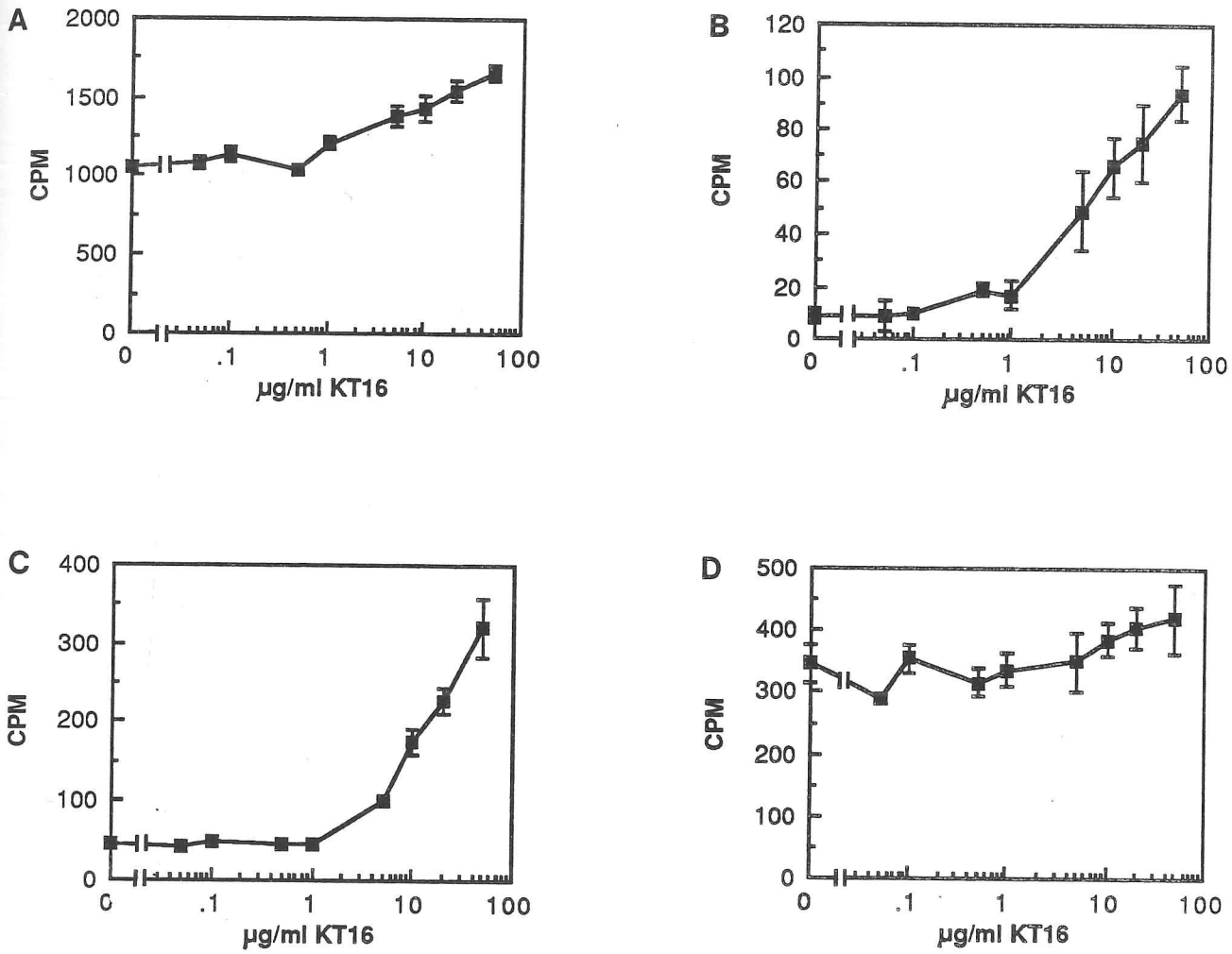


FIGURE 4.10 KT16-stimulated inositol phosphate accumulation in BALB/c thymocytes.

[^{3}H]inositol-labelled thymocytes were incubated with the indicated concentrations of KT16 for 10 mins (Section 2.7.2), reactions terminated and inositol phosphates extracted as described in Section 2.7.3. Inositol phosphates were separated on AG1-X8 resin (Section 2.7.4) and oven-dried eluates counted in 15ml of scintillation fluid (Section 2.8). Each point in this and subsequent thymocyte inositol phosphate determinations is the mean (+/- standard deviation) of triplicate determinations. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

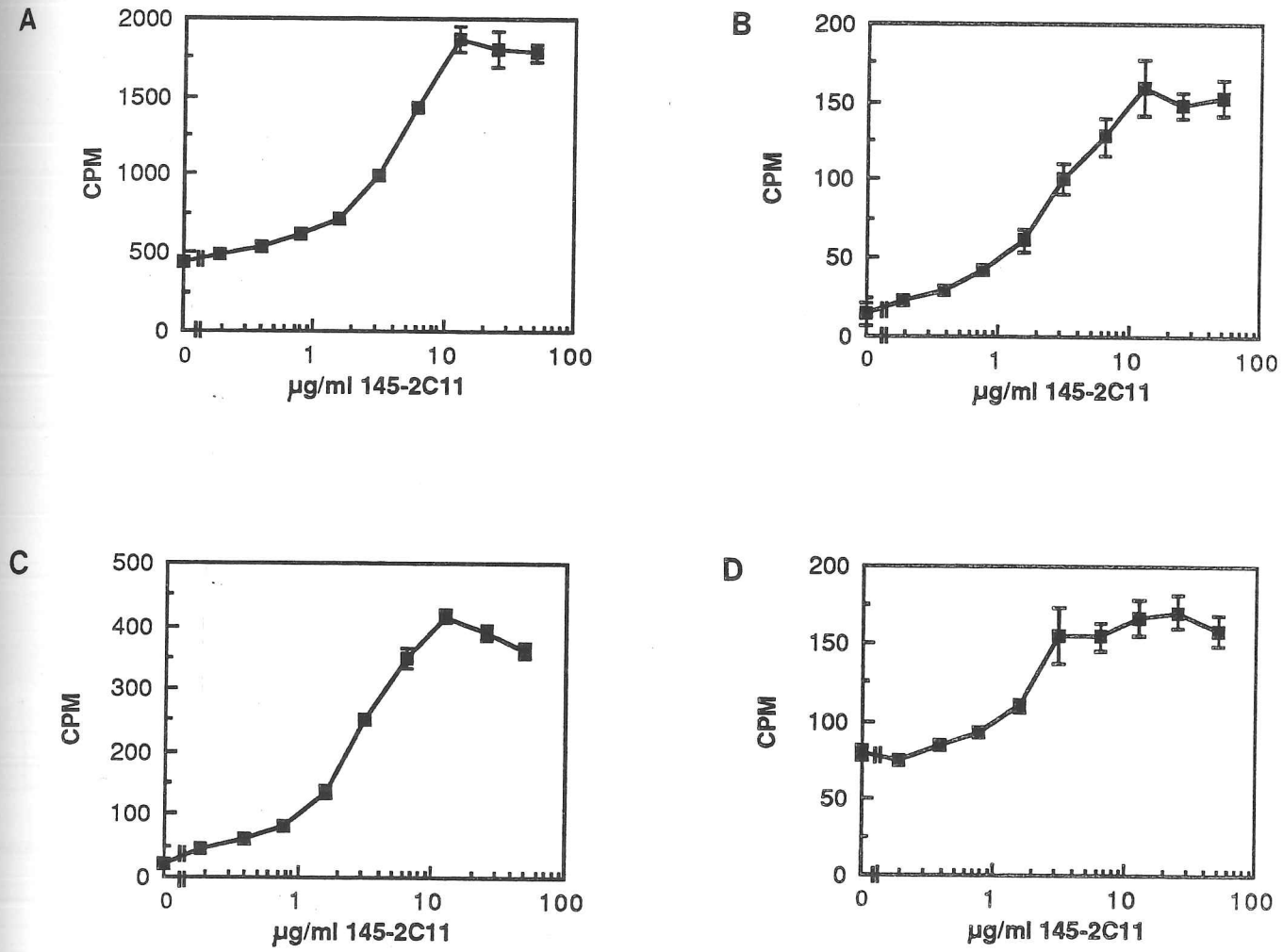


FIGURE 4.11 145-2C11-stimulated inositol phosphate accumulation in BALB/c thymocytes

[^{3}H]inositol-labelled thymocytes were incubated with the indicated 145-2C11 concentrations for 10 mins, then reactions terminated and inositol phosphates assayed as described in the legend to Fig. 4.10. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

The similarities in concentration dependence of ligand-induced inositol phosphate accumulation and DNA synthesis suggest a role for $PtdInsP_2$ hydrolysis in mitogenesis. However the threshold requirement must be quite low as 145-2C11 and KT16 were of similar mitogenic potency in the presence of IL2 or TPA (Section 3.2.2), but KT16 was a far less potent agonist of $PtdInsP_2$ hydrolysis than was 145-2C11 in either the absence (Fig. 4.12) or presence (Fig. 4.13) of IL2 or TPA.

It is of interest that 145-2C11-induced $InsP_3$ and $InsP_4$ responses were slightly inhibited by 10 min pre-treatment with 10nM TPA (Fig. 4.13), as TPA had a similar effect on the Con A response (see Section 4.1.2). These data are consistent with the idea formulated in Chapter 3 that Con A evokes responses via interaction with T3. However, it is clear that Con A has additional effects not mimicked by anti-T3 antibody as Con A, but not 145-2C11, is capable of inducing appreciable thymocyte proliferation in the absence of IL2 and TPA.

Taken together these data suggest that $PtdInsP_2$ hydrolysis may be necessary for activation of DNA synthesis by KT16, Con A and 145-2C11, although the threshold requirement may be quite low. However, since both KT16 and 145-2C11 are poor mitogens in the absence of exogenous IL2, $PtdInsP_2$ hydrolysis may be insufficient to stimulate appreciable IL2 secretion. In turn this suggests that $PtdInsP_2$ hydrolysis is not the primary response capable of inducing all subsequent responses required for the lymphocyte G_0 -> S transition.

4.2.2 $[Ca^{2+}]_i$ responses

The effect of KT16 and 145-2C11 on $[Ca^{2+}]_i$ was examined using ligand concentrations optimal for both DNA synthesis and inositol phosphate accumulation. Both 20μg/ml KT16 and 10μg/ml 145-2C11 induced $[Ca^{2+}]_i$ increases (Fig. 4.14). 145-2C11 induced a response of similar magnitude to that generated by 10μg/ml Con A (1μM cf 700nM), but the response to KT16 was slower than that evoked by 0.8μg/ml Con A and reached a maximum of less than 250nM.

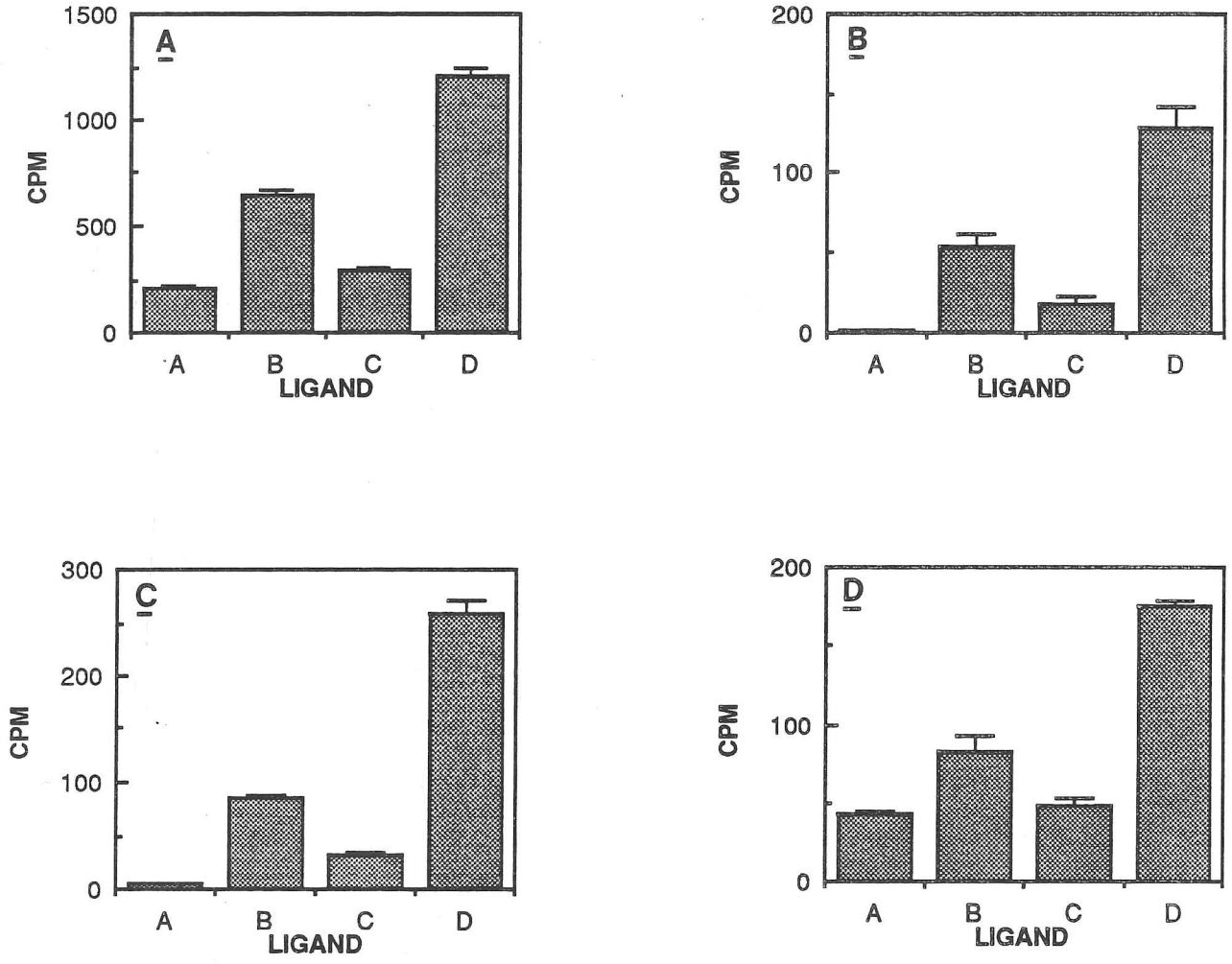


FIGURE 4.12 Comparison of inositol phosphate responses to optimal mitogenic ligand concentrations.

[^{3}H]inositol-labelled thymocytes were incubated with no addition (A), 0.8μg/ml Con A (B), 20μg/ml KT16 (C) or 10μg/ml 145-2C11 (D) for 10 mins, then reaction terminated and inositol phosphates assayed as described in the legend to Fig. 4.10. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

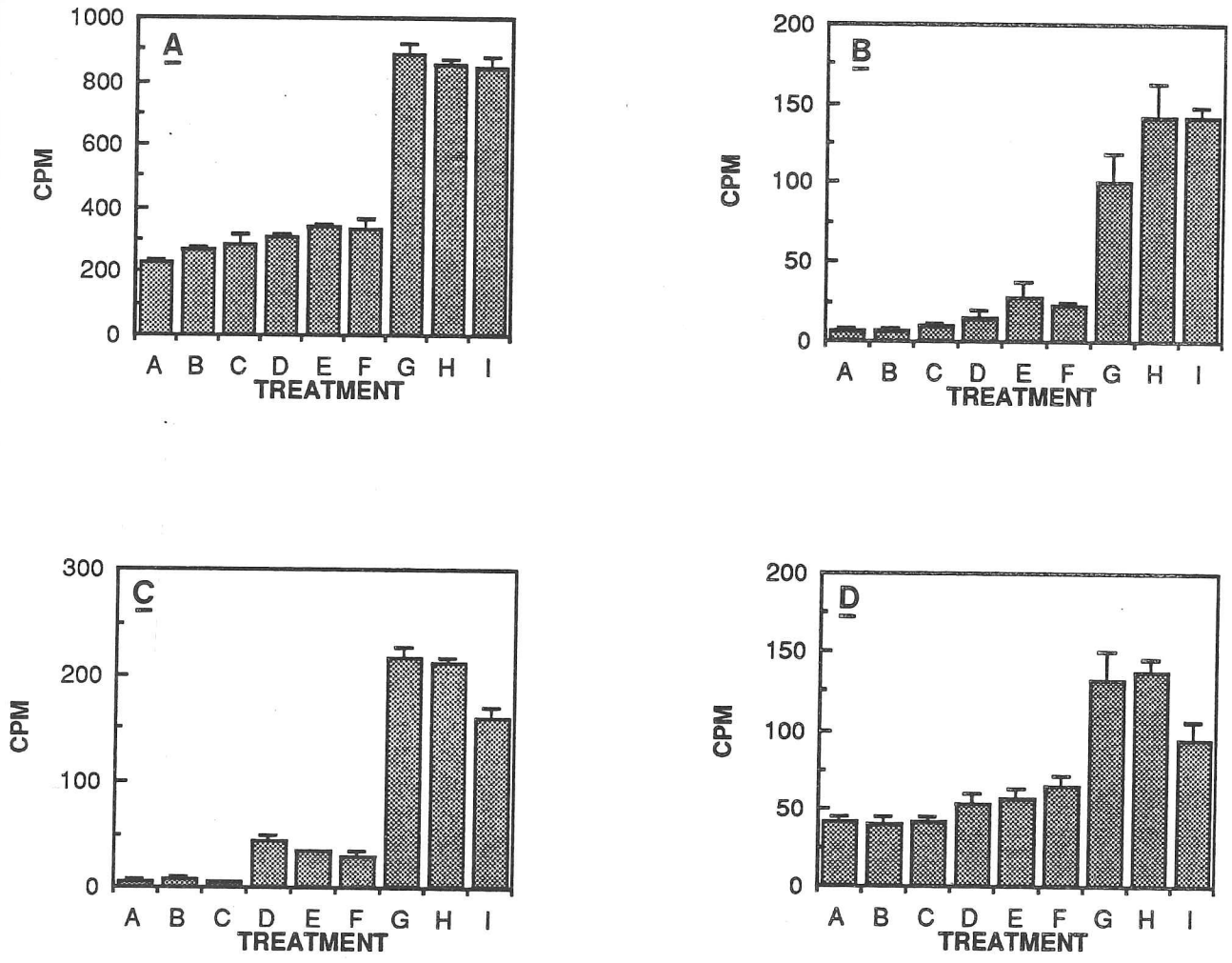


FIGURE 4.13 Effect of IL2 and TPA on ligand-stimulated inositol phosphate accumulation in thymocytes.

[^{3}H]inositol-labelled thymocytes were incubated with the following agents: no addition (A), 20U/ml IL2 (B), 10nM TPA (C), 20µg/ml KT16 (D), KT16 + IL2 (E), KT16 + TPA (F), 10µg/ml 145-2C11 (G), 145-2C11 + IL2 (H), 145-2C11 + TPA (I). Reactions were terminated after 10 mins and inositol phosphates assayed as described in the legend to Fig. 4.10. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

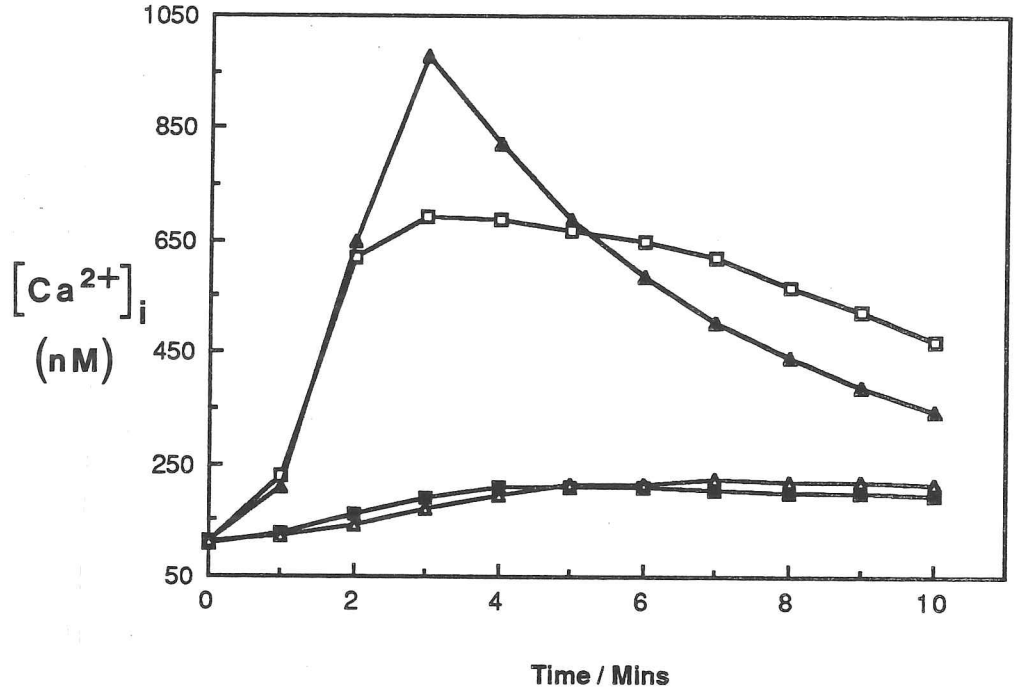


FIGURE 4.14 Comparison of $[Ca^{2+}]_i$ responses to Con A, KT16 and 145-2C11. Quin 2-loaded thymocytes prepared as described in Section 2.6.1 were challenged with 10μg/ml Con A (□), 0.8μg/ml Con A (■), 10μg/ml 145-2C11 (▲) or 20μg/ml KT16 (△). $[Ca^{2+}]_i$ was measured as described in the legend to Fig. 4.3.

The time course of $[Ca^{2+}]_i$ elevation was compared with that of inositol phosphate generation in response to KT16 and 145-2C11. $InsP_3$ was unchanged 1 min after KT16 addition, consistent with the slow initial increase in $[Ca^{2+}]_i$, then increased monotonically between 1 and 20 mins (Fig. 4.15). $InsP_4$ increased rapidly between 1 and 2 mins, presumably due to partial activation of $InsP_3$ kinase by Ins(1,4,5)P_3-released Ca^{2+}, then remained approximately constant. InsP and $InsP_2$ production were delayed relative to $InsP_3$ and $InsP_4$, consistent with their being dephosphorylation products of $InsP_3$ and $InsP_4$, but increased monotonically between 5 and 20 mins.

145-2C11 stimulated much larger, more rapid increases in all inositol phosphates (Fig. 4.16), correlating with the rapid 145-2C11-induced $[Ca^{2+}]_i$ increase. Amounts of InsP, $InsP_2$ and $InsP_3$ increased up to 10 mins. Subsequently $InsP_2$ and $InsP_3$ declined, while InsP increased.

The ability of each ligand to mobilise $[Ca^{2+}]_i$ was in proportion to its ability to evoke inositol phosphate accumulation i.e. 145-2C11 was a potent agonist for both responses, while KT16 was only a weak agonist for either response. Furthermore the time courses of both responses were similar. Thus these data are consistent with a mechanism of $[Ca^{2+}]_i$ elevation involving inositol phosphates. However, the inositol phosphate (if any) responsible for Ca^{2+}_o influx could not be determined from these data. As concluded in relation to the inositol phosphate data, it is clear that only low levels of $[Ca^{2+}]_i$ elevation are required for mitogenesis since KT16 and 145-2C11 are of approx. equal mitogenic potency.

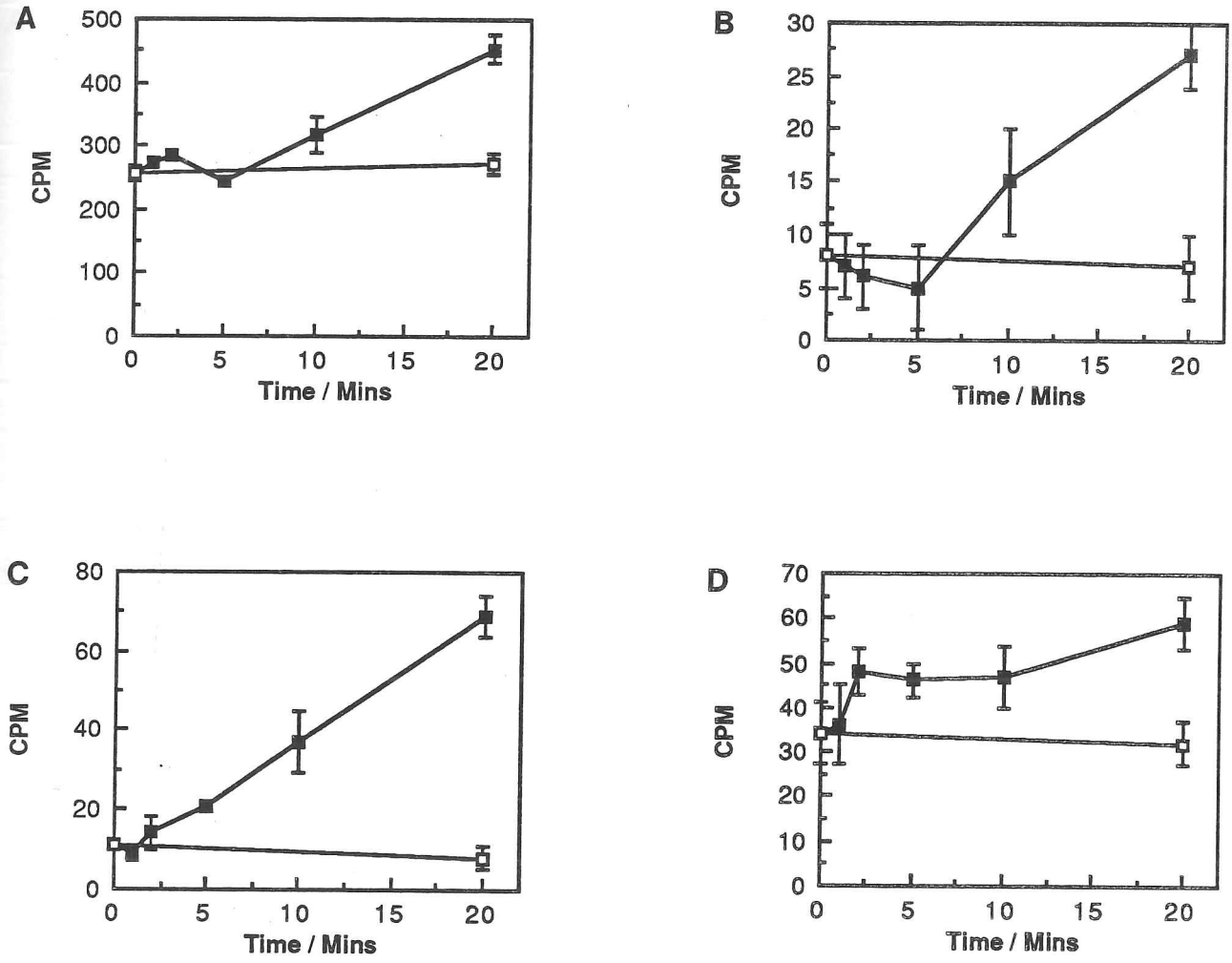


FIGURE 4.15 Time course of KT16-stimulated inositol phosphate accumulation in thymocytes.

[^{3}H]inositol-labelled thymocytes were incubated with medium (□) or 20μg/ml KT16 (■) and reactions terminated at the times indicated. Inositol phosphates were assayed as described in the legend to Fig. 4.10. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

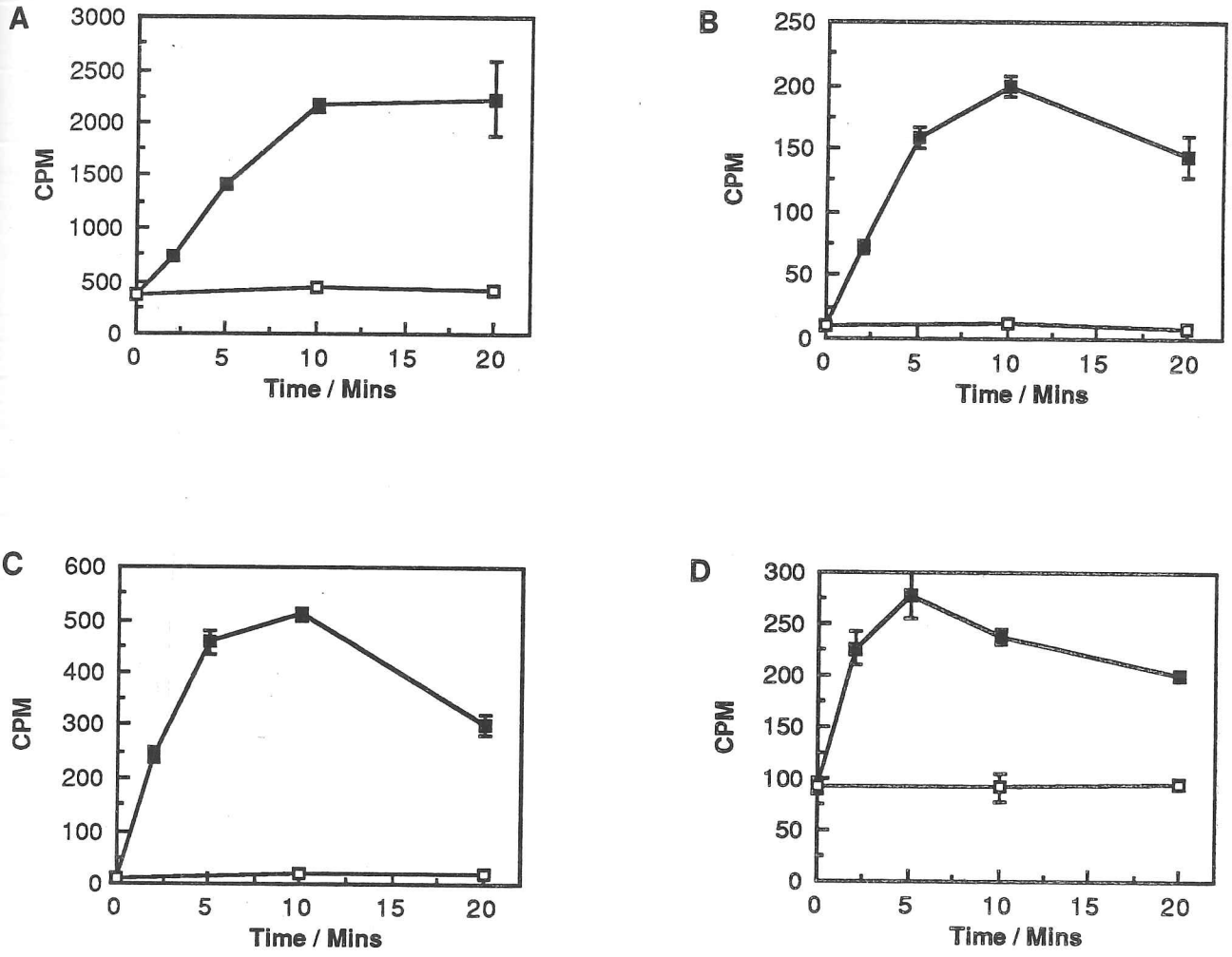


FIGURE 4.16 Time course of 145-2C11-stimulated inositol phosphate accumulation. [^{3}H]inositol-labelled thymocytes were incubated with medium (□) or 10μg/ml 145-2C11 (■) and reactions terminated at the indicated times. Inositol phosphates were assayed as described in the legend to Fig. 4.10. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

4.3 Ligand-induced increases in inositol phosphates and $[Ca^{2+}]_i$ in clone 4

4.3.1 Inositol phosphate responses

It was of interest to determine the effect of IL2 on inositol phosphates in clone 4, as IL2 stimulates clone 4 proliferation. The effect of a range of IL2 concentrations on inositol phosphate accumulation was examined in two experiments. 10 min incubation with 10U/ml or 50U/ml IL2 had no significant effect on any inositol phosphate, but a supramitogenic concentration (500U/ml) slightly increased inositol phosphate amounts. In experiment I, InsP, $InsP_2$ and $InsP_4$ were increased 32%, 24% and 29% respectively; and $InsP_3$ was marginally reduced (8%). Qualitatively similar but even smaller effects of 500U/ml IL2 were seen in experiment II.

	IL2 Concentration (U/ml)			
	0	10	50	500
Experiment I				
InsP	1076+/-67	1272+/-105	1115+/-9	1425+/-91
$InsP_2$	126+/-9	117+/-10	143+/-30	156+/-3
$InsP_3$	171+/-2	179+/-8	202+/-13	157+/-33
$InsP_4$	356+/-8	368+/-7	336+/-2	459+/-37
Experiment II				
InsP	756+/-6	737+/-7	780+/-1	830+/-32
$InsP_2$	135+/-6	155+/-6	129+/-6	156+/-7
$InsP_3$	130+/-6	137+/-9	108+/-4	119+/-3
$InsP_4$	241+/-24	246+/-6	244+/-8	284+/-11

TABLE 4.1 The effect of IL2 on inositol phosphate accumulation
[^{3}H]inositol-labelled clone 4 cells were incubated in salts solution with recombinant IL2 (0, 10, 50 or 500U/ml) for 10 min, after 10 min pre-incubation with 5mM LiCl. Reactions were quenched and inositol phosphates extracted and separated on AG1-X8 as described in Section 2.7. Inositol phosphate fractions were counted for ^{3}H as described in Section 2.8.

These data suggest that at concentrations relevant to mitogenesis, IL2 does not stimulate $PtdInsP_2$ hydrolysis, although supramitogenic concentrations may elicit a small

response. Similar findings have been reported by Kozumbo et al. (1987) and Wickremasinghe et al. (1985). In contrast, Bonvini et al. (1987) demonstrated IL2-induced inositol phosphate accumulation. However as these workers used a high IL2 concentration (500U/ml) and failed to relate this to the concentration required for mitogenic stimulation, IL2-induced phosphoinositide metabolism may be unrelated to its ability to activate DNA synthesis. Therefore, at least in IL2r-expressing cells, $PtdInsP_2$ hydrolysis is not obligatory for mitogenesis.

In contrast, both KT16 and 145-2C11 did stimulate inositol phosphate accumulation in clone 4. KT16 stimulated inositol phosphate accumulation over a similar concentration range in clone 4 as in thymocytes (Fig. 4.17 cf Fig. 4.10). Significant responses were observed at concentrations greater than 3μg/ml, and the magnitude of these increased up to 20μg/ml, but declined at higher concentrations. Because KT16 concentrations required for $PtdInsP_2$ hydrolysis were 100 times greater than those optimal for DNA synthesis, the relationship between this response and KT16-induced mitogenesis is unclear. However, the differing cell density in the two assays (100-fold greater in the inositol phosphate assays) renders direct comparison impossible. Therefore it is not possible to rule out a role for $PtdInsP_2$ hydrolysis in KT16-stimulated proliferation of clone 4, particularly since in the thymocyte system (where both assays are performed at equivalent cell density) the optimal KT16 concentrations required for $PtdInsP_2$ hydrolysis and DNA synthesis are coincident (Section 4.2.1).

Figure 4.18 shows total inositol phosphates accumulated in response to 145-2C11. Individual inositol phosphate fractions are not shown because labelling was too low in this experiment for the $InsP_2$ and $InsP_3$ data to be significant. However it is clear that 145-2C11 stimulated inositol phosphate accumulation with the maximal response evoked by 10μg/ml. The optimum concentration was similar to that for thymocytes (12.5μg/ml; see Fig 4.11).

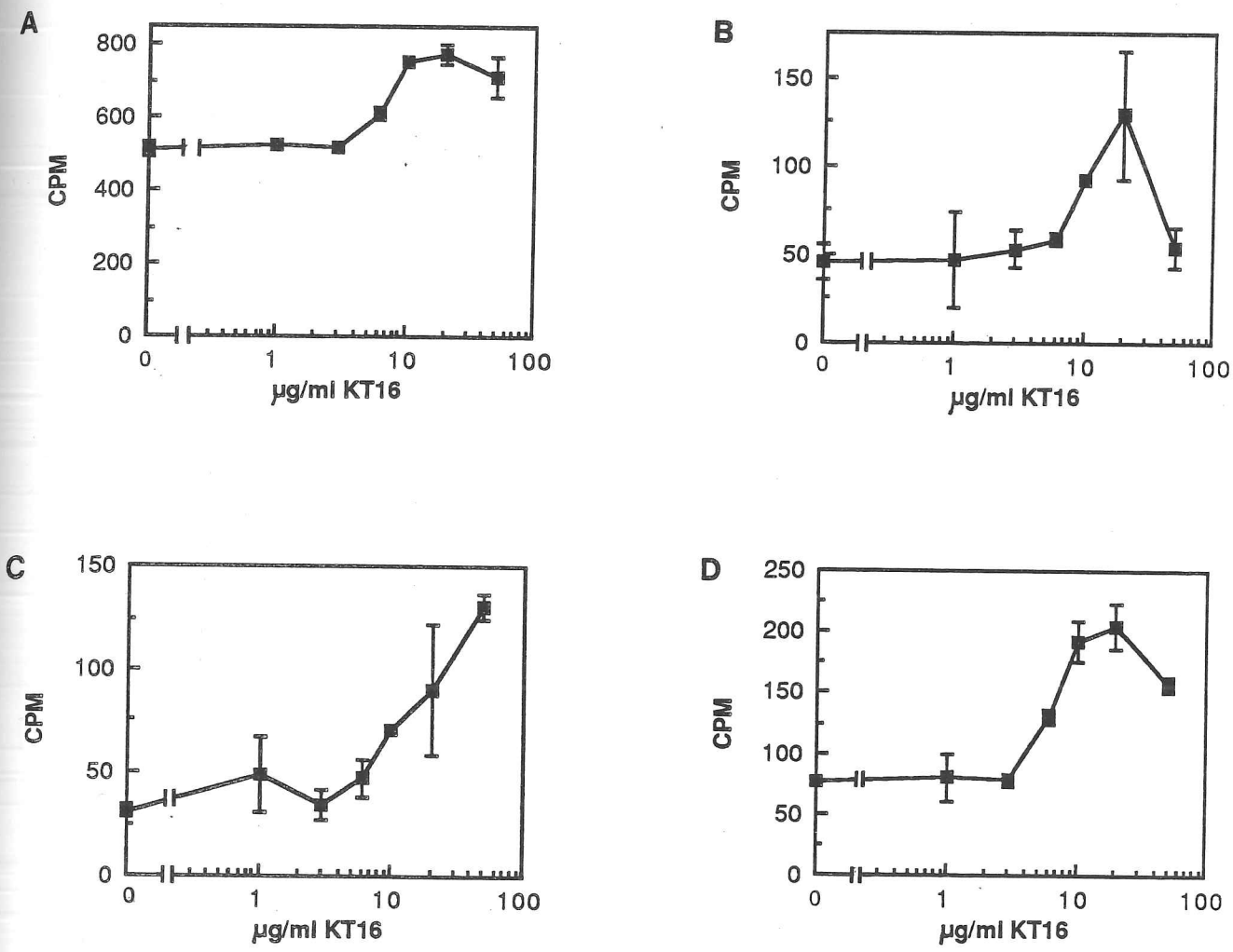


FIGURE 4.17 KT16-stimulated inositol phosphate accumulation in clone 4
[^{3}H]inositol-labelled clone 4 cells (Section 2.7.1) were incubated with the indicated concentrations of KT16 for 10 mins (Section 2.7.2) then reactions terminated and inositol phosphates assayed as described in the legend to Fig. 4.10. Each point in this and subsequent clone 4 inositol phosphate determinations is the mean (+/- standard deviation) of duplicate determinations unless otherwise stated. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

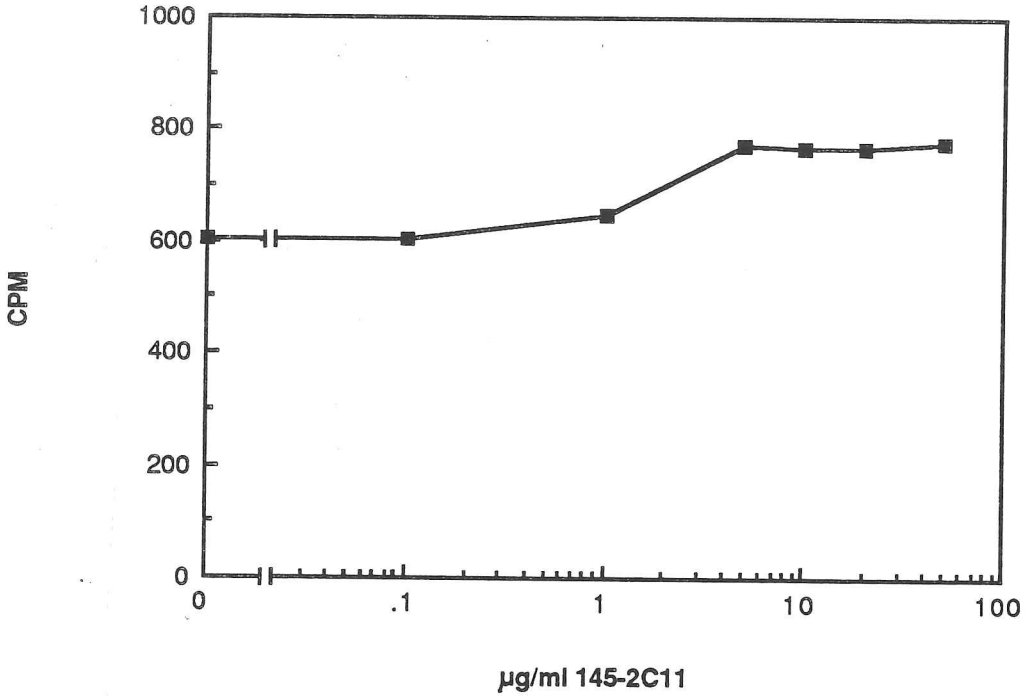


FIGURE 4.18 145-2C11-stimulated inositol phosphate accumulation in clone 4. [^{3}H]inositol-labelled clone 4 cells were incubated with the indicated concentrations of 145-2C11 for 10 mins, reactions terminated and inositol phosphates assayed as described in the legend to Fig. 4.10. Each point is the mean of duplicate determinations of total inositol phosphates.

The time courses of inositol phosphate accumulation in response to 145-2C11 and KT16 were also determined. KT16 stimulated greater $InsP_4$ accumulation (relative to unstimulated amounts) in clone 4 than in thymocytes, but correspondingly less $InsP_3$, suggesting that in thymocytes either $Ins(1,4,5)P_3$ phosphorylation or dephosphorylation was more rapid. In clone 4, little $InsP_3$ and $InsP_4$ accumulation occurred within 2 minutes of addition of 20μg/ml KT16 (Fig. 4.19), although sufficient InsP accumulated to suggest that it might have been generated directly from PtdIns, or that the rate of $InsP_3$ dephosphorylation exceeded the rate of $PtdInsP_2$ hydrolysis. The rapid increase in amounts of $InsP_3$ and $InsP_4$ between 2 and 5 mins was succeeded by a small $InsP_3$ decline between 5 and 10 mins. The amount of $InsP_4$ remained approx. constant between 5 and 10 mins. InsP and $InsP_2$ increased monotonically between 2 and 10 mins.

The patterns of inositol phosphate accumulation in response to 10μg/ml 145-2C11 were fairly similar in clone 4 and thymocytes (Fig. 4.20 cf Fig. 4.16), but initial increases in inositol phosphate accumulation were slower in clone 4. Between 2 and 10 mins inositol phosphate accumulation in clone 4 was approximately monotonic.

Con A (10μg/ml) evoked a virtually identical pattern of inositol phosphate accumulation to 145-2C11 (Fig. 4.21). Slow initial rates of inositol phosphate increase were followed by monotonic accumulation up to 10 minutes. However the stimulation index was significantly greater with Con A than with 145-2C11, which was itself a more potent agonist of $PtdInsP_2$ hydrolysis than KT16. However this could not be related to their efficacy as mitogens, nor could the concentration dependence profiles of DNA synthesis and inositol phosphate responses be compared since neither Con A nor 145-2C11 was mitogenic for clone 4. These data are consistent with two earlier suggestions.
i $PtdInsP_2$ hydrolysis may be necessary but is not sufficient to activate DNA synthesis.
ii The threshold amount of $PtdInsP_2$ hydrolysis required for DNA synthesis activation is quite small in comparison with the maximal amounts of $PtdInsP_2$ hydrolysis evoked by Con A.

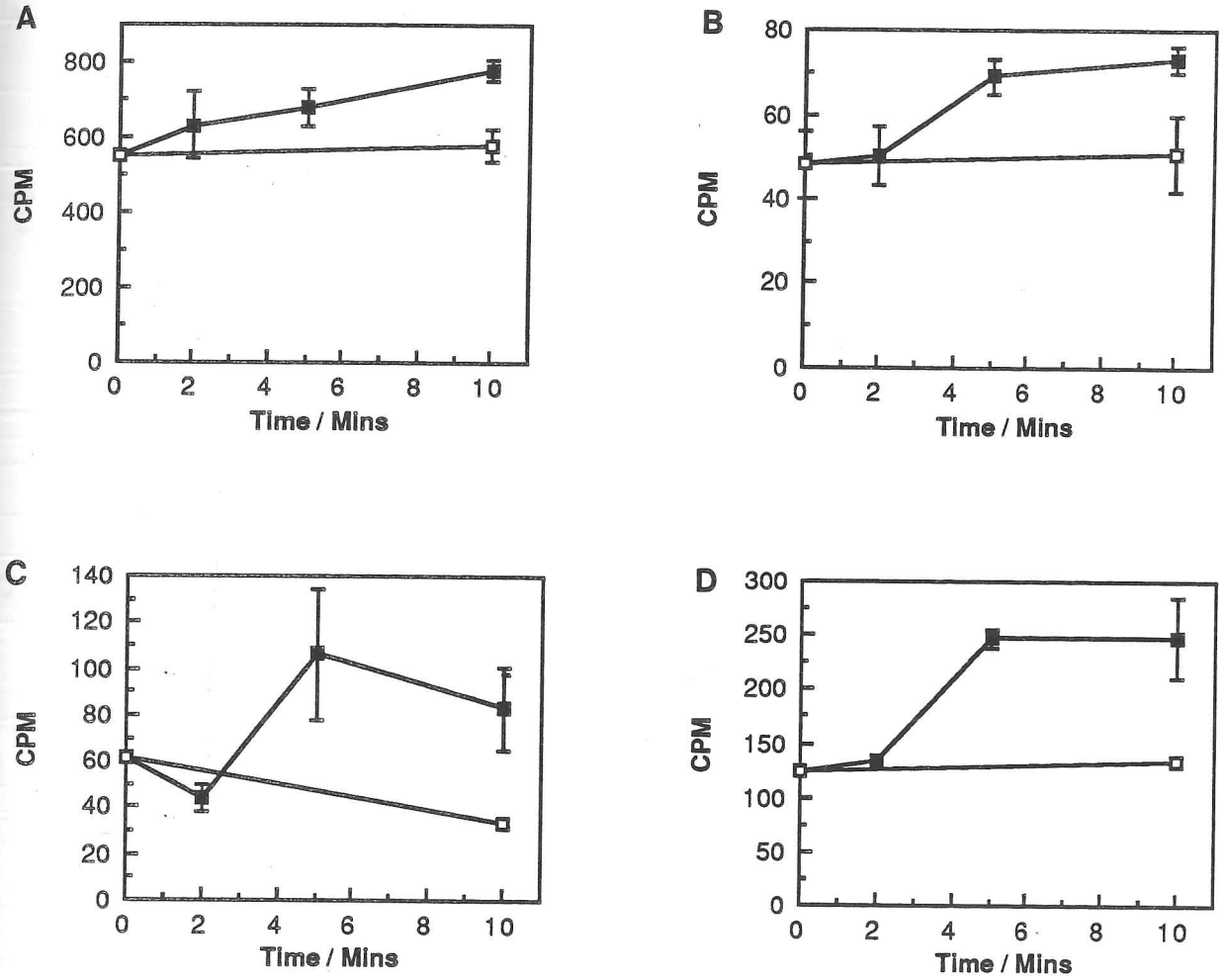


FIGURE 4.19 Time course of KT16-stimulated inositol phosphate accumulation in clone 4.

[^{3}H]inositol-labelled clone 4 cells were incubated with medium (□) or 20μg/ml KT16 (■) and reactions terminated at the indicated times. Inositol phosphates were assayed as described in the legend to Fig. 4.10. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

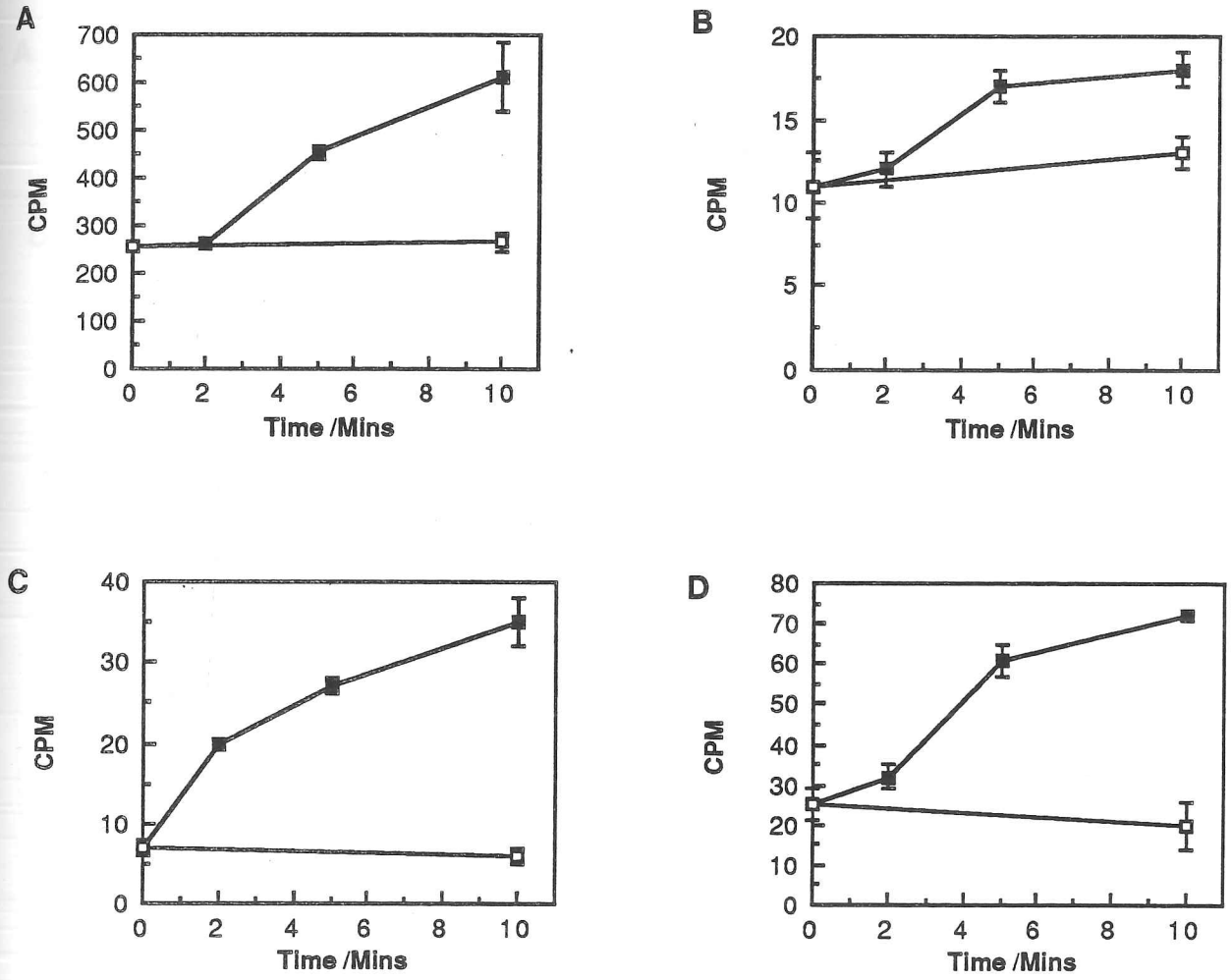


FIGURE 4.20 Time course of 145-2C11-stimulated inositol phosphate accumulation in clone 4.

[^{3}H]inositol-labelled clone 4 cells were incubated with medium (□) or 10μg/ml 145-2C11 (■) and reactions terminated at the times indicated. Inositol phosphates were assayed as described in the legend to Fig. 4.10. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

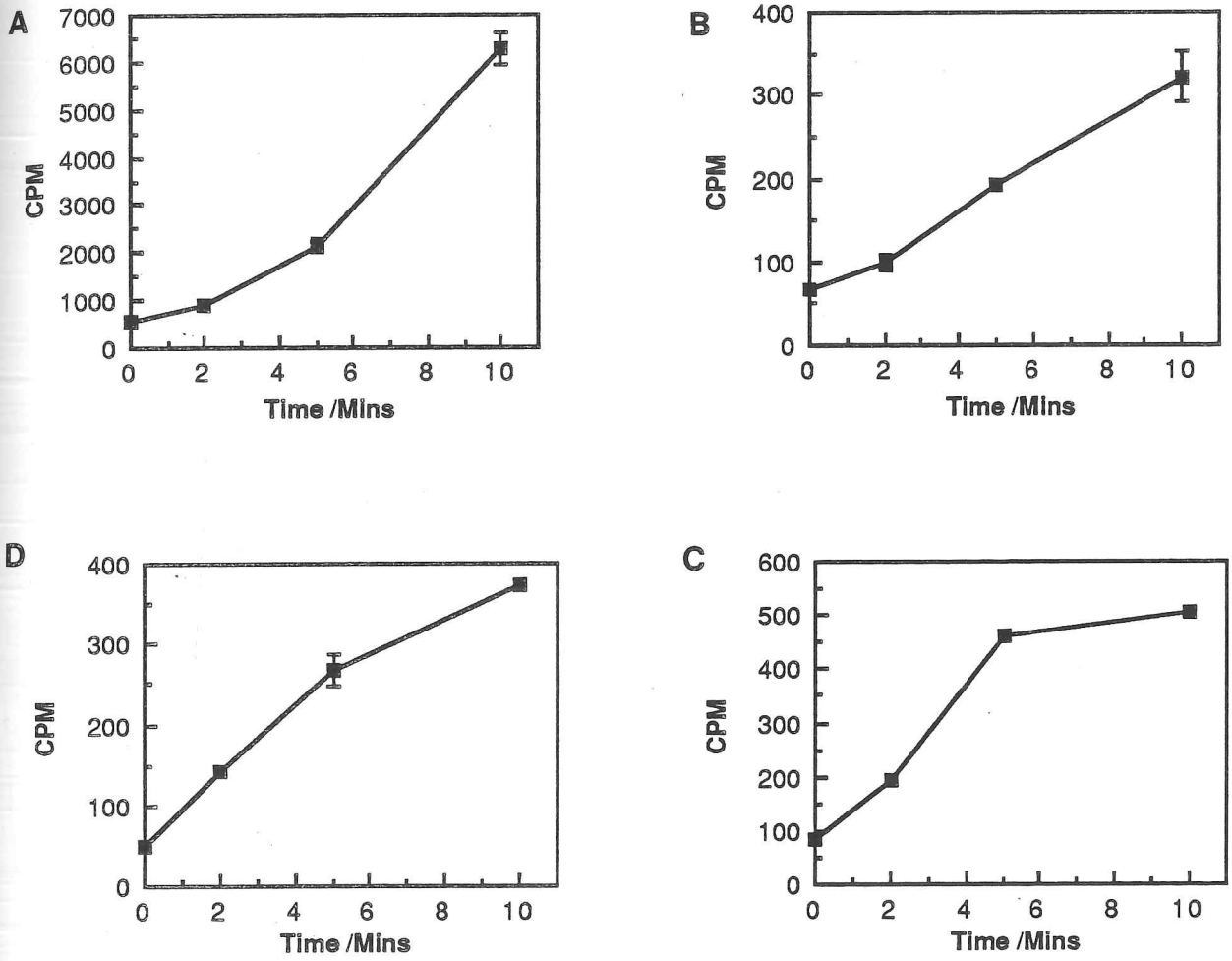


FIGURE 4.21 Time course of Con A-stimulated inositol phosphate accumulation in clone 4

[^{3}H]inositol-labelled clone 4 cells were incubated with 10μg/ml Con A and reactions terminated at the times indicated. Inositol phosphates were assayed as described in the legend to Fig. 4.10. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

4.3.2 $[Ca^{2+}]_i$ responses

The ability of these ligands to elevate $[Ca^{2+}]_i$ in clone 4 was also determined. $[Ca^{2+}]_i$ was unperturbed from its resting value of 110nM by 50U/ml or 500U/ml IL2 (data not shown). The inability of IL2 to elicit a $[Ca^{2+}]_i$ response is consistent with its failure to increase $InsP_3$ accumulation.

In contrast, KT16 and Con A increased $[Ca^{2+}]_i$ to 250nM and 450 nM respectively within 3-4 mins of ligand addition (Fig. 4.22), consistent with their ability to cause $PtdInsP_2$ hydrolysis. There was a 45-90s delay after ligand addition before onset of $[Ca^{2+}]_i$ elevation, consistent with the slow initial increases in inositol phosphate, then a rapid rise succeeded by a gradual decline. At 10 mins after Con A or KT16 addition, $[Ca^{2+}]_i$ was 333^+/-40nM or 122^+/-10nM respectively. The persistent elevation with Con A might be related to its greater ability compared with KT16 to induce $PtdInsP_2$ hydrolysis. In this experiment Con A caused significantly greater maximal $[Ca^{2+}]_i$ than KT16. However in some experiments, maximal $[Ca^{2+}]_i$ stimulated by Con A and KT16 were similar.

Both Con A and KT16 caused transient $[Ca^{2+}]_i$ increases in the presence of 1mM extracellular EGTA demonstrating that both could cause Ca^{2+}-release from intracellular stores, consistent with the ability of both to elevate $InsP_3$.

An attempt was also made to determine the effect of 145-2C11 on $[Ca^{2+}]_i$ in clone 4. However, inconsistent with its effect on inositol phosphate accumulation in clone 4, and on thymocyte $[Ca^{2+}]_i$, no effect of 145-2C11 on clone 4 $[Ca^{2+}]_i$ could be detected. It appeared possible that this difference could be an artefact due to the fact that clone 4 was routinely cultured with irradiated CBA splenocytes and IL2, but for $[Ca^{2+}]_i$ determinations cells were stimulated with IL2 alone to avoid quin 2-AME partitioning into dead CBA cell membranes. Therefore the ability of ligands to evoke inositol phosphate accumulation was compared in cells grown in the presence or absence of CBA cells (Table 4.2).

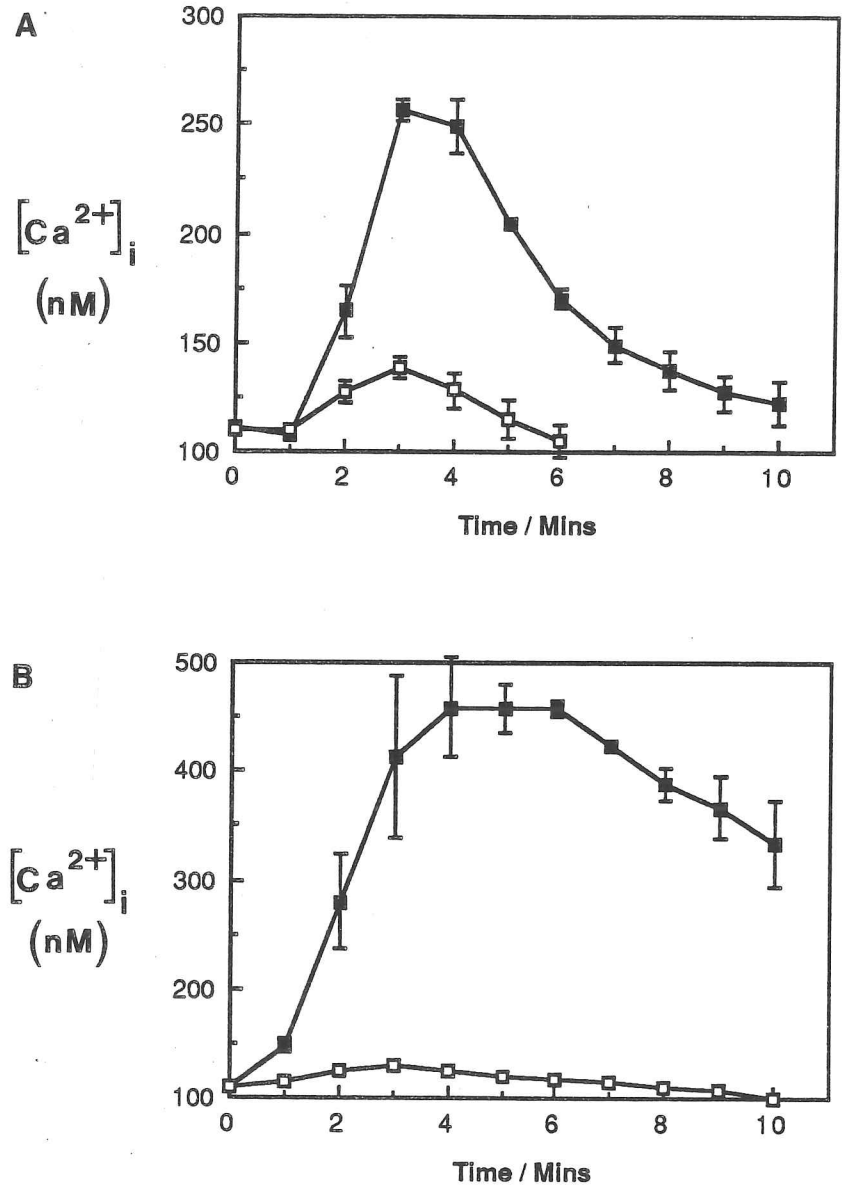


FIGURE 4.22 $[Ca^{2+}]_i$ responses to Con A and KT16 in clone 4.
Quin 2-loaded clone 4 cells (Section 2.6.3) were challenged with 10μg/ml Con A (A) or 20μg/ml KT16 (B) in medium containing 0.43mM free Ca^{2+} (■) or 0.1μM free Ca^{2+} (□). $[Ca^{2+}]_i$ was measured as described in the legend to Fig. 4.3. Each point is the mean (+/- standard deviation) of duplicate determinations.

Inositol phosphate responses to KT16 were similar in cells grown with IL2 plus or minus CBA splenocytes. But despite the ability of 145-2C11 to evoke substantial inositol phosphate accumulation in cells stimulated with CBA splenocytes, the 145-2C11 response of IL2-grown cells was virtually non-existent, suggesting that T3 may be down-regulated or inactivated in cells cultured solely with IL2. In contrast, the response to Con A was similar to that observed in CBA-grown cells (data not shown), suggesting that Con A elicits its responses at least partially by interaction with receptors other than T3.

		Stimulation index of inositol phosphate fractions			
Ligand	Growth stimulus	InsP	$InsP_2$	$InsP_3$	$InsP_4$
KT16	IL2 + CBA cells	1.65	2.13	1.48	1.78
KT16	IL2 alone	1.63	2.38	1.70	2.11
145-2C11	IL2 + CBA cells	3.58	3.06	2.95	2.91
145-2C11	IL2 alone	1.25	1.50	0.90	1.47

TABLE 4.2 Effect of growth stimulus on inositol phosphate responses to KT16 and 145-2C11
Clone 4 cells cultured by stimulation with IL2 +/- CBA splenocytes were labelled with [^{3}H]inositol, washed and pre-incubated as described in Section 2.7.1. Cells were challenged with anti-Thy 1 (20μg/ml) or 145-2C11 (10μg/ml) for 10 mins, then reactions quenched, and samples processed as described in Section 2.7.3. Inositol phosphate fractions were separated, dried and counted for ^{3}H (Section 2.8). The stimulation index is the ratio; cpm in stimulated cells / cpm in unstimulated cells.

Since Thy 1 responses are unaltered in IL2-grown cells, these data are inconsistent with reports that T3 is necessary for Thy 1 functioning. Alcover et al. (1988a) have suggested that in the case of CD2 (another receptor apparently dependent on T3-T_i expression for its activity; Section 1.3.4) only a very low level of T3-T_i expression (<1,000 molecules/cell) is required for responsiveness to CD2-directed ligands. However, these authors fail to report whether these cells retain responsiveness to T3-directed ligands. Therefore it is not possible to draw comparisons between their data and the results described here. However it seems unlikely that the T3-T_i complex would be

able to transduce signals in response to Thy 1 ligands but not anti-T3 antibodies. In contrast the demonstration by Kroczek et al. (1986a) that Thy 1 can elevate $[Ca^{2+}]_i$ in Thy1-transfected B cells suggests that T3-T_i expression is not obligatory for Thy 1-mediated $[Ca^{2+}]_i$ increases, and thus concurs with the data presented here. However it should not be inferred from the ability of KT16 to induce inositol phosphate accumulation in the absence of T3-responsiveness that Thy 1 can also stimulate proliferation of T3-unresponsive cells. Indeed it has proved impossible to demonstrate proliferation in response to KT16 in IL2-grown cells.

4.4 Ligand-induced increases in c-myc mRNA in T lymphocytes

The amount of c-myc mRNA in BALB/c thymocytes and clone 4 was assayed by Northern blotting fractionated RNA extracted from cells as described in Section 2.10. These experiments were performed in collaboration with Garry Menzel and are included with his permission.

4.4.1 Ligand-induced increases in c-myc mRNA in BALB/c thymocytes

Quiescent BALB/c thymocytes contained a small amount of c-myc mRNA, which was unaltered by IL2, consistent with the virtual absence of IL2r from the resting thymocyte surface. However, increases in c-myc mRNA were stimulated by Con A, 145-2C11 and KT16. Con A-stimulated increases in c-myc mRNA were detectable within 1h of Con A addition, and were maximal after 4-8h (Fig 4.23). The elevation of c-myc mRNA was sustained for at least a further 4h .

The effect of 145-2C11 on c-myc mRNA is shown in Figure 4.24. Maximal increases were observed 1h after 145-2C11 addition and this increase was sustained up to 2h (the latest time point assayed). In contrast, c-myc mRNA accumulation was far slower in response to KT16. C-myc mRNA was assayed 0.5, 1 and 2h after KT16 addition, but only at 2h was any increase detected (Fig. 4.25). The relative rates of c-myc mRNA accumulation in response to KT16 and 145-2C11 were in the same order as the relative rates of $[Ca^{2+}]_i$ and inositol phosphate accumulation stimulated by these ligands.

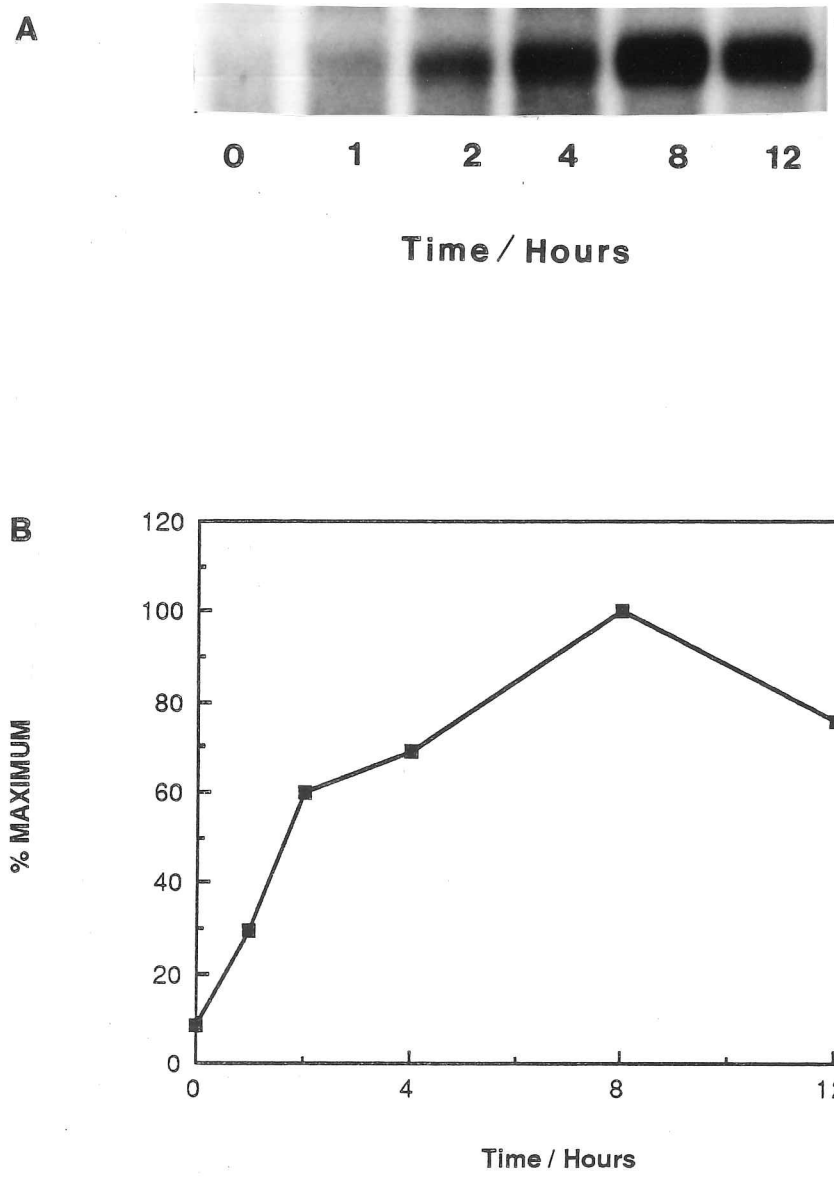


FIGURE 4.23 Time course of Con A-stimulated c-myc mRNA accumulation in thymocytes

Thymocytes were incubated with 0.8μg/ml Con A for the indicated times, then reactions were terminated, and RNA was isolated, fractionated, transferred to nitrocellulose membrane, and probed with ^{32}P-labelled c-myc cDNA as described in Section 2.10. ^{32}P-labelled nitrocellulose membranes were autoradiographed and the intensities of the major band determined by scanning densitometry.
A. Autoradiograph. B. Results from scanning densitometry (expressed as a percentage of the maximum response observed in this and all subsequent experiments). Similar results were obtained in several experiments.

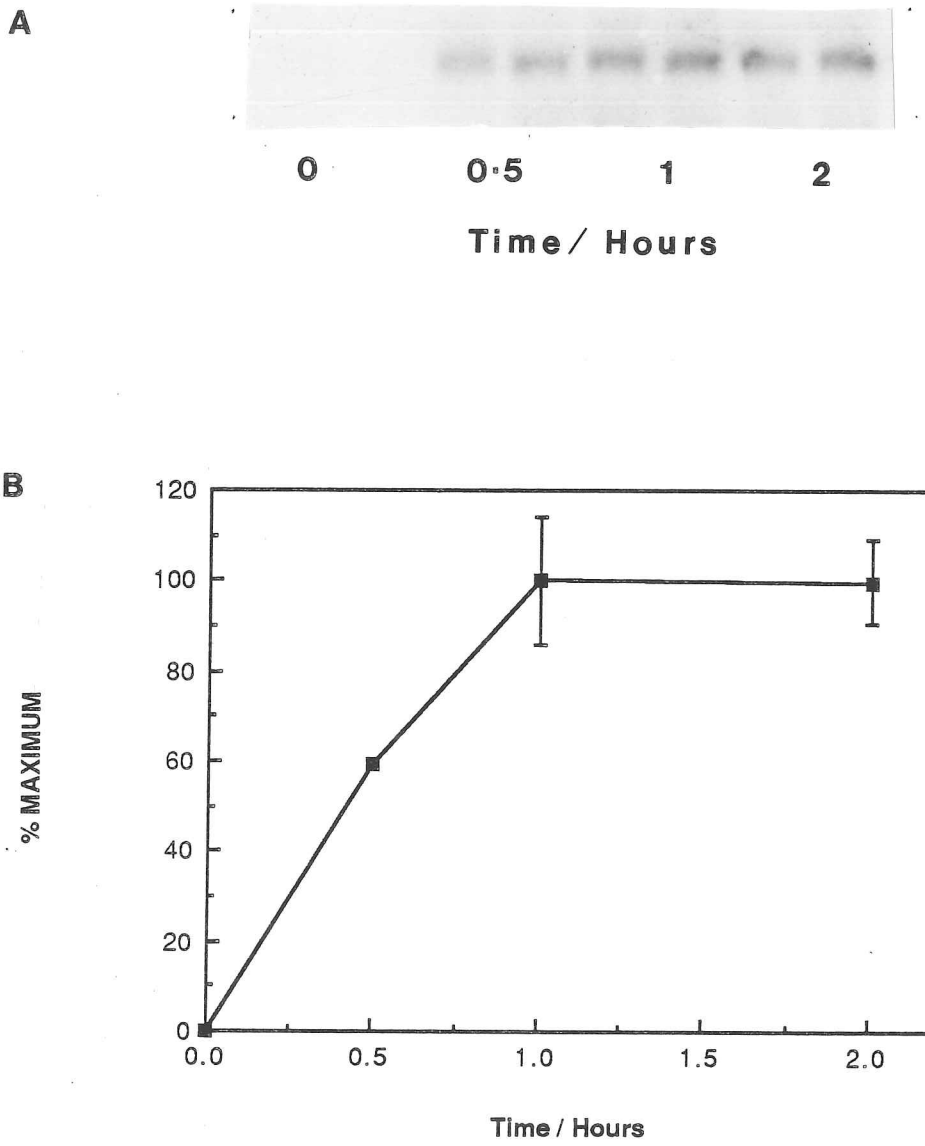
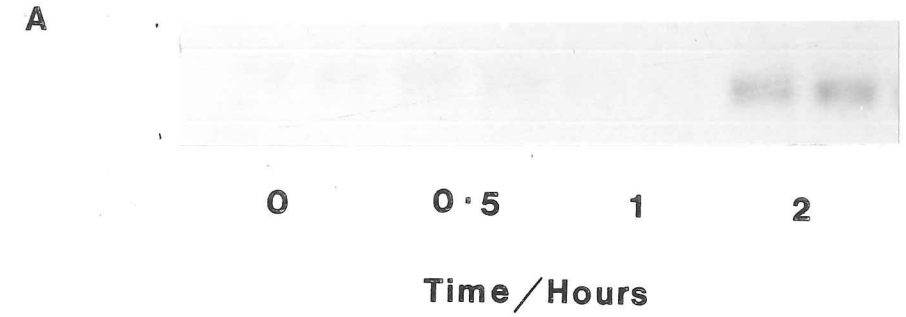


FIGURE 4.24 Time course of 145-2C11-stimulated c-myc mRNA accumulation in thymocytes.

Thymocytes were incubated with 10μg/ml 145-2C11 for the times indicated, then c-myc mRNA assayed as described in the legend to Fig. 4.23.

A. Autoradiograph. B. Results from scanning densitometry. Each point is the mean (+/- standard deviation) of duplicate determinations in this and subsequent c-myc experiments.



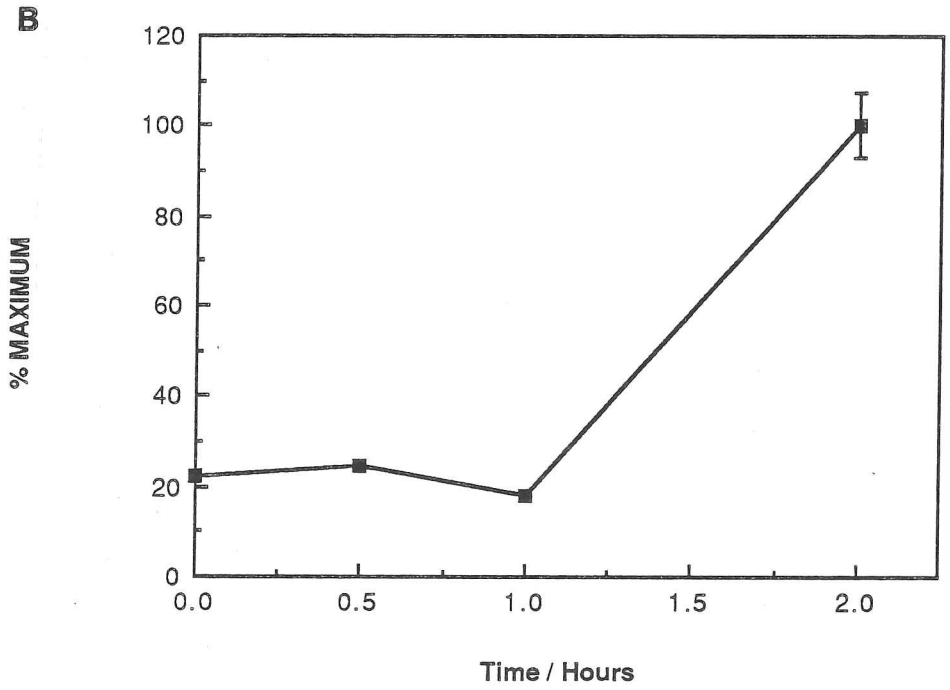


FIGURE 4.25 Time course of KT16-stimulated c-myc mRNA accumulation in thymocytes.

Thymocytes were incubated with 20µg/ml KT16 for the indicated times, then c-myc mRNA assayed as described in the legend to Fig. 4.23.
A. Autoradiograph. B. Results from scanning densitometry.

4.4.2 Ligand-induced increases in c-myc mRNA in clone 4

Quiescent clone 4, like resting thymocytes, contained a low level of c-myc mRNA. But clone 4 c-myc mRNA levels increased in a concentration-dependent manner in response to IL2. 50U/ml and 100U/ml IL2 stimulated 64% and 94% respectively of the c-myc mRNA accumulation stimulated by 500U/ml IL2 after 1h (Fig. 4.26a&b). The time course of c-myc mRNA accumulation in response to 50U/ml IL2 was also measured (Fig. 4.26c&d). An increase was first detectable at 30 mins, and accumulation was maximal at 1h. By 2h the amount of accumulated c-myc mRNA had declined to 59% of the maximum observed.

Time course data in response to 145-2C11 and KT16 are shown in Figs. 4.27 and 4.28. The times taken to achieve maximal response were in the order; 145-2C11 < IL2 < KT16. The 145-2C11-stimulated increase in c-myc mRNA was maximal at 30 mins, and declined to 40% of maximum by 2h (Fig. 4.27). In contrast, KT16-stimulated c-myc was maximal at the latest time point measured (4h; Fig. 4.28). The relative rates of responses to KT16 and 145-2C11 were thus similar for Ca^{2+}, inositol phosphate and c-myc mRNA responses. Interestingly, although ligand-stimulated $[Ca^{2+}]_i$ and inositol phosphate responses were slower in clone 4 than in thymocytes, the time taken to achieve maximal c-myc mRNA accumulation was less in clone 4 than in thymocytes. However as these data do not permit quantitation of absolute amounts of c-myc mRNA, it is not possible to compare initial rates of c-myc mRNA accumulation. Therefore the earlier maxima in clone 4 compared with thymocytes may simply represent greater RNAse activity in clone 4.

The ability of both 145-2C11 and KT16 to cause c-myc mRNA accumulation was consistent with their ability to mobilise $[Ca^{2+}]_i$ since c-myc mRNA accumulation can be stimulated by increased $[Ca^{2+}]_i$ both in BALB/c thymocytes and 3T3 fibroblasts (Moore et al., 1986; Yamashita et al, 1986; Kaibuchi et al., 1986). However, the mechanism by which IL2 elevates c-myc mRNA is not clear, despite previous reports of c-myc responses to IL2 (Farrar et al., 1987; Depper et al., 1985; Reed et al., 1985), since IL2 does not stimulate $[Ca^{2+}]_i$ elevation.

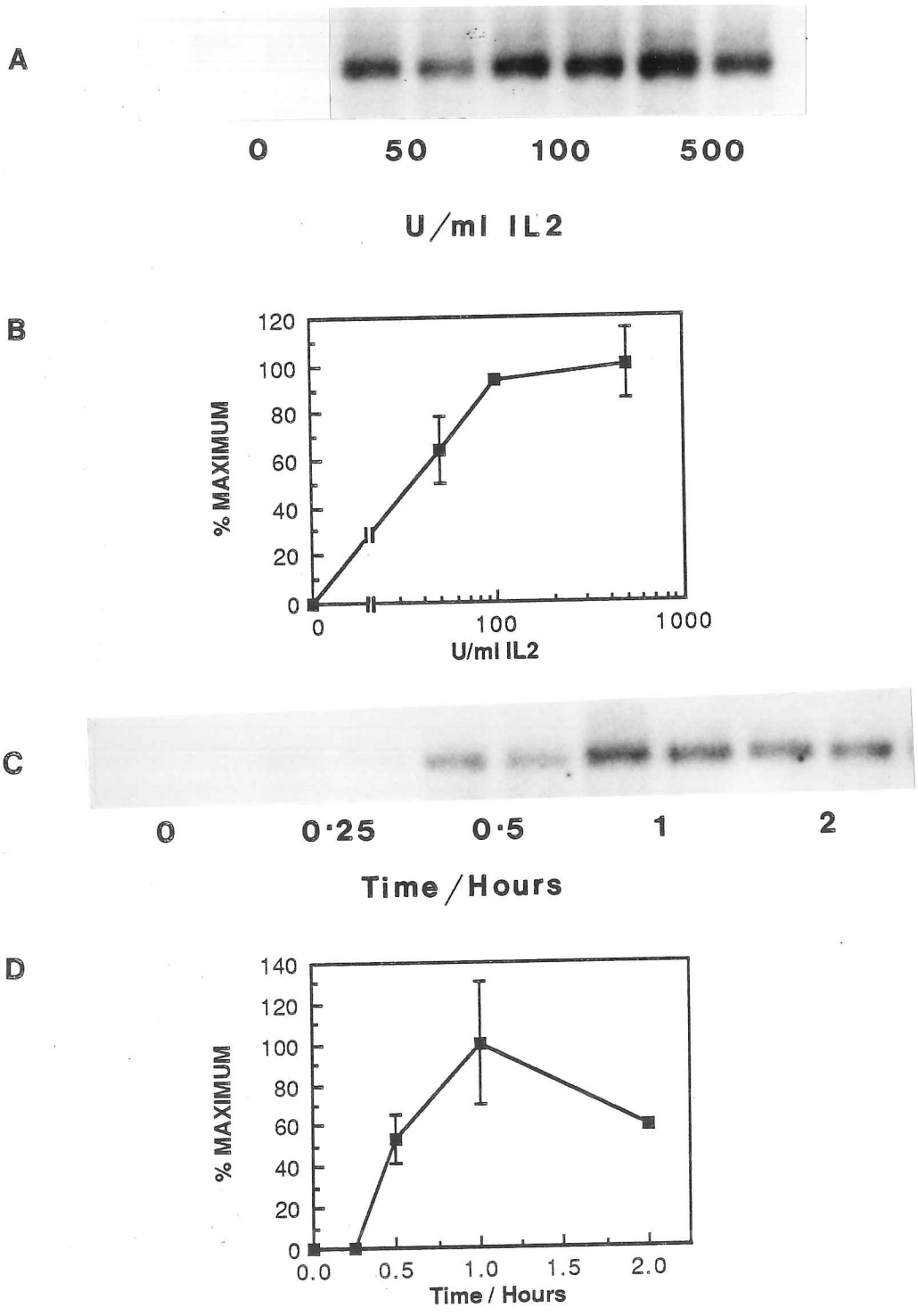


FIGURE 4.26 Effect of IL2 on c-myc mRNA accumulation in clone 4
Clone 4 cells were stimulated for 1h with the IL2 concentrations indicated (A and B) or with 50U/ml IL2 for the indicated times (C and D) then c-myc mRNA assayed as described in the legend to Fig. 4.23. A and C show the autoradiographs and B and D show the scanning densitometry data.

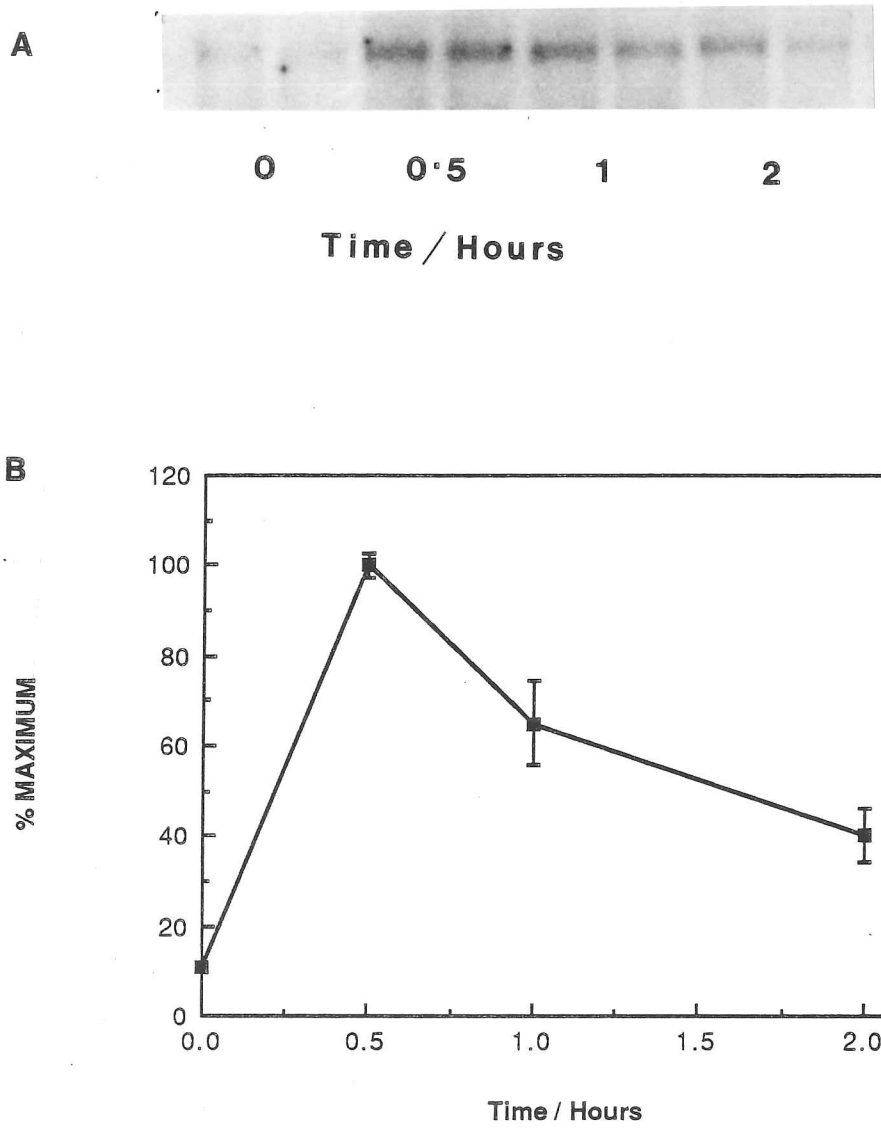
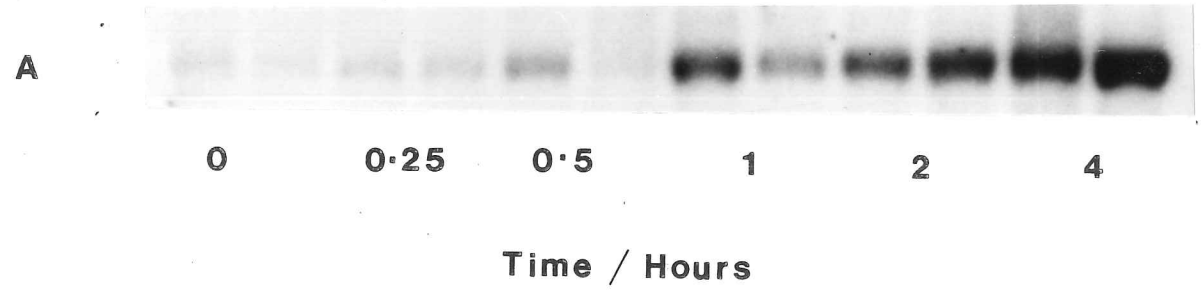


FIGURE 4.27 Time course of 145-2C11-stimulated c-myc mRNA accumulation in clone 4.

Clone 4 cells were incubated with 10μg/ml 145-2C11 for the times indicated, then c-myc mRNA assayed as described in the legend to Fig. 4.23.
A. Autoradiograph. B. Results from scanning densitometry.



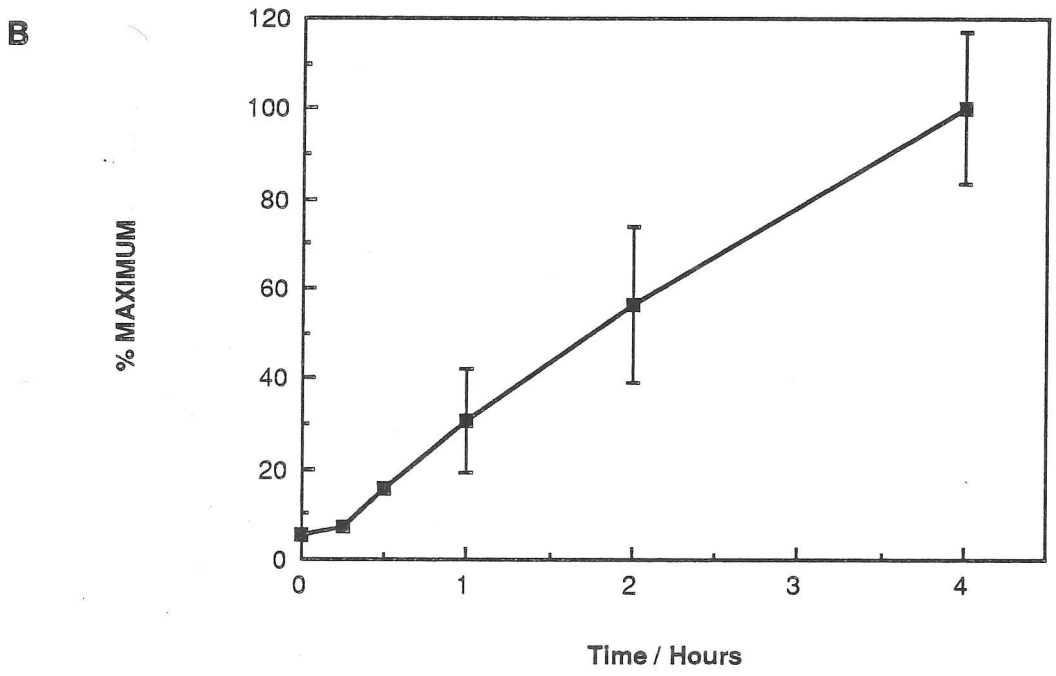


FIGURE 4.28 Time course of KT16-stimulated c-myc mRNA accumulation in clone 4.
Clone 4 cells were incubated with 20µg/ml KT16 for the indicated times, then c-myc mRNA assayed as described in the legend to Fig. 4.23.
A. Autoradiograph. B. Plot of the numbers obtained by scanning densitometry of all lanes except lane 6 where RNA was degraded during preparation.

There is evidence that activated protein kinase C can increase c-myc transcription (Moore et al., 1986; Lindsten et al., 1988). however the ability of IL2 to activate protein kinase C is controversial (Beckner and Farrar, 1986, 1987; Valge et al., 1988; Mills et al., 1988). Therefore it cannot be assumed that IL2-stimulated c-myc mRNA accumulation is mediated by activated protein kinase C.

The ability of all the ligands tested to induce c-myc mRNA accumulation in cells for which they are mitogenic, despite their failure to produce identical responses, strongly suggests that increases in c-myc mRNA may be important for DNA synthesis. This conclusion is supported by experiments utilising antisense myc oligonucleotides and anti-myc antibodies (see Section 1.6). However, the rapid c-myc mRNA accumulation induced by 145-2C11 was apparently insufficient to activate DNA synthesis, as 145-2C11 was not mitogenic for clone 4. Therefore c-myc mRNA accumulation may be necessary but is not sufficient to activate DNA synthesis in T lymphocytes.

4.5 Summary

Ligand-induced modulations of T lymphocyte $[Ca^{2+}]_i$, inositol phosphates and c-myc mRNA have been examined in two murine T cell populations; BALB/c thymocytes and clone 4, a helper T cell line. Ligands employed were first screened for their mitogenic potential (Chapter 3), then ligand effects on early responses were characterised. Table 4.3 summarises the results of these studies, and for comparison also shows the mitogenic response to each ligand.

i) THYMOCYTES

Response	LIGAND Con A	KT16	145-2C11	IL2
$InsP_x$	+++	+	++	-
Ca^{2+}	+++	+	+++	-
c-myc mRNA	++	+	+	-
DNA synthesis:				
Ligand alone	++	+	+	-
+IL2	+++	++	++	-
+TPA	+++	++	+++	+++

ii) CLONE 4

Response	LIGAND Con A	KT16	145-2C11	IL2
$InsP_x$	+++	+	++	-
Ca^{2+}	++	++	n.d.	-
c-myc mRNA	n.d.	+	+	+++
DNA synthesis:				
Ligand alone	-	+	-	+++
+IL2	-	-	n.d.	n.d.
+anti-CD4	n.d.	-	n.d.	+++

TABLE 4.3 T cell responses to a panel of ligands

Key: +++ = large response
++ = moderate response
+ = small response
- = no response
n.d. = not determined

CHAPTER 5

THE EFFECT OF INHIBITORY RECEPTOR INTERACTIONS ON EARLY RESPONSES TO MITOGENS IN T LYMPHOCYTES

CHAPTER 5

THE EFFECT OF INHIBITORY RECEPTOR INTERACTIONS ON EARLY RESPONSES TO MITOGENS IN T LYMPHOCYTES

During characterisation of the mitogenic responses of clone 4 and BALB/c thymocytes (Chapter 3), it became apparent that the response to one ligand was often abrogated by a second ligand. IL2 and the anti-Thy 1 antibody KT16, although both mitogenic for clone 4 individually, were mutually antagonistic. Furthermore, clone 4 proliferation induced by KT16, but not by IL2, was sensitive to inhibition by the anti-CD4 antibody KT6. In contrast, thymocyte proliferation evoked by Con A (or KT16 plus TPA) was insensitive to KT6, and IL2 synergised with Con A, KT16, and the anti-T3 antibody 145-2C11. 145-2C11 (a poor mitogen for thymocytes in the absence of IL2 or TPA) reduced Con A-stimulated [^{3}H]thymidine incorporation into BALB/c thymocytes.

The antagonism between 145-2C11 and Con A was interpreted as evidence of a common binding site for the two ligands on the thymocyte cell surface (Section 3.2.4), but the mechanism by which the other inhibitory receptor interactions occurred was not immediately apparent. This was investigated by examining the effect of inhibitory receptor interactions on the early biochemical responses described in Chapter 4. These studies aimed not only to identify the molecular mechanisms by which inhibition was achieved, but in so doing to identify responses obligatory for activation of DNA synthesis.

5.1 The effect of inhibitory receptor interactions on stimulated inositol phosphate accumulation

5.1.1 The effect of IL2 on ligand-stimulated inositol phosphate accumulation

The failure of IL2 to elicit appreciable inositol phosphate accumulation (Section 4.3.1) made it possible to determine the effect of IL2 on the inositol phosphate response to KT16. IL2 caused partial inhibition of the increases in all inositol phosphate fractions in

response to KT16 (Fig. 5.1). 50 and 500U/ml IL2 caused similar reductions in inositol phosphate accumulated 10 mins after KT16 addition, but less inhibition was observed with 10U/ml IL2. 50U/ml IL2 inhibited stimulation of InsP, $InsP_2$, $InsP_3$ and $InsP_4$ accumulation by 63%, 50%, 63% and 71% respectively.

The effect of IL2 on responses to Con A was also determined, despite the failure of Con A to induce clone 4 proliferation, to assess the generality of IL2-mediated inhibition of $PtdInsP_2$ hydrolysis. Con A-stimulated inositol phosphate accumulation in clone 4 was also inhibited by IL2 (Fig. 5.2), but the inhibition was not as pronounced as the effect on the KT16 response. 50U/ml IL2 inhibited Con A-stimulated accumulation of InsP, $InsP_2$, $InsP_3$ and $InsP_4$ by 15%, 7%, 31% and 18% respectively. Similar inhibitions were observed at all IL2 concentrations tested. The ability of IL2 to inhibit $PtdInsP_2$ hydrolysis stimulated by Con A demonstrated that IL2-mediated inhibition was not peculiar to stimulation through the Thy 1 molecule. The mechanism by which IL2 antagonised $PtdInsP_2$ hydrolysis was unknown. However, as KT16 evoked small inositol phosphate responses (relative to Con A or 145-2C11), it is possible that IL2-mediated inhibition reduced $PtdInsP_2$ hydrolysis to below a critical threshold level necessary for activation of DNA synthesis. Therefore, the IL2-mediated reduction in $PtdInsP_2$ hydrolysis might be sufficient to explain the ability of IL2 to inhibit KT16-stimulated clone 4 proliferation.

5.1.2 The effect of anti-CD4 antibody on ligand-stimulated inositol phosphate accumulation

In addition to the antagonistic effect of IL2 on clone 4 mitogenesis, KT16-activated DNA synthesis was also inhibited by the anti-CD4 antibody KT6. Therefore the effect of KT6 on KT16-stimulated inositol phosphate accumulation in clone 4 was determined. An anti-CD8 antibody (15D5) of identical isotype to KT6 (rat IgG2a) was used to control for effects of any interaction of the F_C portion of KT6 with F_C receptors on clone 4.

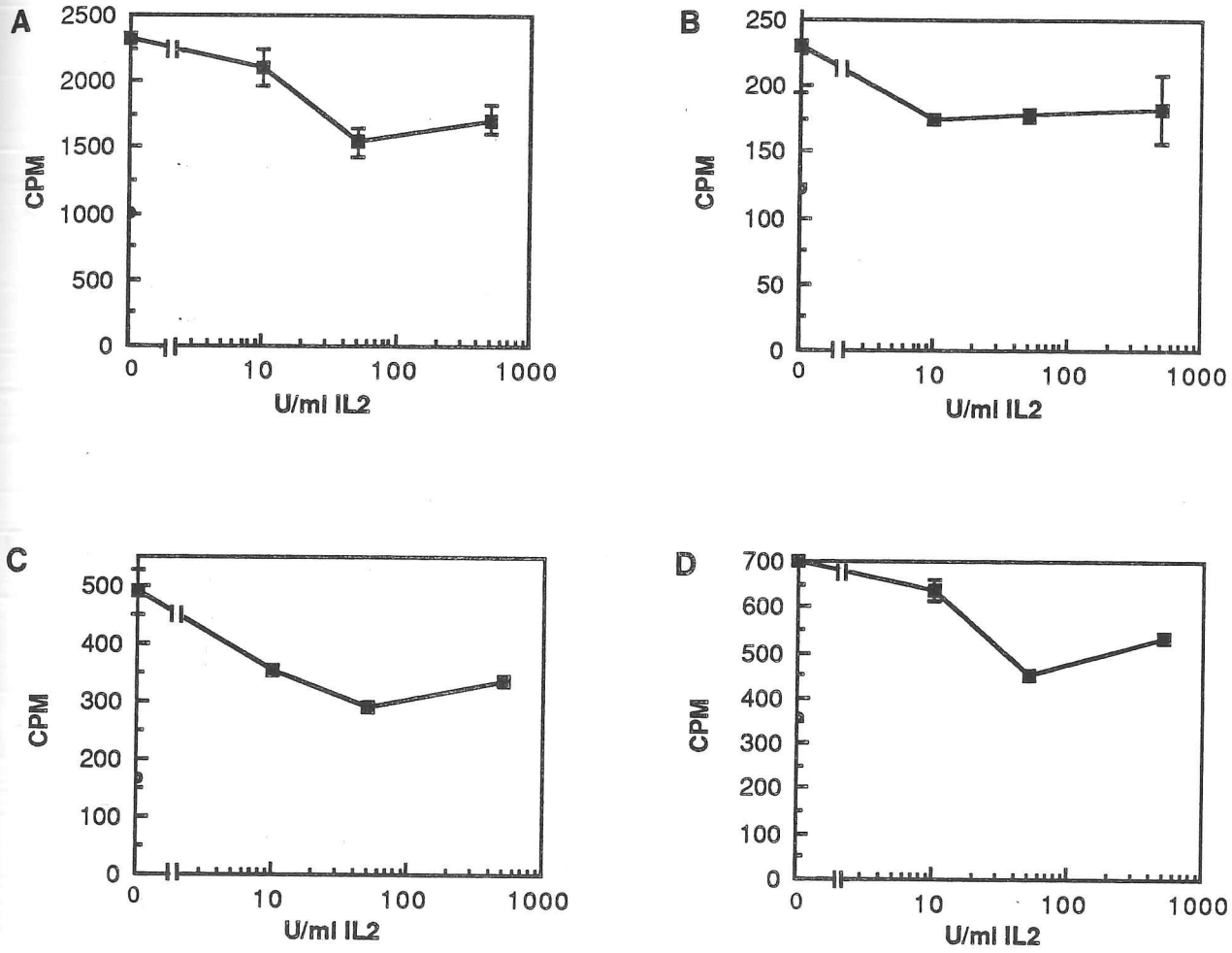


FIGURE 5.1 Effect of IL2 on KT16-stimulated inositol phosphate accumulation. [^{3}H]inositol-labelled clone 4 cells were incubated for 10 mins with medium (○) or 20μg/ml KT16 (■) and the indicated concentrations of IL2. Reactions were terminated and inositol phosphates assayed as described in the legend to Fig. 4.10. A, B, C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

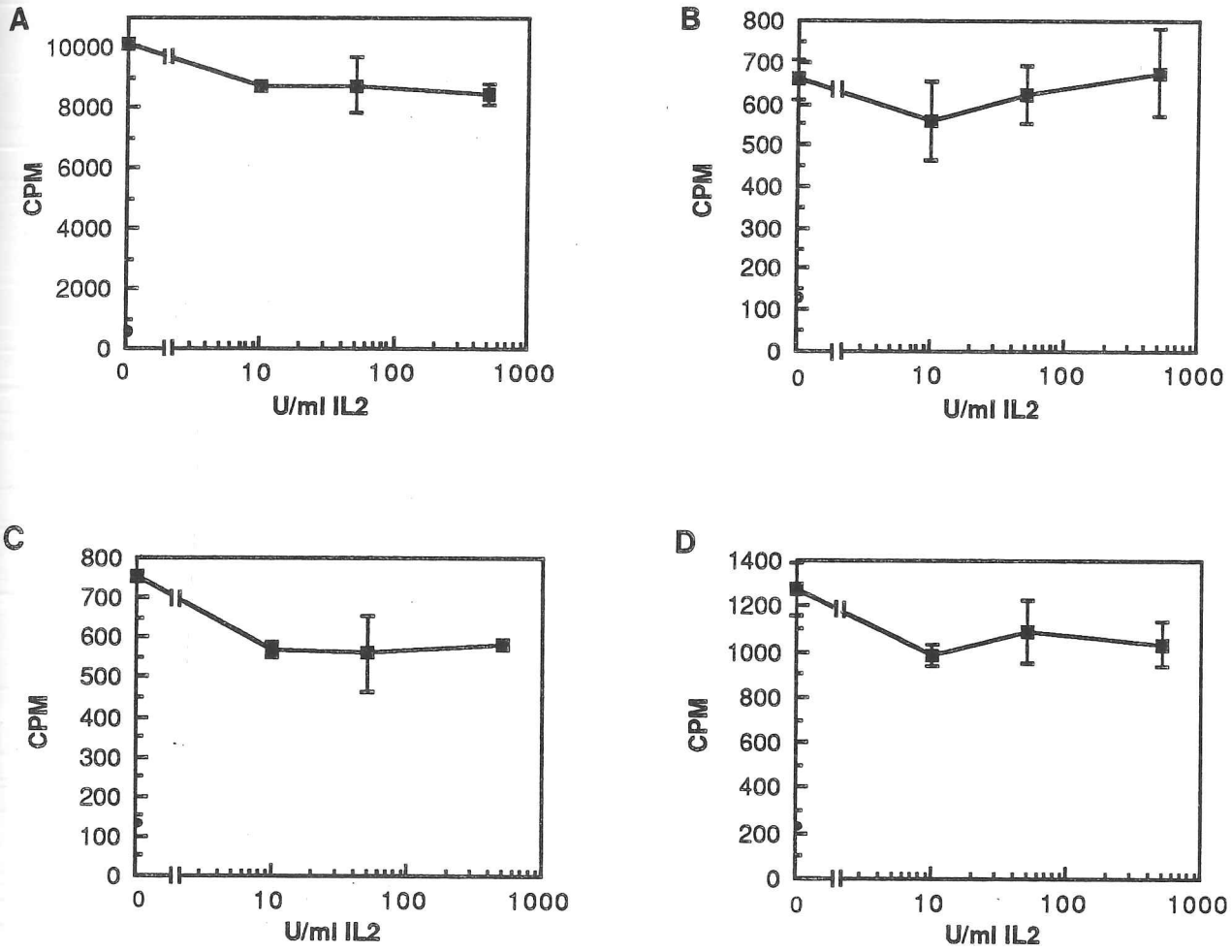


FIGURE 5.2 Effect of IL2 on Con A-stimulated inositol phosphate accumulation. [^{3}H]inositol-labelled clone 4 cells were incubated for 10 mins with medium (○) or 10μg/ml Con A (■) and the indicated concentrations of IL2. Reactions were terminated and inositol phosphates assayed as described in the legend to Fig. 4.10. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

15D5 had no effect on Con A-stimulated inositol phosphate accumulation over the concentration range 1-100μg/ml (Fig. 5.3). Therefore all effects of KT6 were assumed to be due to its specific interaction with CD4.

KT6 alone (5μg/ml) caused no $PtdInsP_2$ hydrolysis but totally inhibited KT16-stimulated accumulation of all inositol phosphate fractions at all time points tested (Fig. 5.4). However, the small response to KT16 made it difficult to detect any small residual response in the presence of KT6. Therefore the effect of KT6 on the much greater responses to 145-2C11 and Con A was also determined.

KT6 was a potent antagonist of Con A-stimulated inositol phosphate accumulation. In the presence of KT6 little increase was detectable up to 2 mins, but subsequently slow monotonic accumulation occurred up to 10 mins, when amounts of inositol phosphates were 10-20% of those in cells not treated with KT6 (Fig. 5.5). The time of KT6 addition relative to Con A addition was not critical for inhibition. The percentage inhibition of each inositol phosphate was very similar when KT6 was added simultaneously with or 5 mins prior to Con A (Table 5.1).

KT6 addition	Percentage inhibition of inositol phosphate fraction			
	InsP	$InsP_2$	$InsP_3$	$InsP_4$
Simultaneous	84%	71%	66%	65%
5 min pre-treatment	86%	77%	74%	68%

TABLE 5.1 Comparison of the effect on inositol phosphate accumulation of KT6 added at different times

[^{3}H]inositol-labelled clone 4 cells were washed into salts solution and pre-incubated as described in Section2.7, then challenged with 10μg/ml Con A for 10 mins. KT6 (5μg/ml) was added simultaneously or 5 min prior to Con A. Reactions were quenched and inositol phosphates extracted and separated using AG1-X8 resin as described in Sections 2.7.2-4. Inositol phosphate fractions were dried and counted for ^{3}H (Section 2.8). Percentage inhibition was calculated as [(cpm in Con A sample-cpm in inhibited sample)x100] / [cpm in Con A sample-cpm in unstimulated cells]

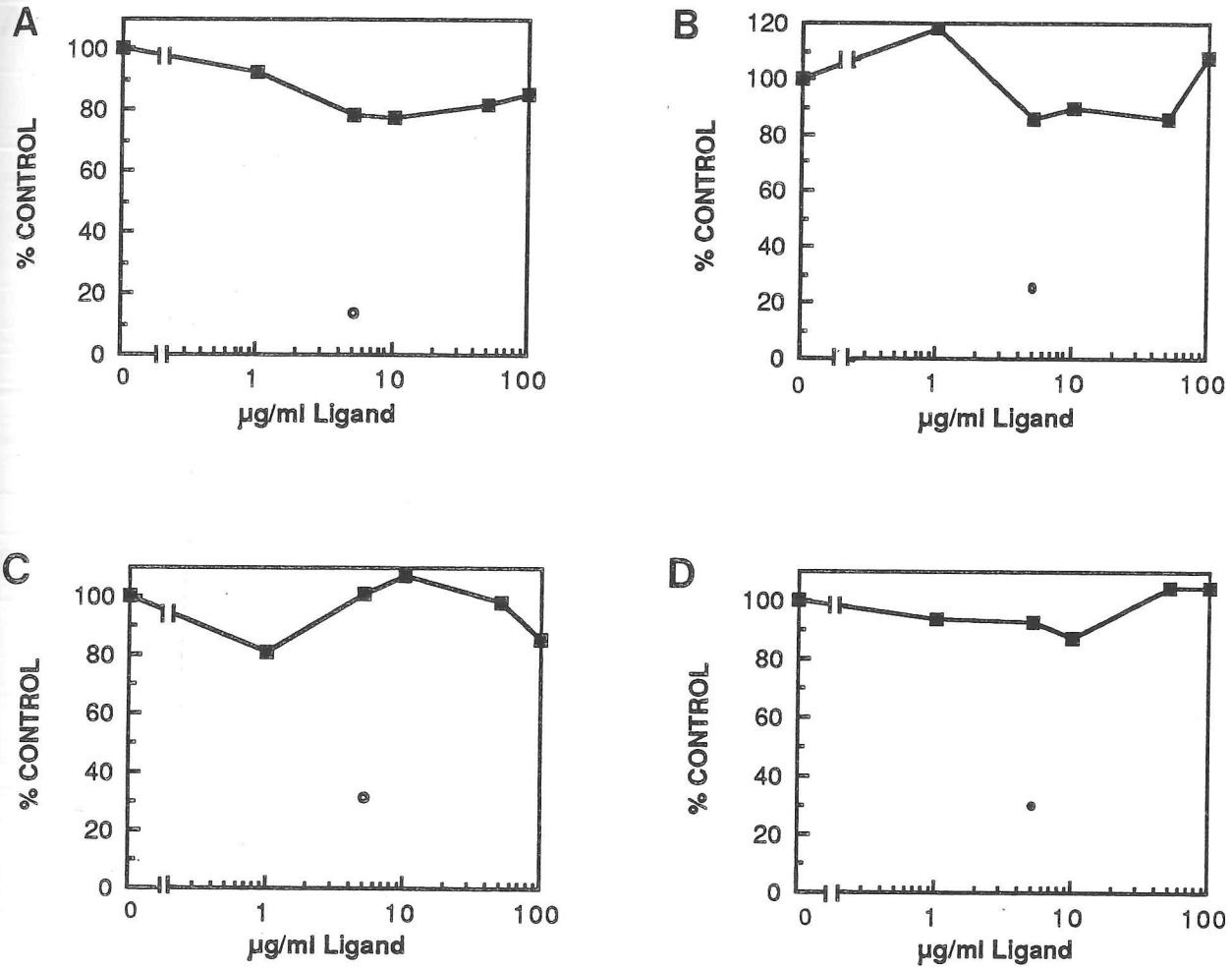


FIGURE 5.3 Effect of anti-CD8 antibody on Con A-stimulated inositol phosphate accumulation.

[^{3}H]inositol-labelled clone 4 cells were incubated for 10 mins with 10µg/ml Con A after 5 min pre-incubation with the indicated concentrations of 15D5 (■) or KT6 (○). Reactions were terminated and inositol phosphates described as in the legend to Fig. 4.10. Results are expressed as a percentage of the stimulated increases seen in cells treated with Con A alone, and are the means of duplicate determinations. A, B, C and D show the results for InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

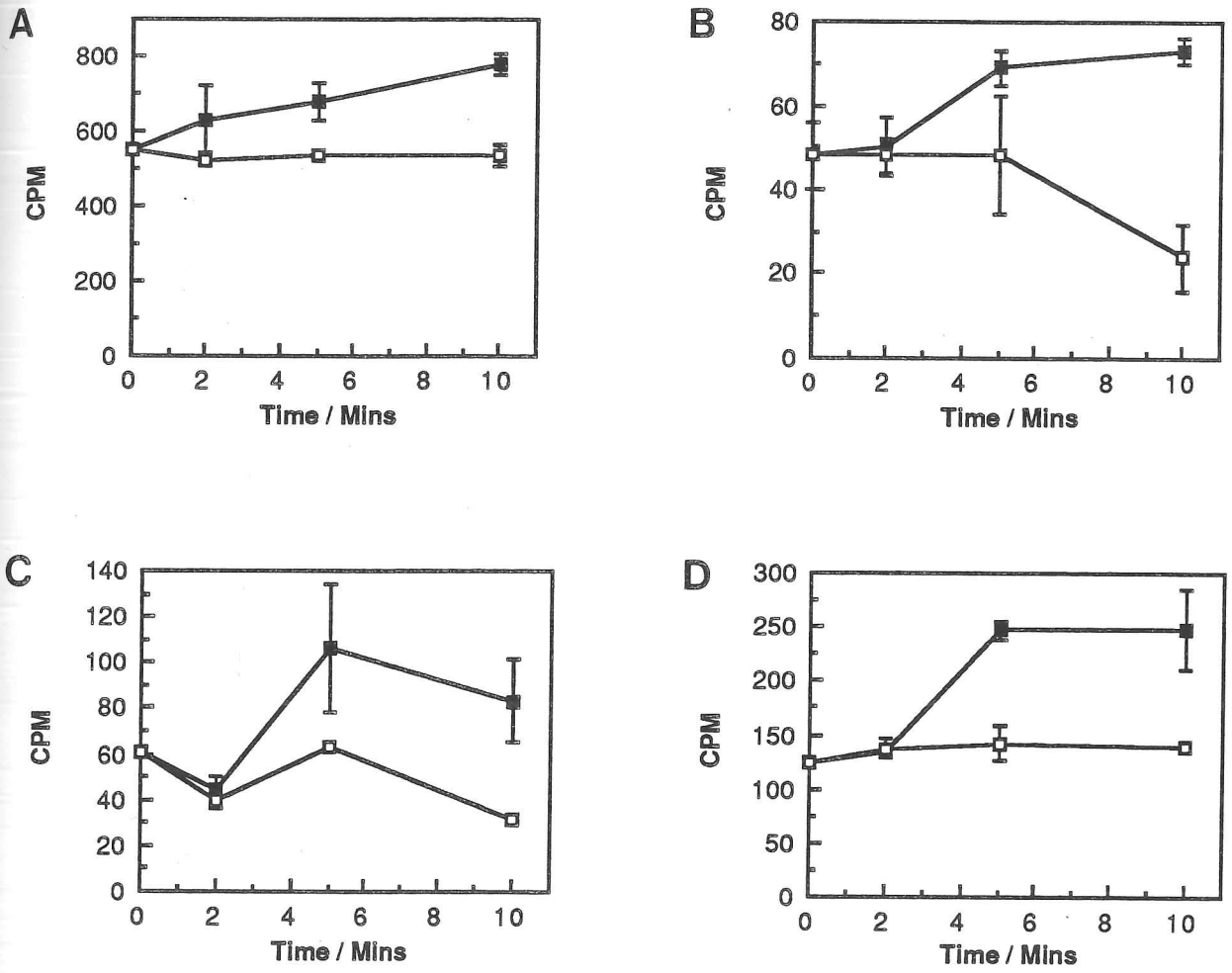


FIGURE 5.4 Effect of KT6 on KT16-stimulated inositol phosphate accumulation. [^{3}H]inositol-labelled clone 4 cells were incubated with 20µg/ml KT16 in the presence (□) or absence (■) of 5µg/ml KT6 for the times indicated. Reactions were terminated and inositol phosphates assayed as described in the legend to Fig. 4.10. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

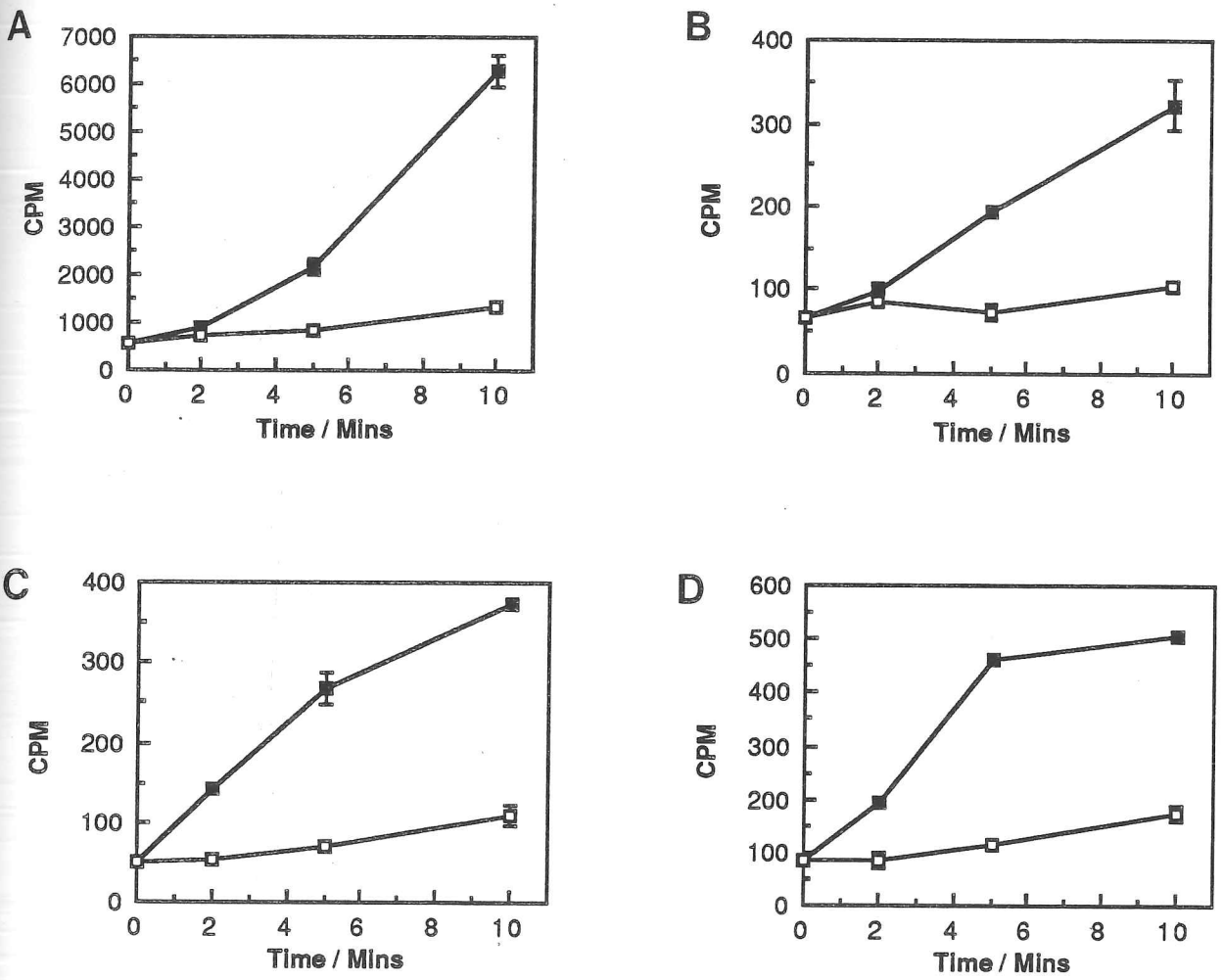


FIGURE 5.5 Effect of KT6 on Con A-stimulated inositol phosphate accumulation. [^{3}H]inositol-labelled clone 4 cells were incubated with 10μg/ml Con A in the presence (□) or absence (■) of 5μg/ml KT6 for the times indicated. Reactions were terminated and inositol phosphates assayed as described in the legend to Fig. 4.10. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

KT6 also antagonised the inositol phosphate response to 145-2C11 (Fig. 5.6). Similar to its effect on the response to KT16, KT6 totally abolished 145-2C11-stimulated inositol phosphate accumulation. This was interesting by comparison with the partial inhibition of the response to Con A, since data presented earlier in the thesis suggested that Con A might trigger responses by interacting with T3 (Section 3.2.4) However, the ability of Con A to elicit inositol phosphate responses in IL2-grown cells in the absence of a response to T3 (Section 4.3.2) suggested that Con A might stimulate $PtdInsP_2$ hydrolysis via interaction with receptors other than or in addition to T3. This would be consistent with the difference in effect of KT6 on responses to Con A and 145-2C11.

The additional Con A receptor(s) could be less sensitive or even insensitive to CD4-mediated inhibition. To investigate this, the effect of KT6 concentrations on Con A-stimulated inositol phosphate accumulation (measured at 10 mins) was determined. Increasing KT6 concentrations up to 100μg/ml mediated no greater inhibition than that caused by 1μg/ml (Fig. 5.7). Concentrations greater than 10μg/ml increased the amount of $InsP_4$ to greater than that stimulated by Con A alone, but did not have similar effects on the other inositol phosphates.

The enhanced $InsP_4$ accumulation at high KT6 concentrations might be due to activation of $InsP_3$ kinase by CD4-mediated increases in $[Ca^{2+}]_i$, as cross-linking of CD4 with antibody has been shown to cause $[Ca^{2+}]_i$ increases (Ledbetter et al., 1987b; Carrel et al., 1988). This is not inconsistent with the failure to observe KT6-induced $[Ca^{2+}]_i$ increases (Section 5.2.2) since these experiments only examined the effect of 5μg/ml KT6. These data suggest that Con A may cause inositol phosphate accumulation by interaction with at least two receptors, distinguishable on the basis of sensitivity to CD4-mediated inhibition.

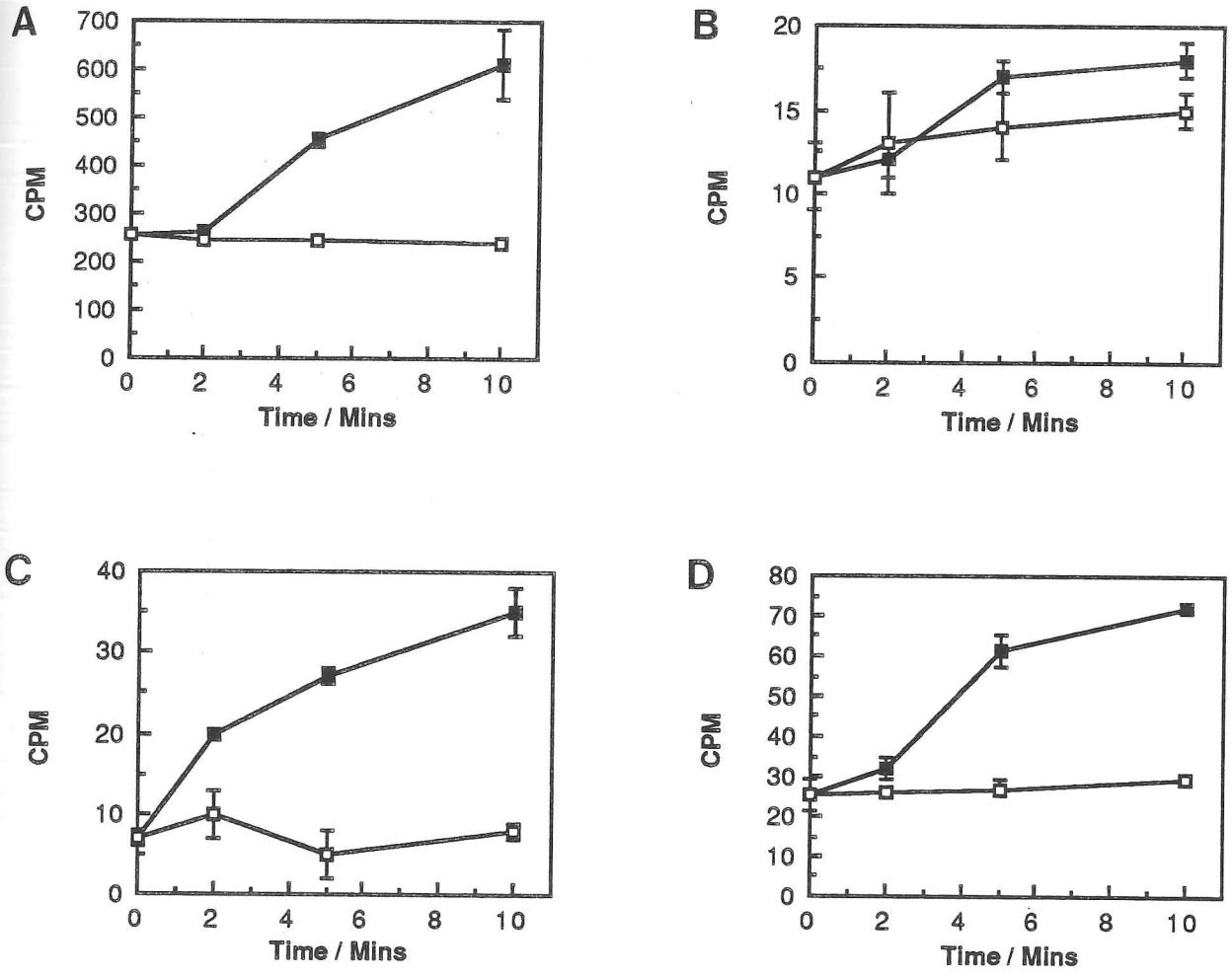


FIGURE 5.6 Effect of KT6 on 145-2C11-stimulated inositol phosphate accumulation. [^{3}H]inositol-labelled clone 4 cells were pre-incubated with medium (■) or 5μg/ml KT6 (□) for 5 mins, then 10μg/ml Con A added and reactions terminated at the indicated times. Inositol phosphates were assayed as described in the legend to Fig. 4.10. A,B,C and D show cpm in InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

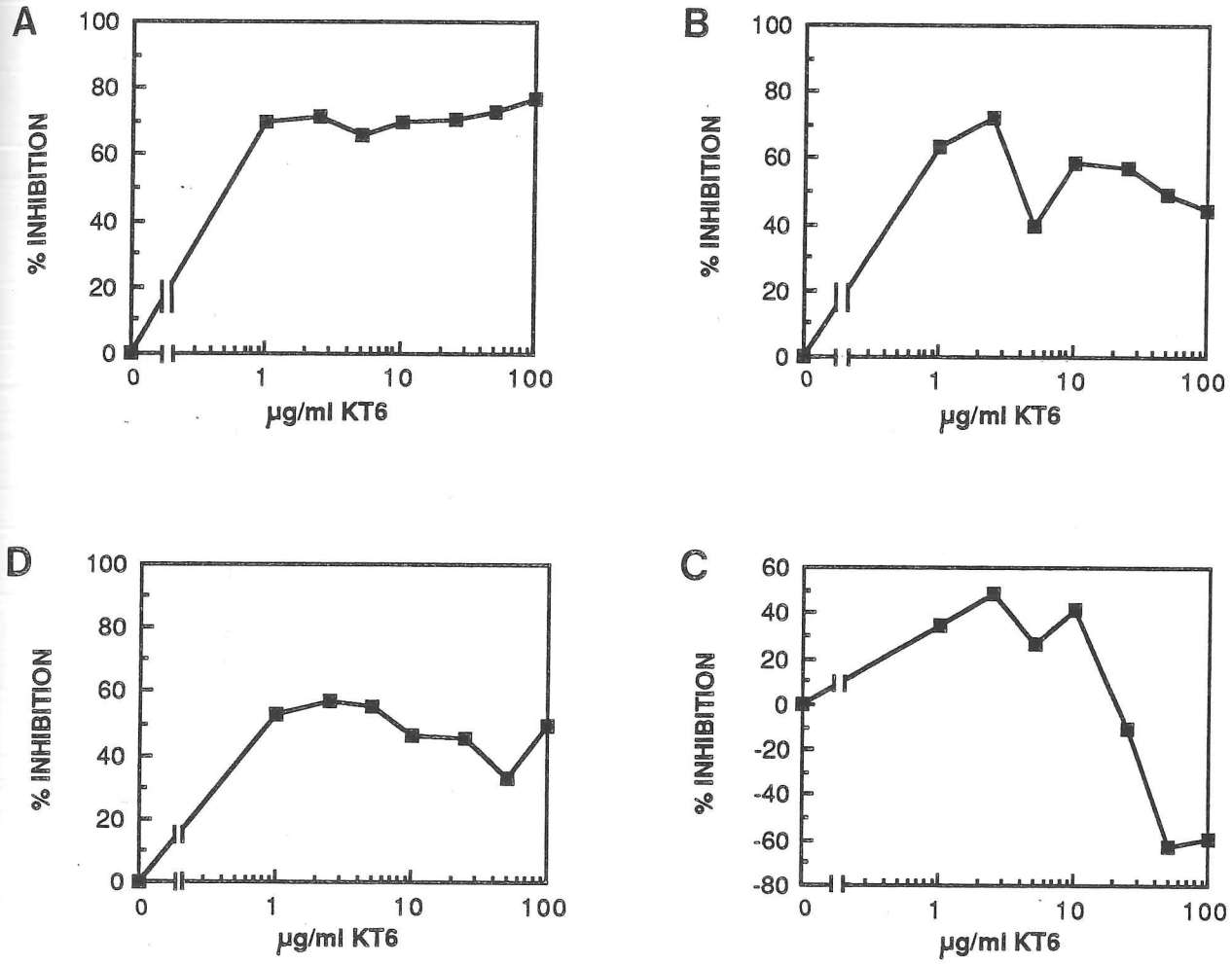


FIGURE 5.7 Concentration dependence of KT6-mediated inhibition of inositol phosphate accumulation.
[^{3}H]inositol-labelled clone 4 cells were pre-incubated for 5 mins with the indicated KT6 concentrations, then incubated for a further 10 mins with 10µg/ml Con A. Reactions were terminated and inositol phosphates assayed as described in the legend to Fig. 4.10. Results are expressed as percentage of inhibition relative to cells treated with Con A alone, and are the means of duplicate determinations. A, B, C and D show the results for InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

These results are reminiscent of data from fibroblasts indicating that different mitogen receptors couple to $PtdInsP_2$ hydrolysis via G-proteins of differing sensitivities to inhibition by pertussis toxin (Taylor et al., 1988), and suggest that different Con A receptors might couple to $PtdInsP_2$ hydrolysis via G-proteins of differing sensitivity to CD4-mediated modulation. However there are no data to support the hypothesis that the antagonistic effects of KT6 are due to modulation of the G-protein(s) responsible for receptor coupling to $PtdInsP_2$ hydrolysis. Furthermore the failure of KT6 to cause inhibition of Con A-stimulated $PtdInsP_2$ hydrolysis in BALB/c thymocytes (Fig. 5.8) comparable to that observed in clone 4 is consistent with the hypothesis that KT6-mediated inhibition is due to destabilisation of interaction between CD4 and MHC class II molecules (Section 3.3). There are however no data to support this hypothesis other than differential sensitivity of class II-expressing (clone 4) and non-expressing cells (thymocytes) to CD4-mediated inhibition.

5.2 The effect of inhibitory receptor interactions on stimulated $[Ca^{2+}]_i$ increases

5.2.1 The effect of IL2 on ligand-stimulated $[Ca^{2+}]_i$ increases

The ability of IL2 to antagonise ligand-stimulated inositol phosphate accumulation suggested that IL2 might also reduce stimulated $[Ca^{2+}]_i$ increases. However, despite substantial inhibition of KT16-induced inositol phosphate accumulation, IL2 had only a small effect on KT16-stimulated $[Ca^{2+}]_i$ elevation (Fig. 5.9a). Maximal $[Ca^{2+}]_i$ was reduced 15nM by simultaneous addition of 50U/ml IL2, but the initial rate of increase and subsequent rate of decline were unaffected.

The initial rate of $[Ca^{2+}]_i$ increase in response to Con A was also unaltered by IL2 (Fig. 5.9b). However, in the presence of simultaneously added IL2, $[Ca^{2+}]_i$ declined more rapidly between 5 and 10 mins. This effect was more marked when IL2 was added 5 mins prior to Con A, suggesting that IL2 might activate a response antagonistic to $[Ca^{2+}]_i$ elevation in a time-dependent manner.

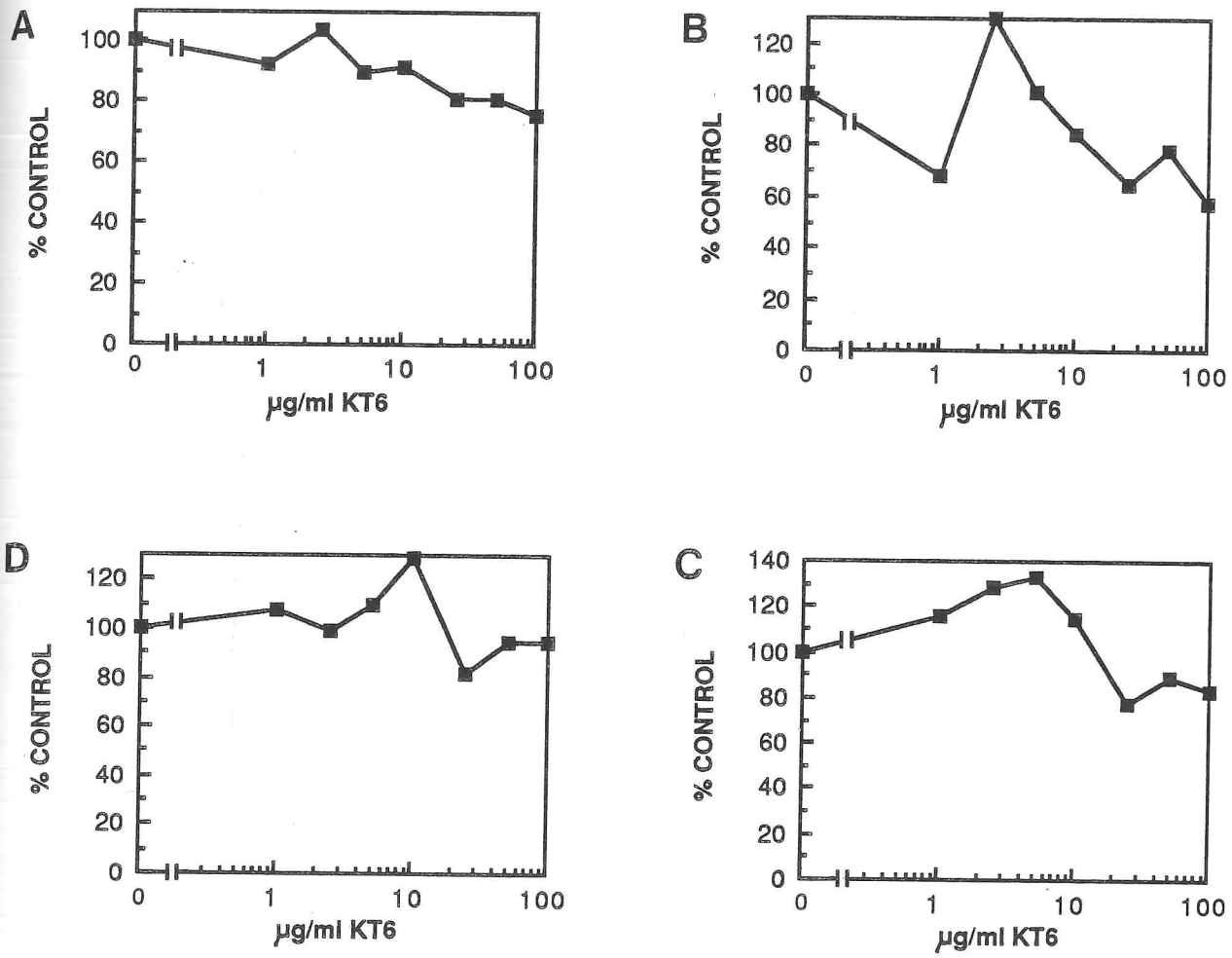


FIGURE 5.8 Effect of KT6 on Con A-stimulated inositol phosphate accumulation in BALB/c thymocytes.

[^{3}H]inositol-labelled thymocytes were pre-incubated for 5 mins with the indicated KT6 concentrations then incubated for a further 10 mins with 10µg/ml Con A. Reactions were terminated and inositol phosphates assayed as described in the legend to Fig. 4.10. Results are expressed as a percentage of the stimulated increases seen in cells treated with Con A alone, and are the means of triplicate determinations. A, B, C and D show the results for InsP, $InsP_2$, $InsP_3$ and $InsP_4$ respectively.

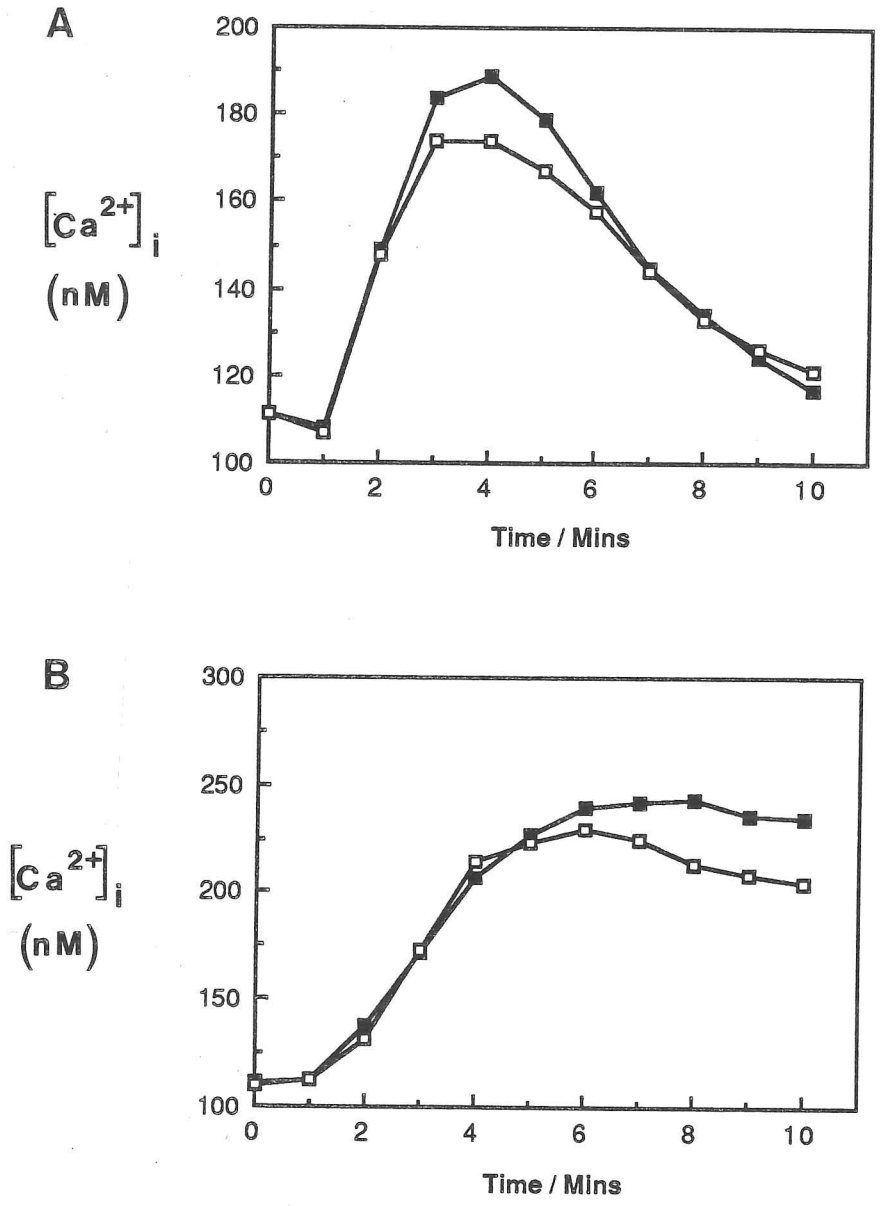


FIGURE 5.9 Effect of IL2 on ligand-stimulated increases in $[Ca^{2+}]_i$.
Quin 2-loaded clone 4 cells (Section 2.6.2) were challenged with 20µg/ml KT16 (A) or 10µg/ml Con A (B) added simultaneously with medium (■) or 50U/ml IL2 (□). $[Ca^{2+}]_i$ was measured as described in the legend to Fig. 4.3. Each point is the mean of triplicate determinations.

5.2.2 The effect of anti-CD4 antibody on ligand-stimulated $[Ca^{2+}]_i$ increases

The effect of KT6 on KT16-induced $[Ca^{2+}]_i$ elevation was examined by pre-treating clone 4 cells with 5μg/ml KT6 for 5 mins prior to KT16 addition. KT6 antagonised both total Ca^{2+} responses and $[Ca^{2+}]_i$ increases occurring in the presence of 1mM EGTA (Fig. 5.10), but itself had no effect on $[Ca^{2+}]_i$. The initial rate of $[Ca^{2+}]_i$ increase and maximum $[Ca^{2+}]_i$ achieved were both reduced by KT6 pre-treatment and the time taken to attain the maximum $[Ca^{2+}]_i$ was increased. The maximum response was reduced from 262^{+}/-23nM to 164^{+}/-17nM, and the intracellular response was reduced from a maximum of 138^{+}/-5nM to 122^{+}/-5nM.

Con A-induced $[Ca^{2+}]_i$ elevation was also antagonised by KT6. Pre-treatment with KT6 reduced the initial rate of Con A-induced $[Ca^{2+}]_i$ increase, and increased the time taken to reach maximum from 5 mins to greater than 10 mins. Little $[Ca^{2+}]_i$ increase was observed up to 2 mins, consistent with the delay in inositol phosphate accumulation. Intracellular Ca^{2+} release was also slowed and the maximum reduced from 129^{+}/-3nM to 115^{+}/-4nM. Neither KT16- nor Con A-induced $[Ca^{2+}]_i$ increases were inhibited by the control antibody 15D5.

KT6 pre-treatment had no significant effect on $[Ca^{2+}]_i$ increases evoked by 0.8 or 10 μg/ml Con A in thymocytes (Fig. 5.11), consistent with the failure of KT6 to inhibit Con A-stimulated inositol phosphate accumulation in thymocytes.

The ability of KT6 to antagonise $[Ca^{2+}]_i$ increases in the presence of EGTA suggests that KT6 can inhibit release of intracellular Ca^{2+} stores, consistent with its ability to inhibit $InsP_3$ accumulation. However, the residual KT16-stimulated $[Ca^{2+}]_i$ increase in the presence of EGTA is inconsistent with the total inhibition of inositol phosphate accumulation. A possible explanation for this inconsistency might be that in the presence of KT6, KT16 stimulates a residual accumulation of inositol phosphates of insufficient magnitude to be detected above basal levels, but sufficient for a limited mobilisation of intracellular Ca^{2+} stores.

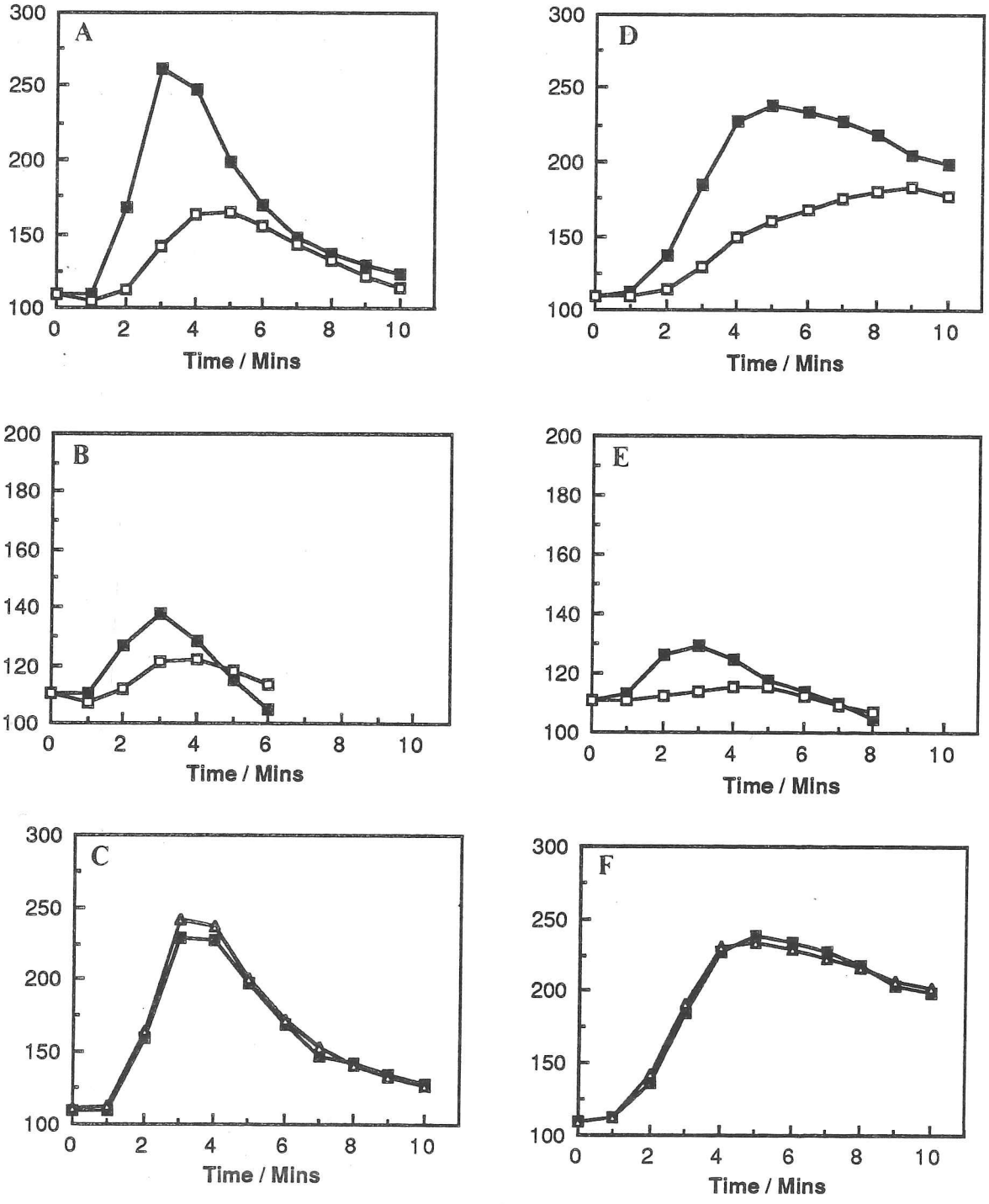


FIGURE 5.10 Effects of KT6 and 15D5 on ligand-stimulated $[Ca^{2+}]_i$ increases in clone 4.

Quin 2-loaded clone 4 cells (Section 2.6.2) were challenged with 20µg/ml KT16 (A-C) or 10µg/ml Con A (D-F) after 5 mins pretreatment with medium (■), 5µg/ml KT6 (□) or 5µg/ml 15D5 (Δ). Experiments were performed in medium containing 0.43mM free Ca^{2+} (A, C, D and F) or 0.1µM free Ca^{2+} (B and E). $[Ca^{2+}]_i$ was measured as described in the legend to Fig. 4.3. Each point is the mean of triplicate determinations.

The abscissas in A-F show intracellular free calcium concentration (nM).

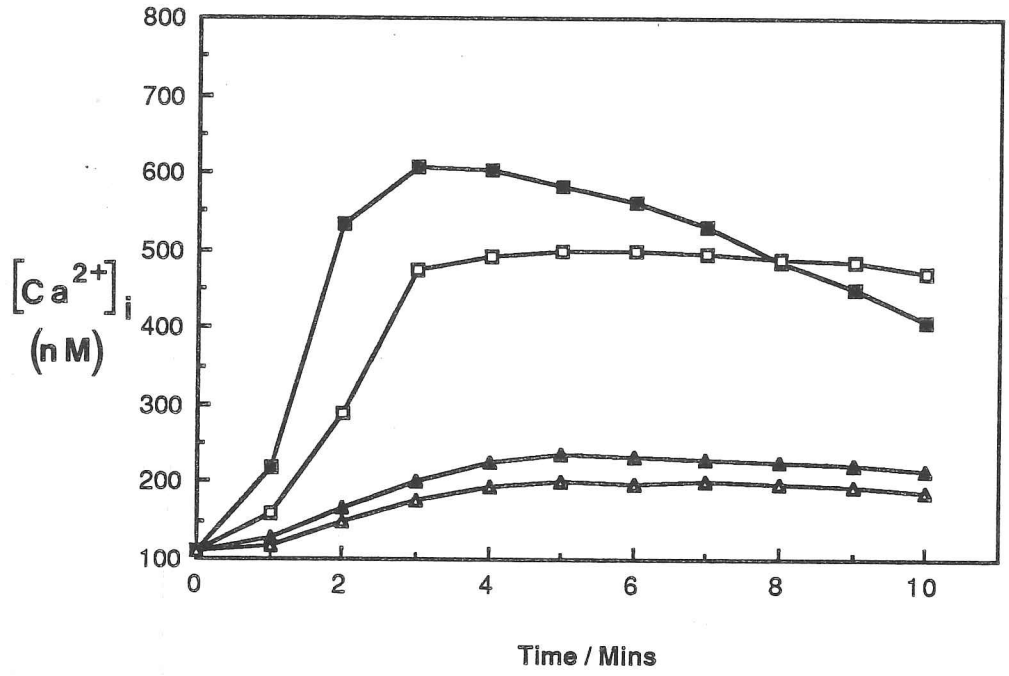


FIGURE 5.11 Effect of KT6 on Con A-stimulated $[Ca^{2+}]_i$ increase in BALB/c thymocytes.

Quin 2-loaded thymocytes (Section 2.6.1) were challenged with 10μg/ml Con A (■,□) or 0.8μg/ml Con A (▲,△) after 5 mins pretreatment with medium (■,▲) or 5μg/ml KT6 (□,△). $[Ca^{2+}]_i$ was measured as described in the legend to Fig. 4.3. Each point is the mean of triplicate determinations.

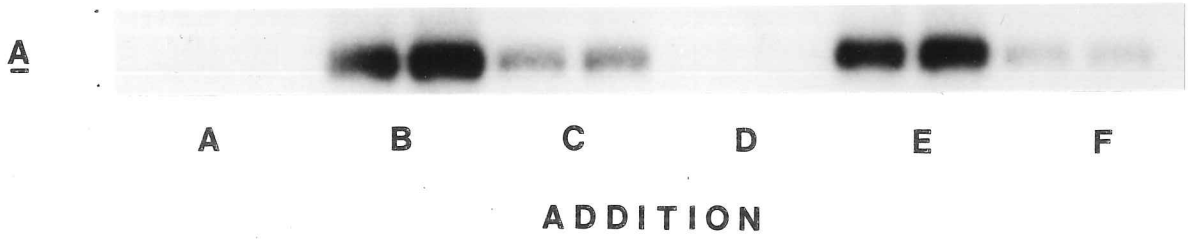
These data contrast with those of Rosoff et al. (1987a) who found that the anti-CD4 antibody Gk1.5 inhibited extracellular Ca^{2+} entry but not intracellular Ca^{2+} release or inositol phosphate accumulation stimulated by Con A or antigen. From their results Rosoff et al. concluded that Gk1.5 might function by inhibiting a plasma membrane-associated Ca^{2+} transporter. However, this hypothesis is inconsistent with the observed inhibition of $[Ca^{2+}]_i$ increase in the presence of EGTA described above. Despite the probability that Gk1.5 and KT6 recogise different epitopes on CD4, it is difficult to explain their markedly different effects.

5.3 The effect of inhibitory receptor interactions on stimulated c-myc mRNA accumulation

5.3.1 Mutual interactions of anti-Thy 1 antibody and IL2

Determination of the effect of IL2 on ligand-stimulated $[Ca^{2+}]_i$ or inositol phosphate increases in clone 4 was facilitated by the failure of IL2 alone to induce such responses. However assay of the effect of IL2 on KT16-stimulated c-myc mRNA accumulation was complicated by the ability of IL2 to induce a much greater response than KT16 (Section 4.4.2).

IL2-stimulated c-myc mRNA accumulation was virtually abolished by simultaneous addition of the anti-IL2r antibody PC61-53 (7^{+}/-1% of IL2 alone), but was unchanged (91^{+}/-10%) by simultaneous addition of 20 μg/ml KT16 (Fig. 5.12). These data suggest that KT16-mediated inhibition of IL2-activated DNA synthesis in clone 4 is not due to a reduction in c-myc mRNA accumulation (at least at this time of measurement-1h after ligand addition), and is therefore likely to be due to inhibition of an event distal to, or on a separate pathway from, c-myc mRNA accumulation. The identity of this KT16-sensitive event is unclear, as the "signals" required for IL2-induced T cell proliferation are not well understood (see below). The data support the conclusion (Section 4.4) that c-myc transcription may be necessary but is not sufficient for DNA synthesis, as KT16 abrogates IL2-induced proliferation without reducing IL2-stimulated c-myc mRNA accumulation.



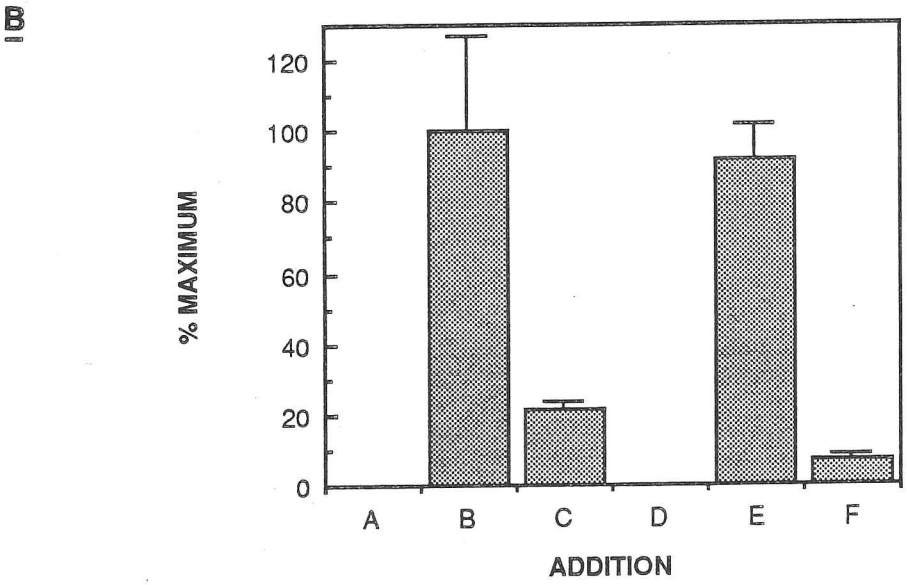


FIGURE 5.12 Effect of ligand combinations on c-myc mRNA in clone 4.
Clone 4 cells were imcubated with medium (A), 50U/ml IL2 (B), 20µg/ml KT16 (C), 20µg/ml PC61-53 (D), IL2 + KT16 (E), or IL2 + PC61-53 (F) for 1h, then c-myc mRNA assayed as described in the legend to Fig. 4.23.
A. Autoradiograph B. Results from scanning densitometry.

The ability of IL2 to antagonise clone 4 proliferation stimulated by KT16 is clearly not due to a reduction in stimulated c-myc mRNA accumulation. However, the increased response in cells challenged with KT16 plus IL2 relative to KT16-stimulated cells could be antagonistic to KT16-mediated proliferation.

5.3.2 The effect of anti-CD4 antibody on stimulated c-myc mRNA accumulation

The effect of KT6 on c-myc mRNA accumulation induced by KT16 or IL2 is shown in Figure 5.13. Consistent with the hypothesis that KT16 and IL2 stimulate c-myc mRNA accumulation via different pathways, KT6 had little effect on the response to IL2 (91^{+}/-1% cf 100^{+}/-5%), but reduced the KT16 response by ~60%. KT6 alone did not stimulate c-myc mRNA accumulation.

This result is consistent with the suggestion (Section 4.22) that KT16 may evoke a c-myc response via elevation of inositol phosphates and $[Ca^{2+}]_i$, since KT6 partially antagonised all three responses. However, as $PtdInsP_2$ hydrolysis is unlikely to be the primary response to KT16 (Section 4.2.1), it is possible that KT6 inhibits an alternative response to KT16 which is capable of activating transcription of the c-myc gene. A possible candidate for this response might be tyrosine kinase activation, which occurs rapidly in T cells in response to T3- and Thy 1-directed antibodies and antigen (Klausner et al., 1987; Section 1.5.3). However specific stimulation of c-myc transcription by an activated tyrosine kinase has not yet been demonstrated.

5.4 Molecular mechanisms of inhibitory receptor interaction

It is not possible to deduce the mechanism by which KT16 antagonises IL2-stimulated clone 4 proliferation from these data, as no effect of KT16 on any IL2 response has been demonstrated. However, the abilities of both IL2 and KT6 to antagonise ligand-stimulated increases in $[Ca^{2+}]_i$ and inositol phosphate suggested that IL2 and/or KT6 might cause cAMP accumulation, since cAMP is known to inhibit $[Ca^{2+}]_i$ and inositol phosphate responses in T cells (Taylor et al., 1984; Lerner et al., 1988).

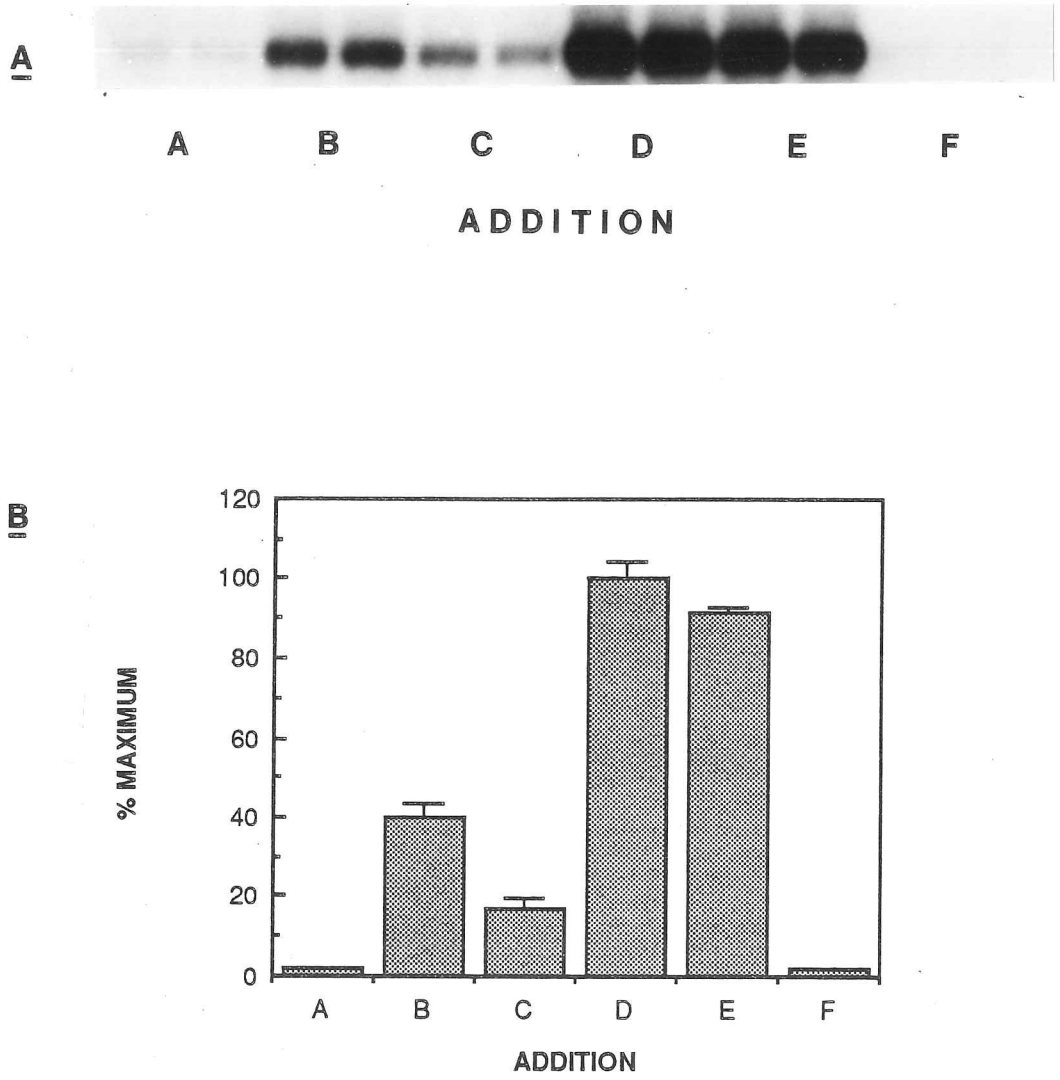


FIGURE 5.13 Effect of KT6 on ligand-stimulated c-myc mRNA accumulation in clone 4.

Clone 4 cells were incubated with medium (A), 20µg/ml KT16 (B), KT16 + 5µg/ml KT6 (C), 50U/ml IL2 (D), IL2 + KT6 (E) or KT6(F) for 1h, then c-myc mRNA assayed as described in the legend to Fig. 4.23.
A. Autoradiograph. B. Results obtained from scanning densitometry.

Cyclic AMP has been characterised as a potent anti-proliferative agent in T lymphocytes stimulated with lectins or antigen, using both cAMP analogues such as 8-BrcAMP and cAMP-elevating agents including forskolin and prostaglandin E_1 (PGE_1) (Novogrodsky et al., 1983; Nel et al., 1988; Ling and Kay, 1975). Therefore the effect of forskolin on KT16-activated clone 4 DNA synthesis was examined. Forskolin inhibited KT16-stimulated [^{3}H]thymidine incorporation into clone 4 by greater than 99% (2,508$^+$/-1,220 and 11$^+$/-3 cpm stimulated by 100ng/ml KT16 $^+$/- 100μM forskolin respectively). Therefore the hypothesis that IL2 and/or KT6 elevate cAMP would be consistent with, and sufficient explanation for, their effects on KT16-stimulated mitogenesis. However, IL2-activated DNA synthesis was also antagonised by forskolin in a concentration-dependent manner (Fig. 5.14). These data suggest that neither KT6 nor IL2 cause cAMP elevation. This was confirmed by direct assay of cAMP levels in cells challenged with a variety of ligands.

IBMX had no effect on basal cAMP levels but enhanced forskolin-stimulated cAMP accumulation; cAMP concentrations were 0.397 and 0.188 pmol cAMP/10^6 cells in cells stimulated with forskolin $^+$/- IBMX respectively. Therefore the ability of ligands to elevate cAMP was assayed in the presence of IBMX to maximise cAMP accumulation, and a forskolin sample was included in each experiment as a positive control. KT6 (5μg/ml) or KT16 (20μg/ml) had no effect on resting [cAMP] (Table 5.2a). However, 50U/ml IL2 caused a reduction of basal [cAMP] (Table 5.2b and other experiments).

The inability of either KT6 or IL2 to elevate [cAMP] is consistent with the conclusions inferred from forskolin effects on clone 4 mitogenesis. The ability of IL2 to reduce basal [cAMP] is consistent with reports that IL2 reduces both basal and PGE_1-stimulated adenylate cyclase activity in the murine T cell clone CT6 (Beckner and Farrar, 1986, 1987). However, since neither IL2 nor KT6 elevate cAMP, the mechanism by which they antagonise KT16-activated DNA synthesis is unclear.

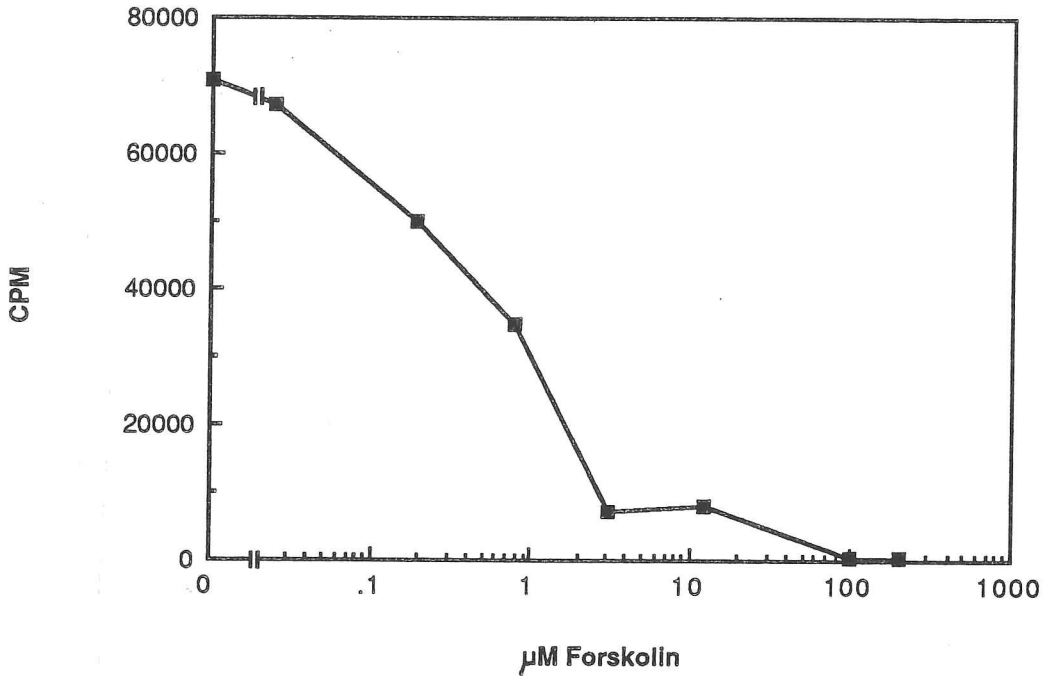


FIGURE 5.14 Effect of forskolin on IL2-stimulated clone 4 proliferation.
Clone 4 cells were cultured with 50U/ml IL2 plus the indicated forskolin concentrations for 96h and DNA synthesis assayed as described in the legend to Fig. 3.1. Each point is a single determination.

	Addition	pmol cAMP / 10^6 cells
a)	0	0.023
	KT6 (5μg/ml)	0.023
	KT16 (20μg/ml)	0.020
	Forskolin	0.764
b)	0	0.032
	IL2 (50U/ml)	0.012
	Forskolin	0.195

TABLE 5.2 Ability of ligands to elevate cAMP in clone 4
Clone 4 cells were incubated for 15 mins with various ligands, then reactions were terminated and cAMP was assayed as described in Section 2.9. Results in a and b are from separate experiments. Each result is the mean of duplicate determinations.

Several responses to IL2 have been described; c-fos, c-myc, and c-myb proto-oncogene mRNA accumulation are well characterised (Farrar et al., 1987; Reed et al., 1985; Depper et al., 1985; Pauza, 1987) and a variety of proteins are phosphorylated in response to IL2 including the ribosomal S6 protein and proteins of M_r 26kDa, 27kDa, 63kDa, 67kDa, 68kDa and 85kDa (Evans and Ferrar, 1987; Evans et al., 1987; Ishii et al., 1987; Kohno et al., 1986; Mire et al., 1985; Mire-Sluis et al., 1989). These proteins are predominantly phosphorylated on serine or threonine although IL2 also stimulates tyrosine phosphorylation (Saltzman et al., 1988; Morla et al., 1988). Thus IL2 activates both an unidentified tyrosine kinase and a serine/threonine kinase. The identity of the serine/threonine kinase is also unclear. While an early report suggested that IL2 activated protein kinase C (Farrar and Anderson, 1985), recent demonstrations of protein kinase C-independent IL2-induced proliferation (Mills et al., 1988; Valge et al., 1988) question the involvement of protein kinase C activation in proliferative responses to IL2. Furthermore, although there are conflicting reports of the ability of IL2 to elevate cAMP in lymphocytes (Knudsen et al., 1987; Beckner and Farrar, 1986, 1987; Wickremasinghe et al., 1986), IL2 failed to elevate cAMP in clone 4. Thus it appears likely that the activated serine/threonine kinase is neither protein kinase C nor cAMP-dependent kinase. Few early responses to IL2 other than these protein phosphorylations and oncogene

accumulation have been reported. Therefore the mechanisms by which IL2 induces proliferation and abrogates KT16-stimulated DNA synthesis are unclear.

The mechanism by which KT6 antagonises responses to KT16, Con A and 145-2C11 is also unknown. It was suggested above that KT6 might destabilise interaction between CD4 and MHC class II molecules thereby abrogating the activation of the tyrosine kinase $pp56^{lck}$. For this to be the sole reason for the inhibitory effects of KT6, activated tyrosine kinase must be sufficient to stimulate $PtdInsP_2$ hydrolysis. However, although the ability of activated tyrosine kinase to stimulate inositol phosphate accumulation has been inferred from experiments involving mutant EGF receptors (Honneger, et al., 1987; Moolenaar et al., 1988), it is clear that $PtdInsP_2$ hydrolysis is not an automatic consequence of tyrosine kinase activation, as both EGF and insulin stimulate the latter but not the former response (Besterman et al., 1986). Therefore it is not certain whether inhibition of tyrosine kinase activation would be sufficient to abrogate all ligand-induced responses. Alternatively KT6 might be inhibitory due to transduction of a negative signal. However other than cAMP there are no obvious candidate biochemical responses capable of antagonising $[Ca^{2+}]_i$ and inositol phosphate responses.

Rosoff et al. (1987a) suggested that anti-CD4 antibody inhibited influx of extracellular Ca^{2+}. However, although consistent with their data, this explanation is inadequate to explain the ability of KT6 to inhibit inositol phosphate accumulation (Section 5.2.2), unless in clone 4 $PtdInsP_2$ hydrolysis is secondary to, and activated by, $[Ca^{2+}]_i$ elevation due to influx of extracellular Ca^{2+}. The ability of ligands to stimulate increases in $[Ca^{2+}]_i$ in the presence of extracellular EGTA (Section 4.3.2) and the ability of KT6 to inhibit these ligand-stimulated increases (Section 5.2.2) is incompatible with a mechanism of Ca^{2+}-stimulated phospholipase C. Therefore it is unlikely that the antagonistic effects of KT6 are due to direct inhibition of a plasma membrane Ca^{2+} channel.

CD4 is associated with a small fraction of T3-T_i on the cell surface, and further association is stimulated by T3-T_i-directed ligands (Anderson et al., 1988; Rivas et al., 1988). Both T3-T_i and CD4 are modulated in response to ligands directed at the antigen receptor (Acres et al., 1986; Saisawa et al., 1987). Conversely CD4-directed ligands

might be expected to cause modulation of the surface expression of the antigen receptor and possibly other mitogen receptors which might also interact with CD4. Therefore the antagonistic effects of KT6 might be due to premature modulation of mitogen receptors.

Of the possible explanations considered above for the ability of KT6 to inhibit responses to a variety of ligands several appear plausible, but it is not possible to distinguish between them on the basis of the data presented here. Nonetheless, KT6 has proved a useful tool for mitogenic analysis. Data presented in Chapter 4 suggested that increases in $[Ca^{2+}]_i$ and inositol phosphates were insufficient to stimulate IL2 secretion and T cell proliferation. However, the observed inhibition of these responses by an antibody antagonistic to mitogenesis suggests these responses may be important for mitogenic stimulation of T cells through Thy 1 and the antigen receptor. Furthermore, the inhibition of c-myc mRNA accumulation by KT6 suggests either that KT6 antagonises an event necessary for both $PtdInsP_2$ hydrolysis and c-myc transcription or that there is a causal link between these two responses. In contrast, study of the mutual antagonism of IL2 and KT16 has so far yielded no information on the mechanism by which these two ligands activate DNA synthesis. However it is possible that further study of the mechanism by which mutual antagonism is achieved may contribute to our knowledge of the events obligatory for IL2-activated DNA synthesis.

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