

THE ROLE OF PROTEIN KINASE C IN IL-2 SIGNAL TRANSDUCTION

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**Thesis submitted to
the School of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy**

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ISBN 0-612-16423-3

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ABSTRACT

Interleukin 2 (IL-2), one of the most important lymphokines secreted by activated helper T-cells, plays a pivotal role in the generation and regulation of the immune response. The role of protein kinase C (PKC) in the signal transduction of IL-2 and the mechanisms of PKC activation were investigated in the cytotoxic T-cell line, CTLL-2. It was initially established that CTLL-2 cells constitute a relevant *in vitro* model to study the role of PKC in IL-2 signaling. It was demonstrated that IL-2 is critical for CTLL-2 cell survival and cytolytic activity. It was then shown that CTLL-2 cells possess measurable levels of PKC activity and this activity was responsive to IL-2 withdrawal and addition. The IL-2 stimulated PKC activation was not due to the PKC translocation from cytosol to the membrane, but rather from the activation of inactive PKC already resident on membranes. Secondly, it was demonstrated that IL-2's ability to maintain cell viability was dependent on PKC activity since specific PKC inhibitors were able to block IL-2's ability to suppress apoptosis. It was also shown that an early and transient PKC activation was needed for the IL-2 induced suppression of apoptosis. Finally, the mechanism(s) responsible for the IL-2 induced activation of PKC in CTLL-2 cells was investigated. It was shown that while tyrosine kinases were activated after IL-2 stimulation, it was unlikely they were linked to the activation of PKC. On the other hand, a pertussis toxin sensitive-G-protein was likely involved in PKC activation since pertussis toxin blocked IL-2 stimulated PKC activation. The observation that DAG, but not IP₃ and intracellular calcium, increased after IL-2 stimulation, indicated that DAG was unlikely generated from the breakdown of PI, but more likely via the PC-PLC or PC-PLD pathways. It was also shown that the increase in DAG by IL-2 was likely responsible for the PKC activation since exogenously applied DAG stimulated PKC activation in both intact cells and in isolated membranes. IL-2 also stimulated the levels of AA in CTLL-2 cells. This increase likely

resulted from increased PLA₂ activity since PLA₂ inhibitors effectively blocked the IL-2 stimulated activation of PKC. As was the case with DAG, the addition of exogenous AA to intact cells and to isolated membranes caused a rapid increase in membrane PKC activity, suggesting that the endogenous production of AA by IL-2R stimulation was likely linked to PKC activation in CTLL-2 cells. The possible involvement of a cytoplasmic factor in IL-2 stimulated PKC activation was investigated. This compound, designated factor X, is likely a protein(s) with a molecular weight of less than 10 kDa. Factor X was rapidly generated following IL-2 stimulation of CTLL-2 cells, and the cytosolic fraction isolated from IL-2 treated, but not control cells, potently stimulated PKC activity in isolated membranes. Its role in the pathway leading to PKC activation after IL-2R stimulation is unclear, although it appears not to act upstream or to be linked to the G-protein or the generation of AA and DAG.

In summary, IL-2 stimulated PKC in CTLL-2 cells, not through the translocation of cytosolic enzyme to membrane, but via activation of inactive membrane associated PKC. This PKC activation was involved in IL-2's ability to suppress apoptosis in these cells. The mechanism(s) of PKC activation by IL-2 is substantially more complicated than we initially thought, and it likely involves multiple second messengers, including G-proteins, DAG, AA and factor X.

DEDICATION

**To my husband, and my daughter
who through their support, encouragement and love have made this work possible.**

ACKNOWLEDGMENTS

I would like express my sincere gratitude to my supervisor Dr. Jon Durkin for his direction and support during this project.

I would like to thank Roger Tremblay and Rita Ball for their technical help.

I would like to thank Dr. Balu Chakravarthy for scientific discussions.

I would like to thank Dr. Paul Morley for measuring intracellular Ca^{++} levels.

I would like to thank MRC for three years financial assistance.

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LIST OF ABBREVIATIONS

AMG	1-o-hexadecyl-2-o-methyl-glycerol
2-ME	2- β -Mercaptoethanol
TPA	12-O-Tetradecanoyl phorbol-13-acetate
BCIP	5-bromo-4-chloro-3-indolylphosphate
ATP	Adenosine triphosphate
BIS	Bisindolylmaleimide GF 109203X
CaMK	Ca ²⁺ /Calmodulin kinase
Cho	Choline
ChoP	Phosphorylcholine
CK-I	Casein kinase I
CK-II	Casein kinase II
cpm	Counts per minute
DAG	Diacylglycerol
DGK	Diacylglycerol kinase
DMSO	Dimethyl sulfoxide
FFA	<i>cis</i> -unsaturated fatty acid
GDP β S	Guanosine 5'-o-(2-o-thio)diphosphate
GTP γ S	Guanosine 5'-(3-o-thio)triphosphate
Gpp(NH)p	Guanosine 5'-(β , γ -imino)triphosphate
K _d	Dissociation constant

LysoPC	Mono-acyl-<i>sn</i>-glycero-3-phosphocholine
kDa	Kilodalton
NBT	Nitroblue tetrazolium
MLCK	Myosin light chain kinase
PCA	Perchloric acid
PC-PLC	Phosphatidylcholine-specific phospholipase C
PC-PLD	Phosphatidylcholine-specific phospholipase D
PE	phosphatidylethanolamine
PLA₂	phospholipase A₂
PK	Phosphorylase kinase
PKA	cAMP-dependent kinase
PKG	cGMP-dependent protein kinase
PMSF	Phenylmethanesulfonyl fluoride
PBS	Phosphate-buffered saline
PS	Phosphatidylserine
PAGE	Polyacrylamide gel electrophoresis
PPH	Phosphatidic acid phosphohydrolase
PtdOH	Phosphatidic acid
SDS	Sodium dodecyl sulfate
TBST	Tris buffered saline +Tween 20
SEM	Standard error of mean
TCA	Trichloroacetic acid
TLC	Thin layer chromatography

GENERAL INTRODUCTION

Having caught measles as a child you became sick. As you obviously survived, you are no longer afraid of catching measles, although you may catch chickenpox. The protection afforded by the immune system is specific to those dangerous antigens you have previously experienced and survived. The basic unit of this specific immune response is the lymphocyte. Lymphocytes differentiate from precursor cells (derived from the yolk sac, fetal liver and bone marrow at different stages of development) which migrate to the so-called central lymphoid organs, i.e. the thymus and the bursa (or its bone marrow equivalent in mammals). When their differentiation is complete, lymphocytes migrate to and populate the peripheral lymphoid organs, i.e. blood, spleen, lymph nodes, Peyer's patches, lymphoid appendix, etc. There are two types of lymphocytes, the B cells and the T cells, and they are responsible for humoral and cellular immunity, respectively. The immune responses are essential for survival, for they constitute the principal means of natural defense against infection by pathogenic microorganisms.

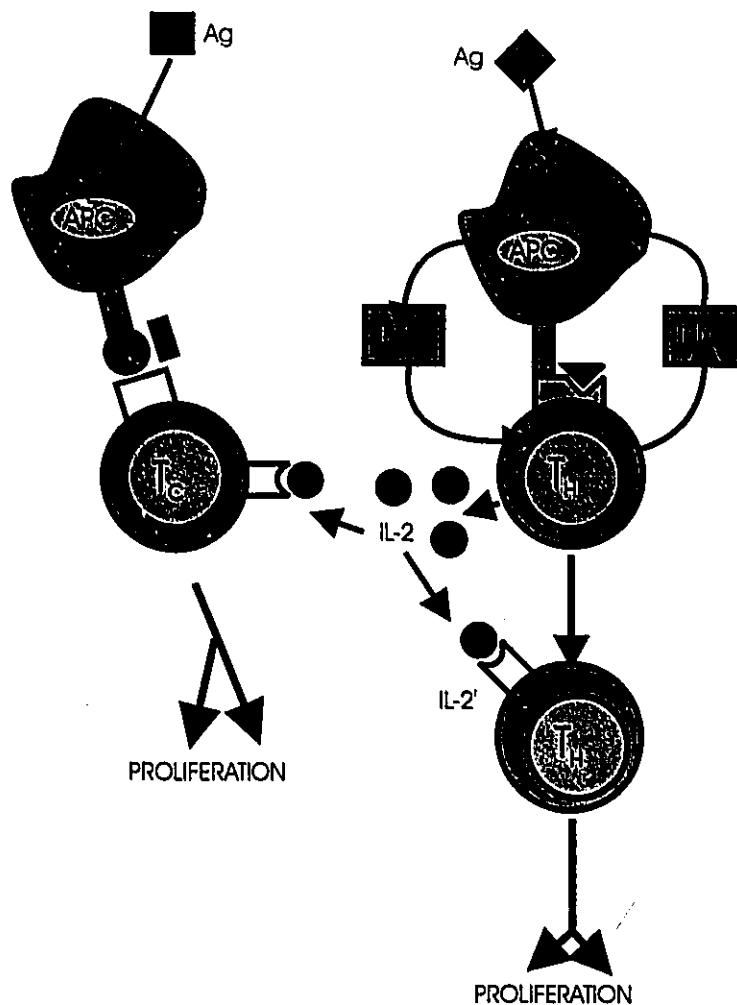
1. Interleukin-2

Interleukin-2 (IL-2), one of the first lymphokines to be identified, plays a pivotal role in the generation and regulation of the immune response (Taniguchi et al., 1986, Smith 1988a). Antigen-specific, clonal proliferation of T cells is initiated *via* a processes of signal transduction, where the binding of T-cell receptor to the antigen/MHC complexes on the accessory cell constitutes the first signal for activation of T-cells (Benjamini and Leskowitz 1988, Eisen 1990). To complete this process of activation, the accessory cell must deliver a second signal (Scheme I-1) in the form of the lymphokine, interleukin-1 (IL-1). This small protein (molecular weight

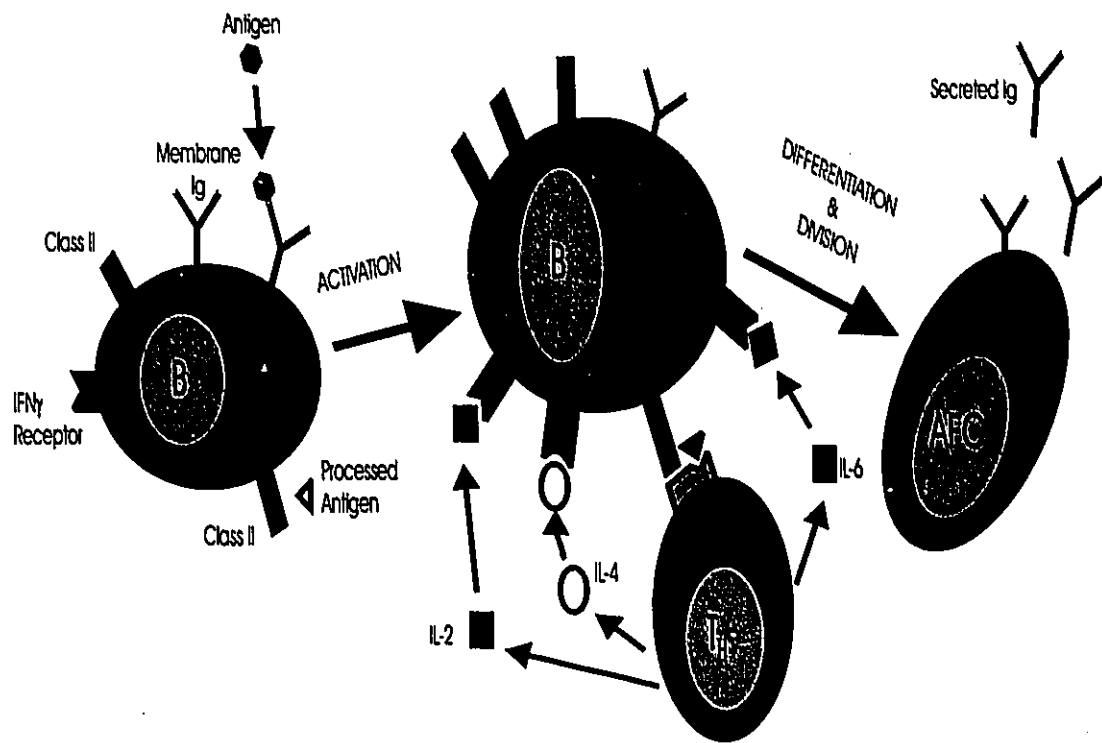
15,000 daltons), which is produced by macrophages, has a variety of other important functions in the body, such as producing fever and causing the release of several acute-phase proteins from the liver (Hamblin 1993). The probable consequence of T-cell contact with IL-1 is the induction, on the surface of the T cell, of specific receptors for IL-2 (Hamblin 1993). Having received the two signals (i.e. antigen/MHC complex and IL-1), the helper T cell undergoes activation and engages in several activities, which include the release of IL-2 and a whole battery of soluble substances, the lymphokines (reviewed by Crabtree 1989), some of which are very important for the triggering of B cells. The IL-2 released serves to stimulate T cells that bear the IL-2 receptor to proliferate in an autocrine fashion (reviewed by Crabtree 1989), which magnifies the overall response initiated by the original T cell population that initially responded to antigen on the accessory cell (Benjamini and Leskowitz 1988, Eisen 1990).

The process of B cell activation, as shown in Scheme I-2, requires a sequence of signaling events (Benjamini and Leskowitz 1988, Eisen 1990), the first of which is the binding of Ag/MHC complexes with the B-cell receptor on the surface of the B cells. Binding alone, however, leads only to low levels of proliferation, without consequent production of antibody (Benjamini and Leskowitz 1988, Eisen 1990). The next stage involves some of the lymphokines produced by the activated helper T-cells in the process of T cell activation. The first lymphokine involved is IL-4 (also called B cell growth factor), which amplifies the signal initiated by Ag/MHC complexes binding to the B-cell receptors (reviewed by Hamblin 1993). Finally, a third signal, also released by helper T cells, IL-6 (B cell differentiation factor), induces the newly dividing B cells to differentiate into plasma cells and to ultimately produce antibody (Hamblin 1993). IL-2 is a co-stimulator for proliferation and differentiation (Hamblin 1993).

Cytotoxic T-lymphocytes (CTL) are a subtype of T-lymphocytes. In contrast to the



Scheme I-1. T cell activation. T cells are activated by a signal of processed antigen plus MHC on an APC followed by specific lymphokines which drive proliferation and differentiation. IFN- γ from T_H cells can enhance MHC expression and antigen presentation on the APCs. IL-2 is required for proliferation of both T_H and T_C cells. IL-1 from macrophages can potentiate activation by increasing IL-2 receptor expression. (Ag = antigen). Modified from Benjamini and Leskowitz's Immunology: a short course 1988.



Scheme I-2. B cell activation. B cells are activated by a sequence of signaling events. First, Ag/MHC complexes on Ag presenting cells binds to the B cell receptor, then IL-4, IL-2 amplify the signal, and finally IL-6 induces the B cells to differentiate into plasma cells and produce antibody. Modified from Benjamini and Leskowitz's Immunology : a short course 1988.

diverse functions of activated T-helper cells, cytotoxic T-lymphocyte actions are much more focused: they destroy only those cells whose surface Ag/MHC complexes they recognize. When a CTL touches another cell, it explores its surface for Ag/MHC complexes that its TcR (T-cell receptor) can recognize. If an Ag/MHC complex is not recognized, the CTL withdraws leaving the explored cell undamaged, but if an Ag/MHC complex is recognized, the CTL is activated to release cytotoxic components which kill the target cell (Benjamini and Leskowitz 1988, Eisen 1990). A CTL can repeat the process many times, killing one target cell after another while generally not affecting "bystander" cells that lack the recognized Ag/MHC complexes. CTL precursor cells are stimulated to proliferate and differentiate into mature CTLs (often termed "effector" cells) by binding to Ag/MHC complexes on the prospective target cells (Benjamini and Leskowitz 1988, Eisen 1990), much in the way that T-cell precursors do. The induction of cytolytic T cells from noncytolytic precursors require the co-ordinate expression of many genes (Gajewski 1989, Mescher 1995). Cytokines from other lymphocytes and accessory cells are also probably necessary (Gajewski 1989, Mescher 1995). Once CTLs have been induced, they can be propagated from single cell and grow for years as cytolytically active CTL cell lines, such as the CTLL-2 cell line used in this thesis study. Unlike other cell lines that grow in culture indefinitely as transformed cells, cultured cytotoxic T cells often retain a normal karyotype and lack the basic characteristics of transformed cells (Eisen 1990).

As mentioned previously, IL-2 plays an important role in both T and B cell activation. The biological activity of IL-2 was initially described as a component of "T-cell growth factor" (TCGF) in the 1960's, a complex mixture of soluble factors secreted by mitogen-activated peripheral blood lymphocytes (Morgan et al., 1976, Mier and Gallo 1980, Ruscetti and Gallo 1981). Human IL-2 was first purified from the culture supernatants of mitogen- or alloantigen-

activated T-cells and the leukaemic cell line, Jurkat, and found to have a molecular weight of 14-17 kDa on SDS-PAGE (Morgan et al., 1976, Mier and Gallo 1980, Ruscetti and Gallo 1981, Gillis et al., 1982). The cDNA consists of a single open reading frame coding for 153 amino acids. The first 20 amino acids of the N-terminus are hydrophobic and constitute the signal sequence which is cleaved to give the mature protein of 133 amino acid with a predicted molecular weight of 15 kDa (Hamblin 1993). IL-2 is a glycoprotein which undergoes O-glycosylation at a threonine residue at position 3 (Hamblin 1993). The disulfide bond between Cys 58 and Cys 102 is critical for biological function since chemical reduction of the bond or site-directed mutagenesis of these residues leads to a complete loss of biological activity (Wang et al., 1984). The tertiary structure of human IL-2 has been determined by X-ray crystallography (Cohen et al., 1986, Brandhuber et al., 1987) and has been shown to consist of six short α -helical segments. A single copy of human IL-2 gene is located on chromosome 4 (Taniguchi et al., 1983, Holbrook et al., 1984, Shows et al., 1986) and is composed of four exons separated by one short and two long introns (Holbrook et al., 1984).

IL-2 has pleiotropic effects on T, B, natural killer (NK) lymphocytes (Taniguchi et al., 1986, Smith 1988a), as well as on nonlymphoid cells (Benvenisto and Merrill 1986). Numerous biological activities have been ascribed to IL-2. IL-2 stimulates T cell proliferation by promoting S-phase progression (Morgan et al., 1976, Farrar et al., 1986). IL-2 has also been implicated in T cell development and function. Using IL-2-deficient mice generated by homologous recombination, the requirement for IL-2 *in vivo* during development was tested (Schorle et al., 1991). It was found that mice homozygous for the IL-2 gene mutation were normal with respect to thymocyte and peripheral T cell subset composition, but that a dysfunctional immune system was

manifested by reduced polyclonal T cell responses *in vitro* and by dramatic changes in the isotype levels of serum immunoglobulins. IL-2 has been shown to be the principle agent which converts large granular lymphocytes into lymphokine-activated killer (LAK) *in vitro* (Lotze et al., 1981, Grimm et al., 1982), a fact which has been exploited in various tumor management strategies (Vujanovic et al., 1988). IL-2 has also been reported to induce cytotoxic T-cell reactivity (Gillis et al., 1979) and, in the process, to induce the synthesis of interferon γ in alloantigen-stimulated T-cells (Farrar et al. 1981). More recently, it has been reported that both $CD4^+$ and $CD8^+$ T cells previously exposed to IL-2 undergo apoptosis after antigen receptor stimulation, suggesting that IL-2 can act negatively in regulating cell growth by programming mature T cells for apoptosis (Lenardo 1991). IL-2 can also influence the growth of glial cells, specifically, the proliferation and differentiation of oligodendrocytes (Benvenisto and Merrill 1986), suggesting that IL-2 may have a role in the growth of brain glia during injury or disease. Finally, IL-2 stimulates B-cell growth and differentiation, and antibody secretion (Kishi et al., 1985, Mond et al., 1985).

2. The IL-2 Receptor

2.1 *Characterization of IL-2 receptor structure, function and distribution*

All of the IL-2 mediated effects on hematopoietic cells are manifested through the IL-2 receptor present on these cells. The IL-2 receptor is unique among growth factor receptors in that it is made up of at least three distinct membrane components: the α chain (IL-2R α) (Leonard et al., 1983, 1985, Robb et al., 1983, Smith 1988b, Farrar et al., 1990, Waldmann 1991), the β chain (IL-2R β) (Sharon et al., 1986, Tsudo, et al., 1986, Teshigawara et al., 1987, Dukovich et al., 1987), and the γ chain (IL-2R γ) (Takeshita 1992). The genes encoding these components have all been cloned and characterised (Farrar et al., 1990, Waldmann 1991, Takeshita 1992).

Expression of the gene encoding IL-2R α is undetectable in resting T cells but is strongly induced upon T-cell activation (Farrar et al., 1990). The IL-2R β gene is expressed constitutively in CD8⁺ cytotoxic T cells but not in CD4⁺ helper T cells; and is further induced upon T cell activation (Sharon et al., 1986, Tsudo, et al., 1986, Teshigawara et al., 1987, Dukovich et al., 1987). IL-2R γ is expressed constitutively in lymphoid cells (Takeshita 1992). It has been demonstrated that IL-2R β plays a critical role in transducing the IL-2-induced mitotic signal(s) in hematopoietic cell lines. When human IL-2R β cDNA is expressed in the murine hematopoietic cell line BAF/B03, which is IL-2R β -negative but IL-2R α - and γ -positive, these cells acquire the ability to proliferate in response to IL-2 (Hatakeyama et al., 1989). Similar results were also obtained when the human IL-2R β cDNA was introduced and expressed in the mouse IL-3-dependent mast cell progenitor cell line, IC-2 (Doi et al., 1989). Both IL-2R α and IL-2R β contain an IL-2 binding site (Farrar et al., 1990).

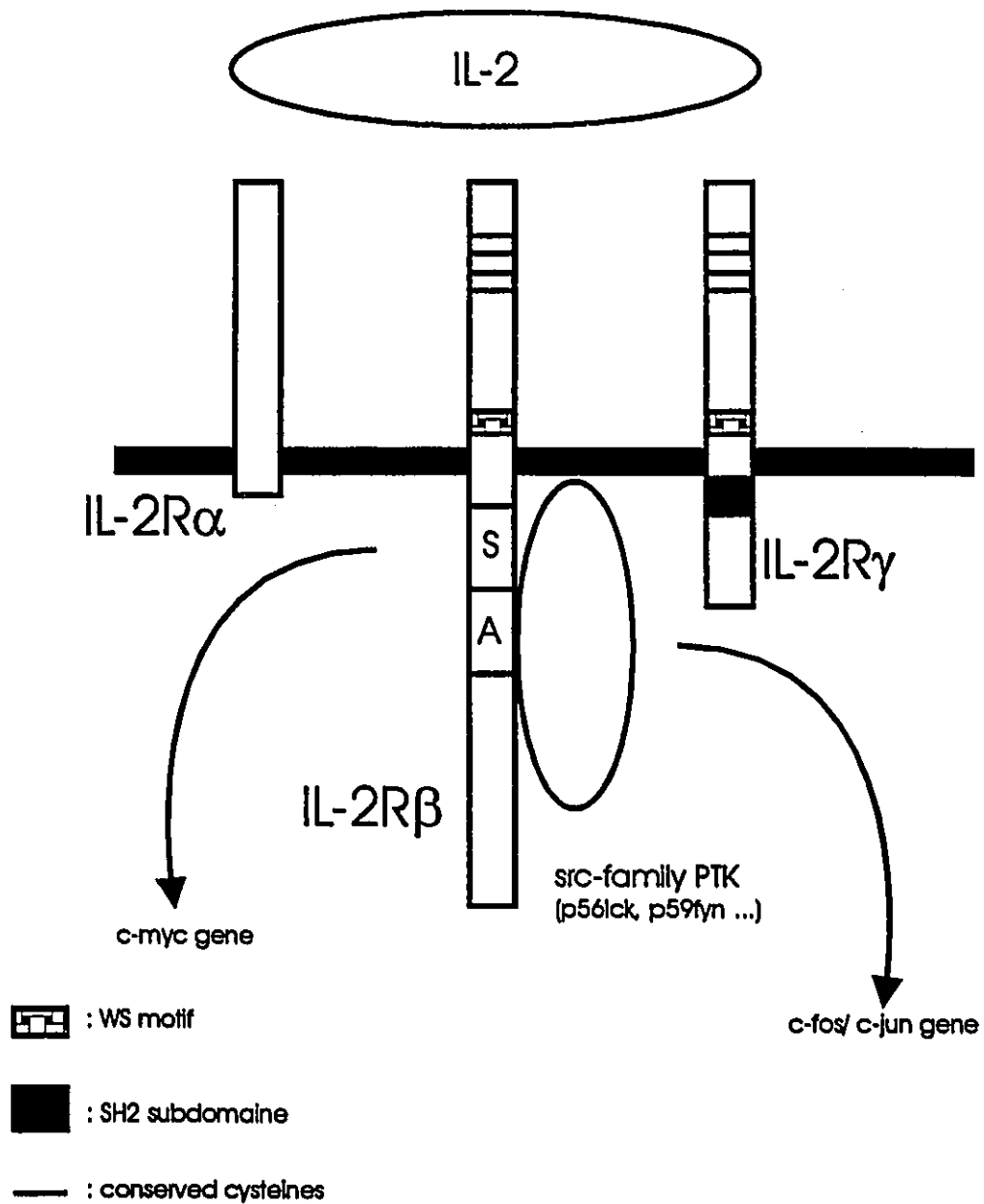
The IL-2 receptor α chain, originally described as the Tac antigen, is a 55 kDa membrane glycoprotein (p55) that binds IL-2 with an equilibrium dissociation constant (K_d) of 10^{-8} M (Leonard et al., 1983, Robb et al., 1983, Smith 1988b, Farrar et al., 1990, Waldmann 1991 and Table 1). The mature protein consists of a 219 amino acid extracellular region, a putative 19 amino acid transmembrane domain, and a 13 amino acid cytoplasmic segment (Smith 1988b, Farrar et al., 1990, Waldmann 1991, and see Scheme I-3). The primary structure of the IL-2R α shows no significant sequence homology with other known receptor molecules. Mutational analysis shows that the N-terminal 83 amino acid residues of the IL-2R α chain, especially residues 1-6 and 35-43, are important for IL-2 binding (Robb et al., 1988).

The IL-2 R β chain was initially identified biochemically by affinity cross-linking experiments (Sharon et al., 1986, Tsudo et al., 1986, Teshigawara et al., 1987, Dukovich et al., 1987). It is a 70-75 kDa protein that binds IL-2 with intermediate affinity (K_d , 10^{-9} M, Table 1). It has a 214 amino acid extracellular portion, a 25 amino acid transmembrane segment, and a 286 amino acid cytoplasmic domain which contains serine-, acidic-, and proline-rich regions (Farrar et al., 1990, Waldmann 1991, see Scheme I-3). Studies employing deletion mutants or site-directed mutagenesis have revealed that the serine-rich region in the cytoplasmic domain of IL-2 receptor β chain is essential for proliferation, but not for IL-2 binding or receptor internalization (Hatakayama et al., 1989, Mori et al., 1991). This region is also required for recruiting Jak1 (Taniguchi 1995). It is noteworthy that the sequence analysis of the "serine-rich" region of the human IL-2R β reveals about 80% homology with the same region found in murine IL-2R β (Kono et al., 1990), and a high degree of homology with the corresponding region of the murine erythropoietin receptor (EPO-R) (Miura et al., 1991). Mutant EPO-R lacking this homologous region fails to respond to EPO (Miura et al., 1991).

The IL-2R γ chain (p64), is a single polypeptide of 347 amino acids in humans (Takeshita et al., 1992). Within the IL-2R γ , sequences of 232 amino acids, 29 amino acids, and 86 amino acids in length constitute the extracellular, membrane-spanning, and cytoplasmic regions, respectively (Takeshita et al., 1992, see Scheme I-3). Interestingly, the sequence spanning positions 288 to 321 appears to be homologous to the Src homology region 2 (SH2), but it does not contain any apparent catalytic motif (Takeshita et al., 1992). The C-terminal 48-amino acid residues of IL-2R γ is required to recruit Jak3 (Taniguchi 1995). It appears that IL-2R γ is required for the receptor-mediated internalization of IL-2 even though the γ chain itself cannot bind IL-2

(Takeshita et al., 1992). In addition, several lines of evidence support the view that IL-2R γ is critical for IL-2-induced signal transduction. A mutant T cell line that has lost expression of the γ chain but retains α and β has been shown to have also lost the ability to proliferate in response to IL-2 (Arima et al., 1992). IL-2 receptor reconstitution studies in fibroblasts have revealed that coexpression of IL-2R α , β , and γ allows the IL-2-mediated induction of *c-fos*, *c-jun*, and *c-myc* to occur (Asao et al. 1993). However, the replacement of the γ chain with a mutant lacking the C-terminal 68 amino acids abrogates proto-oncogene inducibility (Asao et al., 1993). Several studies indicate that IL-2 signaling may require co-operation between the cytoplasmic domains of IL-2R β and IL-2R γ (Nelson et al., 1994, Nakamura et al., 1994).

Expressed together on the cell surface of most, but not all, IL-2-responsive cells, the α , β , and γ chains of the IL-2 receptor can associate noncovalently to form a heterotrimer with high affinity for soluble IL-2 (Kd, 10^{-11} M, Table 1). Studies on activated T-lymphocytes indicates that IL-2 receptor α serves primarily to increase the affinity of IL-2R $\beta\gamma$ for its ligand (Smith 1988b). On the other hand, the heterodimerization of the cytoplasmic domains of IL-2R- β and - γ appears necessary and sufficient to induce signaling in T cells (Nelson et al., 1994, Nakamura et al., 1994, Table 1). It is likely that these two components associate upon ligand binding to the $\alpha\beta$ -subunit and undergo conformational changes in their cytoplasmic domains, similar to that proposed for other membrane-type receptors, such as EGF receptor (Ullrich and Schlessinger 1990). In addition, immunoprecipitation and cross-link studies indicate that other cellular components also associate with the IL-2R, although the nature, structure and function of these components remains unknown (Colamonici et al., 1990, Sharon et al., 1990, Saragovi and Malek., 1990, Saito et al., 1991, Waldmann 1991).



Scheme I-3. Diagram of the IL-2/IL-2 receptor system.

S, serine-rich region of IL-2R β ; A, acidic region of IL-2R β . Signaling pathways associated with IL-2R γ are not yet clear. Copied from Taniguchi and Minami 1993.

2.2 *The Cytokine Receptor Superfamily*

A large number of cytokine receptors have now been cloned. Many of these receptors share significant homology and constitute a novel family of receptors, designated the cytokine receptor superfamily. The family includes IL-2R β , IL-2R γ , IL-3R (α and β -chain), IL-4R, IL-5R (α and β -chain), IL-6R and IL-6R gp130, IL-7R, IL-9R, ciliary neurotrophic factor R (CNTFR), prolactin R (PLR), growth hormone R (GHR), erythropoietin R (EPOR), GM-CSFR (α and β -chain), G-CSFR and leukaemia inhibitory factor receptor α -chain (LIFR α) (reviewed by Bazan 1990a, 1990b, Cosman et al., 1990, Miyajima et al., 1992). It is important to note that the IL-2 receptor complex, IL-2 R α , does not belong to the cytokine receptor superfamily (Hamblin 1993). All the members of this family (except CNTFR) are type I membrane glycoproteins with a single hydrophobic transmembrane domain, oriented with their N-termini exterior to the plasma membrane (Bazan 1990a, 1990b, Cosman et al., 1990, Miyajima et al., 1992). This extracellular domain contains two major regions of homology: one is a region containing four Cys residues located in the N-terminal half of the extracellular domain (Cys-X9-10-Cys-X-Trp-X26-32-Cys-X10-15-Cys). Disulfide bonds reportedly could be formed between the first and the second Cys residues or between the third and the fourth Cys residues in the GHR (Cosman et al., 1990). Another region of sequence homology is the "WS motif" (Trp-Ser-X-Trp-Ser) located proximal to the membrane-spanning domain (Miyazaki et al., 1991). In the case of IL-2R β , the two Trp residues in the "WS motif" play a crucial role in the proper folding of the extracellular domain and in growth signal transduction mediated by IL-2R β (Miyazaki et al., 1991). The ligand binding segment (approximately 200 amino acid residues) of the cytokine receptor superfamily is composed of two discrete folding domains which is highly conserved among the various family

members (Bazan 1990a, 1990b). The C-terminal half of the extracellular domain has been reported to have an evolutionary linkage with the fibronectin type III modules found in a series of cell surface molecules possessing adhesive properties (Bazan 1990a, 1990b). It remains to be determined whether these domains of the cytokines receptor family are indeed involved in ligand binding or association with other extracellular structural components.

Table 1. Different combinations of three IL-2 receptor components give rise to various forms of the IL-2 receptor*

Subunit (combination)	IL-2 Binding (kd)	IL-2 Signaling
α	10^{-8} M	-
β	10^{-7} M	-
γ	Not detectable ^a	-
$\alpha\beta$	10^{-10} M ^a	-
$\alpha\gamma$	10^{-8} M	-
$\beta\gamma$	10^{-9} M	+
$\alpha\beta\gamma$	10^{-11} M	+

* Determined only on cDNA-transfected fibroblasts

* Copied from Taniguchi and Minami et al., 1993

In addition to the structural similarities found in the extracellular domains of the various members of the cytokine receptor superfamily, several conserved motifs have also been reported. For example, a proline-rich motif, referred to as a box 1 (O'Neal et al., 1994) is found in the cytoplasmic region of the human IL-2R β , mouse IL-3R β , mouse IL-4R, human IL-7R, human

GM-CSFR β , mouse EPOR, the recently identified oncogene v-mpl, and its human counterpart, h-mpl (Souyri et al., 1990, Vigon et al., 1992). These similarities in the structures of the cytokine receptors imply that some, if not all, members of this superfamily utilize common or closely related mechanisms to mediate signal transduction.

3. IL-2 Signal Transduction

In contrast to the wealth of information regarding the biological actions of IL-2, the molecular mechanisms involved in the signal transduction of IL-2 remain elusive. Because of the pleiotropic response of cells to IL-2, it is likely that the IL-2 receptor is coupled to a number of different intracellular activation pathways. Since IL-2 has a wide range of effects on T, B, NK cells and has therapeutic value in cancer immunotherapy, autoimmune disorders, chronic infectious diseases, acquired immunodeficiency syndrome (AIDS), and organ-transplant rejection (Smith 1988a), it is absolutely critical to understand the nature of the pleiotropic signals generated by IL-2 which are responsible in altering the behaviour of responsive cells. The following is a description of what is currently known about IL-2 signalling pathways in hematopoietic cells.

3.1 *Role of Tyrosine Kinases*

Tyrosine phosphorylation of cellular substrates is generally believed to play a crucial role in the intracellular signal transduction pathways that regulate cellular activation and differentiation (reviewed by Hunter and Cooper 1985). Compelling evidence has accumulated indicating that tyrosine kinase(s) are involved in signal transduction mediated by cytokine receptors (Koyasu et al., 1987; Murakami et al., 1991; Miura et al., 1991; Isfort et al., 1988; Morla et al., 1988; Saltzman et al., 1988; 1990; Farrar and Ferris 1989; Mills et al., 1990, Merida and Gaulton 1990, Quelle and Wojchowski 1991; Nakajima and Wall 1991; Lord et al., 1991; Uckun et al.,

1991). It has been reported that both serine/threonine and tyrosine kinases are activated in T-lymphocytes stimulated with IL-2 (Gaulton and Eardley 1986; Ishii et al., 1988; Saltzman et al., 1988, Ferris et al., 1989; Mills et al., 1990), and that tyrosine kinases are involved in IL-2 signal transduction mechanisms responsible for cell proliferation (Asao et al., 1990; Mills et al., 1990; Merida and Gaulton 1990). Although the IL-2 receptor components themselves lack protein-tyrosine kinase domains, IL-2 nevertheless induces rapid tyrosine phosphorylation of cellular proteins, including IL-2R β itself (Asao et al., 1990; Mills et al., 1990). This observation may be explained by the fact that IL-2R β interacts both physically and functionally with the nonreceptor tyrosine kinase Src family member p56^{lck} (Veillette et al., 1988; Grassmann et al., 1993). p56^{lck} can be coprecipitated with IL-2R β , and IL-2 stimulation induces p56^{lck} tyrosine kinase activity (Horak et al., 1990; Hatakeyama et al., 1991). cDNA coexpression studies have revealed that the interaction of these two molecules requires a cytoplasmic region of the IL-2 receptor, termed the acidic region, and a region spanning the N-terminal half of the p56^{lck} tyrosine kinase domain (Hatakeyama et al., 1991; Minami et al., 1993). In fact, this molecular interaction appears to be indispensable for IL-2-induced p56^{lck} activation (Hatakeyama et al., 1991; Minami et al., 1993). Interestingly, in BAF/B03 pro-B cell in which p56^{lck} is not expressed, two other Src family members, p59^{lyn} and p53/p56^{lyn}, are activated by IL-2R in an analogous manner to p56^{lck} (Torigoe et al., 1992; Kobayashi et al., 1993), suggesting the importance of the Src family of tyrosine kinases in IL-2R functions.

In many types of cells, Ras protein activity is controlled by a variety of tyrosine kinases including various receptor-tyrosine kinases (e.g., PDGFR, EGFR, M-CSFR, insulin R, and ErbB-2 oncoprotein) and non-receptor tyrosine kinases (e.g., *Src* and *abl* oncoproteins) (Satoh et al.,

1991, 1992; Medema and Bos 1993; Margolis and Skolnik 1994). In the mast cell line PT 18, a specific inhibitor of tyrosine kinases, herbimycin A, can block the accumulation of active Ras•GTP complexes following the addition of IL-3 or GM-CSF, suggesting the involvement of tyrosine kinases in the signal transduction pathway leading to Ras activation by these cytokines (Sato et al., 1991, 1992).

IL-2 can also stimulate the accumulation of active Ras•GTP complexes (Sato et al., 1991). Mutant forms of IL-2R which cannot activate Ras protein are also incapable of inducing *fos* and *jun* mRNAs expression (Sato et al., 1992; Shibuya et al., 1992) suggesting that Ras may play an important role in *fos/jun* induction by IL-2. However, in spite of intensive efforts, the direct downstream targets of Ras have yet to be identified. Evidence suggests that serine/threonine kinases, including Raf-1, MAP kinase kinase (i.e. MEK), and Map kinase, are regulated by the Ras protein (Carroll et al., 1990, Turner et al., 1991, Zmuidzinas et al., 1991) and are thus possible targets for Ras activity induced by IL-2. Indeed, the stimulation of the IL-2, IL-3, or GM-CSF receptors by their respective ligands results in the activation of Raf-1 kinase, possibly as a result of tyrosine phosphorylation (Carroll et al., 1990, Turner et al., 1991, Zmuidzinas et al., 1991). In the case of the IL-2, Raf-1 kinase is the earliest cellular substrate so far identified that is phosphorylated by a tyrosine kinase(s) upon IL-2 stimulation. Thus, this family of cytokines receptors, while lacking a protein-tyrosine kinase domain, can effectively recruit Src family tyrosine kinases and possible other tyrosine kinases to propagate IL-2 signals. However, the biological importance of tyrosine kinase activation in IL-2-signaling is still not fully understood. For example, a mutant cell line lacking p56^{lck} (Karnitz et al., 1992) was found to

retain its strict dependence on IL-2 for both viability and growth, suggesting that p56^{lck} activity may not be a mandatory requirement for the transduction of IL-2-mediated mitogenic signals.

3.2 Role of Janus Kinases (Jaks)

A previously unrecognized direct signal transduction pathway to the nucleus has been uncovered through the study of transcriptional activation in response to interferon α (IFN- α) and interferon γ (IFN- γ). It has been observed that IFN-receptor interactions at the cell surface lead to the activation of kinases of the Jak family which subsequently phosphorylate proteins called STATs (signal transducers and activators of transcription) (Fu 1992a, 1992b; Schindler et al., 1992a, 1992b; Veals et al., 1992; Shuai et al., 1992, 1993). The phosphorylated STAT proteins migrate to the nucleus where they bind to specific DNA elements and direct transcription (Ihle et al., 1994). Knowledge acquired of the IFN- α and IFN- γ signalling pathways has led to the discovery that a number of STAT family members exist, and that other receptor complexes also use the Jak-STAT molecules in transducing their signals.

Four Jaks proteins have been identified to date, namely Jak1, Jak2, Tyk2, and Jak3. Recent studies implicate Jak1 and Jak3 in the signaling pathways coupled to the β and γ chains of IL-2R (Withuhn et al., 1994; Johnston et al., 1994; Miyazaki et al., 1994, Russell et al., 1994). It has also been reported that Jak2-related kinase is activated in response to IL-2 and associates with the β and γ chain of the IL-2 receptor (Tanaka et al., 1994). Six STATs, STAT1 α , STAT1 β , STAT2, STAT3, STAT4, STAT5, and STAT6 have been cloned to date (Fu et al., 1992a, 1992b, Schindler et al., 1992a, 1992b, Darnell et al., 1994, Taniguchi 1995). It also had been reported that IL-2 stimulation causes STAT-like protein phosphorylation (Beadling et al., 1994). However, the biological significance of Jaks and STATs in IL-2 signaling has not been explored.

3.3 Role of Glycosylphosphatidyl Inositol (GPI)

Another signalling mechanism used by hematopoietic cells involves the hydrolysis of glycosyl-phosphatidyl inositol (GPI). IL-2 stimulation has been found to provoke the rapid hydrolysis of GPI in CTLL-2 (Merida and Gaulton 1990b) and B lymphoma cell lines (Eardley and Koshland 1991), generating two potential signal mediators, a myristylated diacylglycerol and an inositol phosphate glycan (Merida and Gaulton 1990b, Eardley and Koshland 1991). Interestingly, IL-4 stimulation failed to induce GPI hydrolysis in the same cells (Eardley and Koshland 1991). Although the functional roles of these mediators have not yet been determined, it is possible that a GPI-specific phospholipase might be activated after IL-2 receptor stimulation.

3.4 Effect on Early Gene Expression

One of the ultimate end products of the signal transduction mechanisms coupled to specific surface receptors is to effect changes in gene expression in the target cells. Nuclear proto-oncogenes have come to be recognized as principle targets for the signals produced by a plethora of surface receptors involved in proliferation, differentiation and other cellular processes (Cantley et al., 1991; Hunter 1991). Most of the products of nuclear proto-oncogenes possess DNA binding properties either individually or in combination and it is well established that most function as regulators of gene transcription (Gutman and Wasylyk 1991).

Cytokines have been shown to induce the expression of many genes, including nuclear proto-oncogenes, in a variety of cell types including T lymphocytes (Reed et al., 1985a, 1985b; Cleveland et al., 1987), B lymphocytes (Kelly et al., 1983), and myeloid cells (Conscience et al., 1986). Following IL-2 stimulation a rapid and transient increase in *c-fos* and *c-jun* expression, as well as a more stable accumulation of *c-myc* and *c-myb* transcripts has been reported in a variety of cell types (Reed et al., 1985a, 1985b; Stern and Smith 1986, Cleveland et al., 1987; Pauza

1987; Trouche et al., 1991; Hatakeyama et al., 1992, Shibuya et al., 1992). IL-2R β is likely linked to at least two intracellular signaling pathways mediating nuclear proto-oncogene induction (Shibuya et al., 1992). One pathway, coupled to tyrosine phosphorylation events and mediated by Src-family PTK(s), leads to the induction of the *c-fos*, *c-jun* and other genes. Another as yet undefined pathway leads to *c-myc* gene induction (Shibuya et al., 1992).

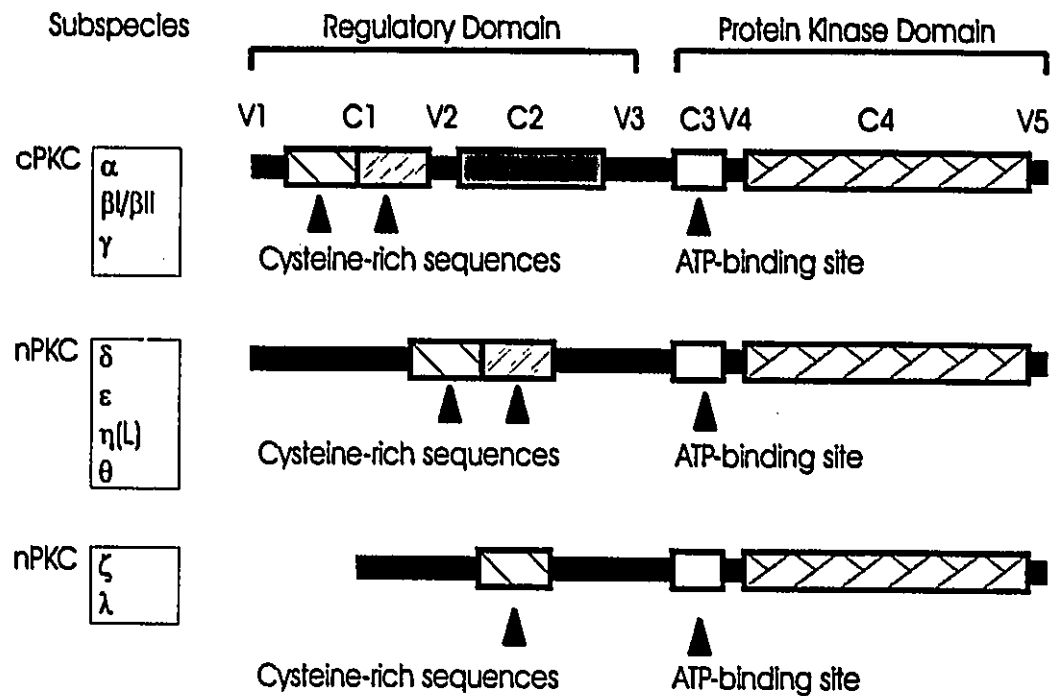
4. Protein Kinase C (PKC)

Protein kinase C (PKC) is a multifunctional serine/threonine protein kinase. It was first identified in 1977 as a proteolytically activated protein kinase present in most tissues (Inoue et al., 1977; Takai et al., 1977). PKC is distributed among several particulate and soluble cellular compartments, but what is clear is that PKC must be membrane associated in order to be active (reviewed by Bell and Burns 1991). As originally defined, PKC is a Ca^{2+} , and phospholipid dependent, diacylglycerol stimutable protein kinase. It is now known that PKC exists as a family of multiple subspecies having closely related structures (Kikkawa et al., 1989, Nelsestuen and Bazzi 1991, Nishizuka 1986). Collectively, the enzymes appear to be composed of single polypeptide chains with a molecular weights of about 80 kDa. At least 12 (mammalian) isoforms with distinct structural and enzymatic properties have been identified to date (reviewed by Dekker and Parker, 1994). All PKC isoforms contain conserved (C1 -C4) and variable (V1 -V5) regions, and are composed of catalytic and a regulatory domains (see Scheme I-4). Proteolytic removal of the regulatory domain by cleavage in the V_3 region renders the catalytic domain constitutively active and unresponsive to the PKC cofactors DAG and Ca^{2+} (Farago and Nishizuka 1990). The amino-terminal half, containing region C1 and C2, constitutes the regulatory domain that interacts with Ca^{2+} , phospholipid and diacylglycerols or phorbol esters (Nelsestuen and Bazzi 1991). This

region also contains an autoinhibitory pseudosubstrate region thought to be involved in the process leading to enzyme activation. It is believed that this pseudosubstrate sequence binds to and blocks the active site in the absence of co-factors needed for enzyme activity (Bell and Burns 1991). The C1 region contains a tandem repeat of a cysteine-rich zinc-finger-like sequence (Ono et al., 1989b). This sequence seems to be essential for the binding of phorbol esters, and by inference, of DAG itself (Ono et al., 1989b). The C2 region is responsible for the Ca^{2+} sensitivity of the enzyme (Bell and Burns 1991). The carboxyl-terminal half of PKC, containing regions C3 and C4, constitutes the protein kinase domain and contains the ATP and protein substrate binding sites. These domains are highly conserved and have been found to share sequence homology with many other protein kinases (Farago and Nishizuka 1990). The catalytic site of the enzyme is embodied within the C3 region of the protein (Bell and Burns 1991).

4.1 PKC Isoforms

Based on their structure, the members of the PKC family can be classified into three groups (Table I-2 and Scheme I-4). The conventional or classical PKC isoforms (α , $\beta_{1/II}$ and γ) conform to the original definition of PKC as a Ca^{2+} - and phospholipid- dependent protein kinase. Although several phospholipids will support cPKCs activity, phosphatidylserine (PS) appears to be the most effective phospholipid for reconstituting enzyme activity *in vitro* (Bell and Burns 1991). Membrane-associated cPKC activation is regulated *in vivo* through the binding of DAG (Nishizuka 1986, Sando et al., 1992, Newton 1993). The Ca^{2+} dependence of cPKCs is also mediated by a binding site located in the C_2 region of the protein (Nishizuka 1986, Sando et al., 1992, Newton 1993). The binding of calcium to the regulatory domain is a prerequisite for the association of cPKCs with membrane phospholipid (reviewed by Bell and Burns 1991, and Sando



Scheme I-4. Structure of PKC subspecies. Four conserved (C₁ to C₄) and five variable (V₁ to V₅) regions of the cPKC group are indicated. The β I and β II subspecies are derived from a single gene by alternative splicing. Details are outlined in the text. Copied from Nishizuka 1992.

et al., 1992). Fully active cPKCs exist as quaternary complexes composed of phospholipid, calcium, DAG and the enzyme; the ternary complex without DAG exhibits kinase activity only at a 100-fold higher concentration of calcium (reviewed by Bell and Burns 1991, Sando et al., 1992).

The novel isoforms (nPKCs δ , ϵ , η (L), θ , and μ) lack the C₂ region, and as a result they are Ca²⁺ independent (Bell and Burns 1991). The atypical forms (aPKCs ζ , τ and λ) are characterized by the fact that they lack one of the two cysteine-rich zinc-finger regions present in the other isoforms. Consequently, when bound to membranes these isoforms are constitutively active since they are unable to bind or be regulated by phorbol esters (Liyanage et al., 1992, McGlynn et al., 1992, Nakanishi and Exton 1992). Collectively, the novel and the atypical isoforms of PKC are referred to in the literature as 'nonconventional' PKC isoforms.

4.2 PKC distribution

PKC is a ubiquitous enzyme present in all eukaryotes (reviewed by Farago and Nishizuka 1990, Nishizuka 1995). Because of its central role in cell signalling, it is not surprising that PKC is found in all mammalian tissues, albeit, as summarised in Table I-2, the distribution of the various isoforms of the enzyme exhibit significant tissue specificity. Until recently, it was believed that PKC- γ is expressed exclusively in the central nervous system, with the highest enzyme activity in the hippocampus, cerebral cortex, amygdaloid complex, cerebellar cortex and spinal cord (Huang et al., 1987; Saito et al., 1988). By contrast, the PKC isoforms β I and β II are present in varying ratios in many but not all tissues and cell types (Table I-2). Normally, the amount of the β II-subspecies far exceeds that of β I (Farago and Nishizuka 1990). PKC- α is widely regarded as being universally distributed in mammalian systems (reviewed by Farago and Nishizuka 1990). The study of nPKCs and aPKCs distribution is ongoing and less is presently

known, however it is apparent that they are also differentially expressed in different tissues and cells (Table I-2). In addition to eukaryotes, PKCs have also been found in lower organisms including *Drosophila* (Schaeffer et al., 1989), *Xenopus leavis* (Chen et al., 1989), *Sea Urchin egg* (Shen et al., 1989), *Dictyostelium Discoideum* (Jimenez et al., 1989) and *Saccharomyces cerevisiae* (Simon et al., 1991).

Evidence is accumulating that PKC isoforms can be differentially distributed within various cellular compartments, including the cytosol, nucleus, cell membranes, and cytoskeleton elements (Hug and Sarre 1993, Newton 1993, Inagaki et al., 1994). It is not possible to establish a general consensus with respect to the subcellular distribution of specific PKC isoforms since each cell type seems to express a defined set of PKC isozymes with unique subcellular patterns of distribution (Hug and Sarre 1993, Newton 1993, Inagaki et al., 1994).

4.3 Endogenous PKC substrates

The fact that multiple PKC moieties are expressed in a single cell suggests the presence of a complex web of PKC-mediated signalling pathways. However, at the present time little is known about the mechanisms by which different PKC isozymes are activated, and there is a paucity of information on the molecular steps between PKC activation and the broad range of biological responses that such activation evokes in cells. One approach to elucidate the molecular pathways downstream of PKC activation is to identify the specific cellular substrates of PKC. PKC substrates have been classified into three broad categories according to their requirements for phosphorylation by PKC (Bazzi and Nelsestuen, 1987). Substrates in the first category, such as protamine, require no additional factors for phosphorylation by PKC. Substrates in the second category, such as myelin basic protein, require phospholipids for PKC directed phosphorylation. Substrates in the third category, which include histone, myelin light protein, MARCKS

Table I-2 PKC subspecies in mammalian tissue^a

Group	Subspecies	Apparent molecular mass (kDa)	Activator ^c	Tissue expression
cPKC	α	76799	Ca^{2+} , DAG, PS, FFA, LysoPC	Universal
	βI	76790	Ca^{2+} , DAG, PS, FFA, LysoPC	Some tissues
	βII	76933	Ca^{2+} , DAG, PS, FFA, LysoPC	Many tissues
	γ	78366	Ca^{2+} , DAG, PS, FFA, LysoPC	Brain only
nPKC	δ	77517	DAG, PS	Universal
	ϵ	83474	DAG, PS, FFA	Brain and others
	$\eta(\text{L})^b$	77972	?	Lung, skin, heart
	θ^a	81571	?	Skeletal muscle (mainly)
aPKC	ζ	67740	PS, FFA	Universal
	λ^a	67200	?	Ovary, testis and others

* Modified from Nishizuka 1992

† The detailed enzymological properties of the η (L)-, θ -, and λ - subspecies have not yet been clarified

‡ The activators for each subspecies are determined with calf thymus H1 histone and bovine myelin basic protein as model phosphate acceptors

(myristoylated alanine-rich C kinase substrate) and troponin I, require the presence of both Ca^{2+} and phospholipids to undergo phosphorylation by PKC.

The best known and the most widely studied endogenous PKC substrate is the MARCKS protein (Stumpo et al., 1989; Graff et al., 1989b; Brooks et al., 1991; Erusalimsky et al., 1991; Harlan et al., 1991; Sakai et al., 1992). MARCKS was originally identified as a protein (initially termed "80K") which was transiently phosphorylated in cells following treatment with phorbol esters such as 12-o-tetradecanoyl-phorbol-13-acetate (TPA) (Rozengurt et al., 1983). It is a widely distributed specific PKC substrate, whose phosphorylation in cells has been used as a marker of PKC activation *in vivo* (Brook 1994). MARCKS, and its phosphorylation site peptide, is at best a poor substrate for a wide range of other protein kinases including cAMP- and cGMP-dependent kinases, and calmodulin-dependent protein kinases I, II, and III (Graff et al., 1991, Hartwig et al., 1992). MARCKS has been found to bind calmodulin in a Ca^{2+} -dependent manner and to be an actin filament cross-linking protein (Graff et al., 1989; Hartwig et al., 1992). It has been demonstrated that the binding of MARCKS to calmodulin and actin is prevented by PKC phosphorylation (Graff et al., 1989; Hartwig et al., 1992). MARCKS has been implicated in a variety of cellular processes, including secretion, membrane trafficking, cell motility, regulation of the cell cycle, and transformation (Rozengurt et al., 1983; Blackshear 1993, Brooks 1994). All of these processes necessitate the regulated rearrangement of the actin cytoskeleton (Aderem 1992),

which has led to the suggestion that MARCKS plays a key role in translating extracellular signals into alterations in actin plasticity and actin-membrane interactions (Aderem 1992).

While much is known about MARCKS protein phosphorylation by PKC, it is an unfortunate truism that despite many years of intensive study, relatively little is known about other endogenous PKC substrates, and what role they play in elucidating the wide range of cellular responses evoked by PKC activation.

4.4 PKC in cell signaling (General)

PKC isoforms are involved in the transduction of a wide variety of cellular signals. Much of the initial interest focused on these enzymes stemmed from the identification of PKC as the high affinity intracellular receptor for phorbol esters, a class of potent tumor promoters (Ashendel, 1985). The direct activation of PKC by phorbol esters was the first indication that PKC is involved in growth control and it is now widely accepted that PKC plays a pivotal role in the regulation of both proliferation and differentiation of many, if not all, cells (Clemens et al., 1992, Nishizuka, 1992). Over the last ten years it has become increasingly clear that many fundamental physiological processes are mediated by changes in cellular PKC activity such as the secretion and release of cellular constituents from endocrine, exocrine and neuronal tissues (reviewed by Nishizuka 1988), as well as smooth muscle contraction, metabolic processes such as steroidogenesis (Pelosin et al., 1987), and the control of proliferation and differentiation of different lymphocytes populations (Nishizuka 1986). In addition to its traditional role in the phosphoinositol signaling pathway (Nishizuka 1984), PKC is also a key player in other Ca^{2+} and cAMP mediated pathways (Nishizuka 1986, Nishizuka 1988). Moreover, an emerging role for PKC appears to lie in its ability to act as a positive and negative regulator of various ion-channel activities (Sigel and Bauer 1988, Shearman et al., 1989). A large body of evidence also indicates

that PKC exerts negative-feedback control or down-regulation of a number of surface receptors that are coupled to inositol phospholipid hydrolysis, such as the epidermal growth factor receptor (EGF) (Schlessinger 1986) and the T-cell surface CD3 complex (Cantrell et al., 1987). One of the key end points of PKC activation is in regulating gene expression. A single, pertinent example is the determination that the sustained activation of PKC is needed in T-cells to induce IL-2 and IL-2R α gene expression, which are necessary steps in T-cell activation and proliferation (Berry et al., 1990a, 1990b).

4.5 The use of PKC inhibitors to elucidate IL-2-signalling

A wide range of protein kinase C inhibitors accessible to cells are now exist, and they have proven to be important biochemical tools in probing the mechanisms by which PKC is regulated in cells. Inhibitors are also used to explore the various cellular activities of PKC and to study the role of protein kinase C in mediating a plethora of biological responses. Cell permeable PKC inhibitors are now available which act on either the catalytic or regulatory domains of the enzyme (Table I-3): for example, calphostin C interacts with the enzyme's regulatory domain (Kobayashi et al., 1989c), isoquinoline (H-7) and staurosporine bind to and block the ATP-binding site (Hidaka et al., 1984; Tamaoki et al., 1986), and inhibitors such as 1-o-alkyl-2-o-methylglycerol (AMG) blocks the DAG site on PKC (Kramer et al., 1989). Staurosporine, a member of a group of antibiotics shown to exhibit protein kinase inhibitory activity, is a remarkably effective inhibitor of protein kinase C (Tamaoki et al., 1986; Gross et al., 1990; Herbert et al., 1990). Kinetic and binding data have shown that staurosporine inhibits protein kinase C with a K_i of 1 to 3 nM (Tamaoki et al., 1986; Gross et al., 1990; Herbert et al., 1990).

However, some caution must be exercised when using staurosporine as a PKC-selective inhibitor, since as shown in Table I-3, several other kinases, including PKA and PKG, are blocked by

Table I-3 PKC inhibitors

Name				K _i or IC ₅₀ (μ M)							
	PKC	PK	TPK	PKA	PKG	MLCK	CaM K	CK- I	CK- II	Isos- yme test-ed	Ref
BIS	0.014	0.7	50 (InsulinR, EGFR, PDGFR)	2.0	-	-	-			α βγ	Toullec et al., 1991
Calpho- stin C	0.05	>50	>50 (p60 ^{src})	>50	>25	>5				α βγ	Kobayash i et al., 1989c
H-7	6.0			3.0	5.8	97		100	780	ND	Ilidaka et al., 1984
Stauro- sporine	0.003	0.06- 6		0.007	0.0085	0.001	0.02			ND	Tamaoki et al., 1986
Sphing- osine	10		EGFR							ND	Hannun et al., 1986
AMG	80			>100			>100			ND	Kramer et al., 1989

staurosporine concentrations that approach that effective in inhibiting PKC activity (Tamaoki et al., 1986). Bisindolylmaleimide is a staurosporine analogue reported to be a potent PKC inhibitor with a higher degree of PKC selectivity (Table I-3) than its parent (Toullec et al., 1991).

AMG was originally detected in cell membranes as a metabolite of the anti-tumor agent 1-o-alkyl-2-o-methylglycero-3-phosphocholine (van Blitterswijk 1987a). AMG is an ether-linked analogue of diacylglycerol, which inhibits the activation of purified protein kinase C by competing for the DAG binding site (van Blitterswijk 1987b). AMG has been found to have no effect on the activity of cAMP-dependent or Ca²⁺/calmodulin-dependent protein kinase in cell-free assay systems (Table I-3), and to inhibit protein kinase C in a dose-dependent fashion (Kramer et al.,

1989). Calphostin C was initially isolated from the soil fungus *Cladosporium* (Kobayashi et al., 1989a, b, c) and has proved to be a highly selective PKC inhibitor which blocks DAG binding with a K_i of about 50 nM (Table I-3). Unfortunately, to date no inhibitors have been identified which inhibit specific PKC isoforms and this fact has severely impaired efforts to elucidate the functions of each of the isozyme of PKC.

4.6 Mechanisms of PKC activation

4.6.1 Conventional view

It has been the traditional view that the stimulation of PKC activity results from the translocation of cytosolic enzyme to membranes (Kraft and Anderson 1983; Anderson et al., 1985; Farrar and Anderson 1985; Abou-Samra et al., 1989; Kondratyev et al., 1990; Kalbag et al., 1991;). The translocation of PKC can be readily measured by immunoblot and conventional activity methods designed to detect the amount of enzyme present on membranes and its soluble compartments (Kraft and Anderson 1983, Kondratyev et al., 1990). In this scenario, increases in intracellular Ca^{2+} , effected by various signalling stimuli, enhances the interaction of cytosolic PKC with the lipid components of membranes (Nelsestuen and Bazzi 1991). Once membrane-associated, the enzyme is activated by the binding of DAG, generated by the hydrolysis PIP_2 or PC (Asaoka et al., 1992, Nishizuka 1992, 1995). Studies on the mechanisms of lipid activation of PKC have established that the enzyme is activated specifically by the sn-1,2-enantiomer of DAG (Boni and Rando, 1985) and that DAGs possessing long-chain, unsaturated acyl groups are the most effective activators of PKC (Boni and Rando, 1985). The regulatory action of DAG's can be mimicked by metabolically stable DAG analogues such as the potent tumor promoter TPA, which has been used extensively to study PKC (Asaoka et al., 1992, Nishizuka, 1992, 1995). This phorbol ester causes the rapid translocation of PKC from the cytosol to membrane compartments

and is a powerful PKC activator (Kraft and Anderson 1983, Nishizuka 1984). Long-term PKC activation by TPA is also known to effect down regulation of the enzyme and in cases can cause a complete loss in cellular PKC (Nishizuka 1984), at least those PKC isoforms known to be dependent on DAG for activity (i.e. cPKC, nPKC, but not aPKC).

4.6.2 Conventional assay methods

This conventional view of PKC activation assumes that if PKC is membrane-associated, it is active, and that translocation is the regulating step controlling the cellular levels of PKC activity. Because of this, conventional methods for measuring PKC activity require that the membranes first be solubilized and their associated PKCs then reconstituted into artificial phospholipid membranes in the presence of maximally effective concentrations of TPA, PS and excess Ca^{2+} (Takai et al., 1979, Kikkawa et al., 1982).

In general, three variations of the conventional assay have been reported and widely applied. In the first method, the enzyme(s) is extracted from membranes with non-ionic detergents, partially purified by column chromatography, reconstituted into an artificial lipid environment using phosphatidylserine, and its activity measured using histone H1, a non-specific PKC substrate, in the presence of ATP, Ca^{2+} and TPA (Takai et al., 1979). In the second method, membrane PKC is also extracted with detergents, but the extracted enzyme is assayed directly, without partial purification, using a modified histone H1 protein believed to be a specific PKC substrate (Halsey et al., 1987). A variation of this assay (House and Kemp 1987, 1990, Smith et al., 1989, Yasuda et al., 1990), using a new generation of PKC selective peptide substrates, is now widely used. In the third method, cells are permeabilized with digitonin to release soluble PKC and the residual membrane-associated enzyme is then partially trypsinized to release the constitutively active catalytic domain, PKM and its activity measured using histone H1

in the presence of a cyclic AMP-dependent protein kinase inhibitor (Pelech et al., 1986).

However, the utility of these assays are becoming increasingly limited as it becomes clear that the premise of the conventional view, namely that all membrane PKC is active, is simplistic, and does not explain many experimental paradigms.

4.6.3 Emerging view of PKC activation

In contrast to earlier views, it is becoming increasingly evident that the mechanisms by which PKC activity is regulated in cells are significantly more complex than previously realized. In many cases, cellular PKC activity can be altered by manipulating pre-existing pools of membrane PKC in the absence of detectable translocation of cytosolic enzyme (Halsey et al., 1987, Mukaida et al., 1988, Pelech et al., 1990a, 1990b, Tang and Houslay 1992) either *via* chemical modification (Pelech et al., 1990a) or by the direct activation of inactive PKC present in membranes (Chakravarthy et al., 1992, 1994, 1995a, Durkin et al., 1992).

Experiments with purified PKCs and phospholipid vesicles (Bazzi and Nelsestuen 1988) or erythrocyte membranes (Wolf et al., 1985; May et al., 1985) have suggested that PKCs might associate with cell membranes in a Ca^{2+} -dependent (i.e. EGTA-sensitive) manner and remain there in an inactive, but still signal-responsive state (Bell 1986; Bell and Burns 1991). However, it was not possible to demonstrate the presence of such an inactive, signal-responsive pool of membrane PKCs because of the inability of conventional PKC assays to distinguish between different activation states of PKC (Takai et al., 1979, Kikkawa et al., 1982). As described previously, conventional methods require that membranes first be solubilized and their associated PKCs then reconstituted into artificial phospholipid membranes in the presence of a maximally effective concentration of a specific activator, the TPA, and excess Ca^{2+} (Kikkawa et al., 1982; Hannun et al., 1985). These manipulations would fully activate all PKCs, including any inactive

enzyme(s) that might be associated with the native cell membranes. Thus, conventional *in vitro* assays can measure changes only in the total amount of membrane-associated PKCs and not changes in the activity of pre-existing membrane-associated PKCs, except in cases where enzyme modification leads to activation (Pelech et al., 1990b). In other word, the stimulation of a pre-existing pool of membrane PKCs without the translocation of enzymes from the cytosol would go undetected by both the conventional activity assay and by any immunoblotting assay.

The above situation has been rectified by the recent development of a 'direct' PKC assay (Chakravarthy et al., 1991) which measures enzyme activity directly in its native membrane-associated state. Using this *in vitro* assay it has been possible to demonstrate that growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), IL-3, and neurotransmitters such as glutamate stimulate PKC activity in target cells, not by inducing the translocation of cytosolic enzyme to membranes, but by activating pre-existing pools of inactive membrane PKC (Chakravarthy et al., 1992, 1995, Durkin et al., 1995). Moreover, PKC that is associated with membranes in an inactive state had been found to require only DAG for activation (Chakravarthy et al., 1994). Although the receptor-mediated hydrolysis of inositol phospholipids was once thought to be the sole mechanism leading to the DAG production and the activation of PKC (Nishizuka 1984), recent studies suggest that several additional cellular mechanisms exist which provide PKC-activating DAGs. For example, the hydrolysis of phosphatidylcholine (Asaoka et al., 1992) and phosphatidylethanolamine (Kester et al., 1989) have been shown to be important sources of diacylglycerol in many cells (Scheme I-5).

Perhaps the most convincing evidence that membrane-associated inactive PKC can be activated by different stimuli without translocation from cytosol to membrane is that the PKC-specific substrate, the 80 kDa MARCKS protein, has be shown to undergo endogenous

phosphorylation under conditions in which translocation of PKC was undetectable, but in which the direct assay showed the activation of inactive membrane PKC (Chakravarthy et al., 1994). Thus, the *in vitro* measurement of PKC activity by the direct assay had been shown to truly reflect the changes in membrane-associated PKC activity occurring in intact cells.

Even the historic role of DAG and Ca^{2+} as the sole regulators of PKC activity are now less certain. For example, it has been reported that arachidonic acid and its metabolites are able to activate some subspecies of PKC under specific conditions. Arachidonic acid can be derived from inositol phospholipids through two consecutive reactions, catalysed by phospholipase C and diacylglycerol lipase (Bell et al., 1979), and it can also be derived from phosphatidic acid by a specific lipase (Billah et al., 1981). However, in most tissues arachidonic acid is released by phospholipase A_2 from phosphatidylcholine and phosphatidylethanolamine (Scheme I-5). The role of arachidonic acid in endogenous PKC activation is not entirely clear but reports indicate that this fatty acid can activate PKC *in vitro* by mechanisms either independent of PS and DAG (McPhail et al., 1984) or working synergistically with DAG (Asaoka 1992b).

4.6.4 Direct *in vitro* PKC assay method

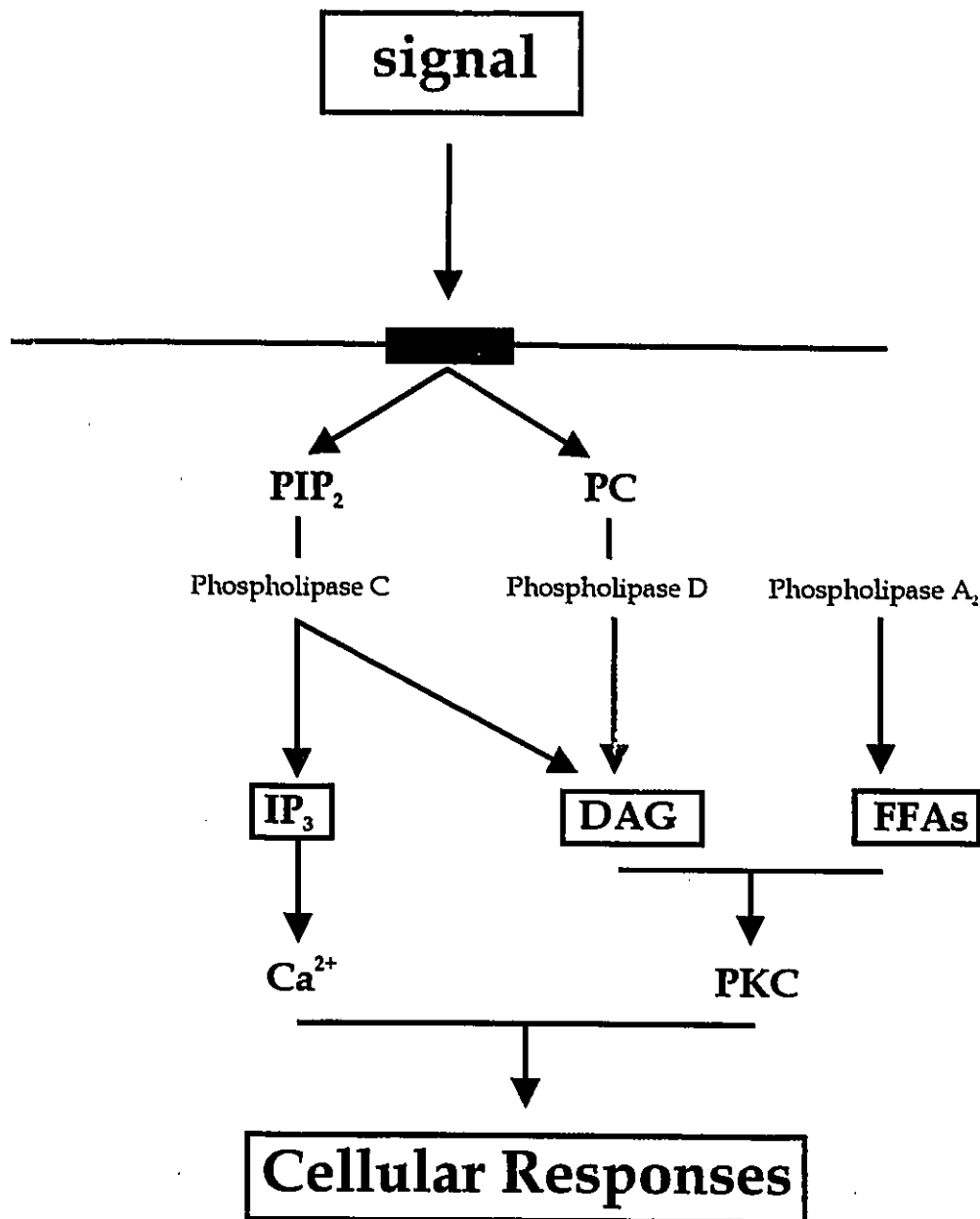
The recently described direct PKC assay (Chakravarthy et al., 1991) measures enzyme activity directly in its native membrane-associated state, without prior detergent solubilization and reconstitution of the enzyme with exogenous PS, TPA, and excess Ca^{2+} . By this method, enzyme activity is measured by using the highly PKC-selective peptide substrate Ac-FKKSFKL- NH_2 , (Chakravarthy et al., 1991, Williams et al., 1992, Orr et al., 1992), recognized by all of the major PKC isoforms (Heemskerk et al., 1993) in the presence of a Ca^{2+} concentration ($1\mu\text{M}$) that closely approximates the intracellular situation. Because no enzyme activation steps are involved in this procedure, the assay measures only that PKC which is present on membranes in an active

state (Chakravarthy et al., 1992, 1994, Durkin et al., 1992). However, if isolated cell membranes also possess an inactive pool of PKC, it can be rapidly and completely activated *in vitro* by adding either 1.0 μM TPA, 50 to 100 μM diacylglycerol, or excess Ca^{2+} (100 μM) to the reaction mixture. The addition of TPA to the membrane preparation gives a measure of total membrane PKC activity (active + inactive), and thus the amount of both active and inactive enzyme can be determined quantitatively by this method.

5. PKC in IL-2 cell signaling

While the importance of PKC in the signaling pathways of many receptors has been established, the role of PKC in IL-2 signalling remains controversial. In some cases it has been reported that IL-2 induces phosphoinositide hydrolysis, increases in intracellular Ca^{2+} and PKC translocation and activation (Farrar et al., 1986; Evans et al., 1987b, Farrar and Anderson 1985). Other reports indicate that other effects of IL-2, such as the reduction in cAMP levels, are mediated through a PKC-dependent pathway (Beckner and Farrar 1986, 1987). Moreover, the PKC inhibitor, H-7, has been shown to block IL-2 stimulated cell proliferation in T-cells (Mukaida et al., 1988). On the other hand, a series of studies have failed to observe such PKC-linked changes associated with IL-2 stimulation of cells (Mills et al., 1985a; 1986; 1988; Gelfand et al., 1987; Valge et al., 1988; Redondo et al., 1988; Paetkau and Mills 1989). In particular, in a widely-referenced study by Mills et al (1988), PKC⁻ mutants were used to show that despite a loss of PKC activity, cells were still able to proliferate in response to IL-2.

Signal routes leading to the activation of PKC may greatly vary with cell types, extracellular signals, and perhaps with the time after cell stimulation. Thus, given what we now know about the complexity of PKC activation, the failure of some studies to demonstrate PKC translocation, PIP_2 hydrolysis, and Ca^{2+} fluxes *does not* necessarily rule out the involvement of



Scheme I-5. A schematic representation of the induction of cellular responses by the signal-induced degradation cascade of membrane phospholipids. PIP₂, phosphatidylinositol bisphosphate; IP₃, inositol triphosphate; PC, phosphatidylcholine; DAG, diacylglycerol; FFAs, free fatty acids; PKC, protein kinase C. Modified from Nishizuka 1992.

PKC activation in IL-2 signalling. Moreover, it must be remembered that most studies of PKC in IL-2 signalling have looked at cell proliferation as the cellular response to the cytokine. Although proliferation is a very important cellular response to IL-2, it is but one of several responses evoked by this cytokine in IL-2 sensitive cells. For example, in addition to its mitogenic effects, IL-2 promotes cytotoxic activity in NK cells (Henney et al., 1981) and in IL-2-dependent cytotoxic T cell lines (Kornbluth and Hoover, 1988; Karnitz et al., 1992), and the production of IgG in B cells (Blackman et al., 1986). The importance of PKC in these IL-2-induced cellular responses has not been explored. Perhaps more important is the fact that aside from the above mentioned study by Mills et al, most studies attempting to link changes in PKC activity with IL-2 stimulation have relied on the traditional dogma that the translocation of cytosolic PKC to the membranes is a necessary step in PKC activation. However, as detailed in Section 4.6 in this Introduction, the fact that quantitative changes in the total amount of membrane PKC were not observed in these cases, does not in itself constitute proof that changes in membrane PKC *in situ* had not taken place.

The objective of this thesis was to determine whether IL-2 indeed stimulates PKC in T-cells, and if so by what mechanism. It was also the purpose of this study to determine what role PKC plays in the pleiotropic responses of cells to IL-2, such as T-cell survival and cytotoxic activity. Studies described in Chapter 1 establish an *in vitro* model for study PKC in IL-2 signalling. In Chapter 2, these models were used to investigate the role of inactive membrane-associated PKC in T-lymphocyte survival and cytotoxic activity. In Chapter 3, the mechanisms responsible for the activation of inactive PKC by IL-2 were explored.

THESIS OBJECTIVES

The overall objective of this thesis is to gain a clearer understanding of the role of PKC in IL-2 signaling by focusing on the involvement of PKC in IL-2 mediated T-cell survival and cytotoxic activity, and to establish mechanisms of IL-2 induced PKC activation. The specific objectives are as follows:

1. To establish an *in vitro* model to study the role of protein kinase C in IL-2 signal transduction (Chapter 1).
2. To determine whether the activation of inactive-membrane PKC is necessary for IL-2-dependent cell survival and cytotoxic activity (Chapter 2).
3. To determine the IL-2 dependent signaling pathways leading to PKC activation (Chapter 3).

CHAPTER ONE: ESTABLISHMENT OF AN *IN VITRO* CELL MODEL TO STUDY THE ROLE OF PROTEIN KINASE C IN IL-2 SIGNAL TRANSDUCTION

1.1 INTRODUCTION

Interleukin-2 (IL-2) is a critical regulator of the immune system. During the last ten years, tremendous work has been done in terms of elucidating the biological functions of IL-2, and exploiting this knowledge for clinical purposes. For example, in preliminary clinical trials, anti-IL-2 receptor (IL-2R) monoclonal antibodies (mAb) and chimeric IL-2 toxins have showed promising signs of efficacy in that they appear to effect improvements in patients with IL-2R⁺ leukemia/lymphoma, as well as in patients with refractory rheumatoid arthritis or new-onset diabetes mellitus (Strom et al., 1993). However, despite these promising findings, the mechanisms underlying IL-2 signaling remain elusive. It is becoming increasingly apparent that aberrations in signaling pathways are at least partially linked to a number of disorders and disease states, including cancers, atherosclerosis, psoriasis, and diseases of the immune system, such as septic shock, tissue rejection and rheumatoid arthritis (reviewed by Levitzki 1994). Thus, it has been suggested that agents which antagonize tyrosine kinases, protein kinase Cs, Ras, Ca²⁺ signaling, as well as estrogen, may be potential tools in cancer management (Levitzki 1994). Clearly, understanding the nature of the biochemical signaling imparted by IL-2 would at the very least help to identify new therapeutic targets to which drugs could eventually be developed.

In order to define the complexity of the signaling pathways coupled to IL-2, it is critical to have experimental models that are both reproducible and relevant. In a global sense, studies related to IL-2 signaling pathways can be conducted both *in vivo* and *in vitro*. It is absolutely evident that studies utilizing mice, rats, and monkeys have been invaluable in demonstrating the

efficacy of immuno-suppression of anti-IL-2R α mAbs for transplantation, graft tolerance, and autoimmune diseases (Strom et al., 1993). On the other hand, animal models are expensive, difficult to control and manipulate, and are increasingly difficult to justify on ethical grounds. As an alternative, much important preliminary work can be conducted using *in vitro* models as long as such models can be shown to be relevant to the *in vivo* case.

In vitro studies can be conducted either in primary cell culture or in immortalized cell lines. In principle, primary cell cultures are more “real” but they usually suffer from problems associated with heterogeneity in that even the most highly purified preparations are likely to be contaminated with other cell types, making the interpretation of data difficult. Another problem with using primary cultures throughout an extended study is that it is often difficult to maintain high levels of experimental reproducibility, a problem arising from the necessity to perform repeated isolation of the cells from animals in which the investigator has only limited control (i.e. diet, stress, supplies, viruses, etc). By comparison, immortalized cell lines which respond to IL-2 in the predicted *in vivo* manner offer several advantages to the study of IL-2 signalling.

Interleukin-2 dependent cytotoxic T cell lines have proved to be very useful systems for investigating IL-2 signaling in T-cells. They provide a unique opportunity to investigate IL-2 linked intracellular signal transduction pathways because many of these cell lines closely represent normal activated cytotoxic T-cells being strictly dependent on IL-2 for growth and viability. The cytotoxic T-cell line, CTLL-2, was originally generated from secondary allogeneic mixed tumor-lymphocyte cultures (MTLC) (Gillis and Smith, 1977). The cells are blastoid in shape and highly vacuolar in appearance, much like normal activated cytotoxic T-cell (Eisen 1990). The CTLL-2 cell line was judged an appropriate *in vitro* model to study IL-2 signal transduction in this study because (1) it is relatively easy to control and manipulate the

conditions of CTLL-2 cell growth, (2) the line has proved over the years to be genetically and phenotypically stable (Saltzman et al., 1988, Sharon et al., 1990) (3) the cells exhibit many of the characteristics of the normal cytotoxic T cells from which they are derived (Gillis and Smith 1977), (4) large amounts of cells can be generated at low cost in a relatively short period of time, a practical necessity given the types of biochemical studies to be conducted and most importantly (5), CTLL-2 cells have functional IL-2 receptors on their cell surface (Cano et al., 1992).

Moreover, they bear endogenous IL-2R $\beta\gamma$ complexes and the IL-2R α subunit can be induced after IL-2 stimulation as occurs in T-cells (Merida et al., 1990a, Sharon et al., 1990, Cano et al., 1992). Since CTLL-2 cells bear IL-2Rs that are indistinguishable to that found *in vivo* and are dependent on IL-2 for viability and proliferation, they were judged to be a suitable model for examining the mechanisms by which intracellular signals are relayed from the membrane to the nucleus following IL-2 stimulation.

CTLL-2 cells have been widely used to study various aspects of T-cell signalling mechanisms. For example, they have been used as a standard cell line to assay IL-2's ability to stimulate cell proliferation (Gillis et al., 1978, Robb et al., 1981). In recent years, CTLL-2 cells have been used to study the role of phosphatidylinositol-3-kinase (Merida et al. 1991), phosphatidic acid (PA) (Cano et al. 1992), and glycosylphosphatidylinositol (GPI) lipids (Merida et al. 1990a) in IL-2 signaling mechanisms. The cell line has also been used to study the role of rapamycin in the inhibition of IL-2-induced T-cell proliferation (Morice et al., 1993), the signaling steps leading to *c-fos* gene expression (Trouche et al., 1991), and the role of PKC translocation and PKC activity in T-cell proliferation (Redondo et al., 1988, Mukaida et al., 1988, Kim et al., 1989). However, the role of PKC in physiological responses other than proliferation to IL-2 have not been explored in CTLL-2 cells.

As discussed in the General Introduction (section 5), the role of protein kinase C in IL-2 induced cell proliferation is controversial in that some researchers believe PKC to be involved in the process, while others discount its role mainly because of the widely-referenced study by Mills et al. (1988) which reported that PKC⁻ mutants were able to proliferate in response to IL-2. However, it must be noted that in the study of Mills, individual clones of cells were isolated from cell populations maintained in the phorbol ester, TPA (10^{-6} M), and high concentration of IL-2 (1000U/ml). Since it was not demonstrated in their study that their PKC⁻ clones did not arise from mutations that allowed cells to bypass a normally PKC-mediated proliferative response, it cannot be concluded categorically that PKC is not part of signalling pathway normally coupled to IL-2 stimulated proliferation in T-cells. Moreover, it must be remembered that although proliferation is a very important cellular response to IL-2, it is but one of several responses evoked by this cytokine in IL-2 sensitive cells. Thus, in addition to its mitogenic effects, IL-2 promotes the cytotoxic activity of NK cells (Henney et al., 1981) and IL-2-dependent cytotoxic T cell lines (Kornbluth et al., 1988, Karnitz et al., 1992), and the production of IgG in B cells (Blackman et al., 1986). The importance of PKC in these IL-2-induced cellular responses is essentially unexplored, and is the subject of this thesis.

1.2 OBJECTIVES

The overall objective of the study described in Chapter I was to establish and characterize several of the key parameters needed to investigate the role of PKC in IL-2 signal transduction in CTLL-2 cells. The specific objectives were:

- 1.2.1 To establish that IL-2 is a mandatory cytokine for CTLL-2 cell survival and proliferation.
- 1.2.2 To establish the importance of serum factors present in the growth medium in IL-2-induced survival and proliferation of CTLL-2 cells.
- 1.2.3 To establish the relative levels of the principle PKC isozymes present in CTLL-2 cells and to determine the distribution of PKC in various cellular compartments.

1.3 METHODS

Cell culture

Stock cultures of CTLL-2 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD20852, USA). The cells were maintained in complete medium, consisting of RPMI 1640 (Flow Laboratories, Irvine, CA) supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY), 100U/ml penicillin, 0.1 mg/ml streptomycin, 0.2% sodium bicarbonate, 57 μ M 2-mercaptoethanol (ME), and 100 U/ml IL-2 (a gift of the Biologic Response Modifiers Program, Division of Cancer Treatment, National Institutes of Health). Cells were typically seeded at a density of $1-2 \times 10^4$ cells/ml in complete medium, grown in a humidified environment of 95% air and 5% CO₂, and used for experiments when cell densities reached 0.5 to 1×10^6 cells/ml. For experiments on cell survival, proliferation, and PKC activity, CTLL-2 cells were washed twice in RPMI medium, then incubated in unsupplemented RPMI medium (i.e. serum and IL-2 free) for specified times before being subjected to the indicated treatments.

1.3.1 Cell lysis and the isolation of membrane and cytosolic fractions

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) (140 mM NaCl, 4 mM KCl, 500 μ M Na₂HPO₄, 150 μ M KH₂PO₄), suspended in 1 ml ice-cold hypotonic lysis medium (1 mM NaHCO₃, 5 mM MgCl₂, and 100 μ M phenylmethyl sulfonylfluoride, pH 7.5) for 10 min, and lysed by vortexing vigorously for 2 min. All subsequent steps were conducted at 4°C. Nuclei and unlysed cells were sedimented at 600 x g for 5 min, and membrane and cytosol fractions were separated by centrifugation at 100,000 x g for 10 min in a Beckman TL-100 ultracentrifuge.

1.3.2 The assay of protein kinase C activity

A) Conventional PKC assay: The amounts of PKC present in the cytosol and membrane fractions were measured by the conventional PKC assay, as described previously (Chakravarthy et al., 1991, Durkin et al., 1992, Chakravarthy et al., 1992). Briefly, for the measurement of membrane PKC, membranes from $5-10 \times 10^6$ cells were solubilized by suspending them in solubilizing buffer (50 mM Tris-HCl buffer [pH 7.5] containing 2 mM EGTA, 0.5 mM EDTA, and 0.1% Triton X-100), followed by incubation for 15 min on ice with periodic vortexing. Under these conditions, more than 95% of membrane-associated PKC was extracted. The membrane extract was clarified at $100,000 \times g$ for 10 min and the supernatant used for the measurement of PKC activity. For the measurement of cytosolic PKC levels, the cytosolic fraction was initially adjusted to 50 mM Tris-HCl (pH 7.5), 2 mM EGTA, 0.5 mM EDTA, and 0.1% Triton X-100. PKC activities in the membrane and cytosolic fractions were measured in a reaction mixture (100 μ l) containing of 5 to 10 μ g of cellular protein and 75 μ M of the PKC-selective peptide substrate (see below) in a conventional assay buffer (50 mM Tris-HCl buffer [pH7.5], 5 mM $MgCl_2$, 2 mM $CaCl_2$, 10 μ g phosphatidylserine, 1 μ M TPA, 100 μ M sodium vanadate, 100 μ M sodium pyrophosphate, 1 mM sodium fluoride, 100 μ M PMSF). The reaction was initiated by the addition of 50 μ M ATP (0.5 μ Ci [32 P] ATP, 6,000Ci/mmol) and after 10 min at 25°C incubation, the reaction was stopped by adding 10 μ l 5% acetic acid and the samples clarified by centrifugation at 16,000 $\times g$ for 5 min in a microfuge. A 90- μ l sample of each supernatant was applied to P81 Whatman paper (2 cm^2)(VWR Scientific of Canada Ltd, Toronto, Ontario), and the papers were washed twice for 10 min in 5% acetic acid (10 ml/ cm^2 of paper) at RT. The radioactivity bound to the washed papers was determined by liquid scintillation counting using Formula-989 scintillation cocktail.

In other cases, membrane and cytosolic PKC were partially purified (Kikkawa et al., 1982, Hannun et al., 1985) before being subjected to the conventional assay described above. Briefly, membrane PKC was detergent-solubilized as described above, and then subjected to DE-52 gel filtration chromatography (1 cm X 0.5 cm) in a buffer consisting of 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 200 μ M sodium vanadate, 200 μ M sodium pyrophosphate, 2mM sodium fluoride, and 100 μ M PMSF. PKC was eluted from the column in the same buffer containing 90 mM sodium chloride, and assayed for PKC activity as described above. Cytosolic PKC was partially purified and assayed in an equivalent manner.

B) Direct PKC assay: Membrane-associated PKC activity was measured directly in isolated membranes (Chakravarthy et al. 1991), without previous extraction and reconstitution of the enzyme in artificial phospholipid membranes, by the incorporation of ³²P into the PKC-selective peptide substrate Ac-FKKSFKL-NH₂. This peptide, which corresponds to the region of the phosphorylation site of the MARCKS protein, a cellular substrate of PKC (Blackshear 1993), has been shown to be a highly specific substrate for PKC in *in vitro* assays (Chakravarthy et al. 1991, Williams et al. 1992, Orr et al. 1992, Jouishomme et al., 1992, Heemskerk et al. 1993). Both the peptide and MARCKS itself are only poorly phosphorylated by CaM kinase, phosphorylase kinase, and protein kinase A (PKA) (Chakravarthy et al. 1991, Blackshear 1993) and in the conventional PKC assay, the peptide is not phosphorylated by kinase present in crude cell extracts in the absence of PS/TPA (Chakravarthy et al., 1991, Orr et al., 1992). Moreover, in the direct assay the peptide is not phosphorylated by membranes isolated from cells in which PKC had been down-regulated by long-term TPA exposure (Jouishomme et al., 1992), and the phosphorylation of the peptide by membrane-associated kinases is abolished completely in the presence of the pseudosubstrate PKC-specific inhibitor, RFARKGALRQKNVHEVKN, a

peptide corresponding to the pseudosubstrate region of PKC (House and Kemp 1987, Chakravarthy et al., 1991).

For the measurement of active membrane PKC by the direct method, isolated membranes from $5-10 \times 10^6$ cells were suspended in direct assay buffer (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1 μM $CaCl_2$, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride, and 100 μM PMSF), and vigorously vortexed to obtain a homogeneous suspension. The reaction mixture (100 μl) consisted of 5 to 10 μg membrane protein and 75 μM peptide substrate in direct assay buffer. The reaction was initiated by the addition of 50 μM ATP (0.5 μCi [^{32}P] ATP, 6000 Ci/mmol) and after 10 min at 25°C the reaction was stopped by the addition of 10 μl of 5% acetic acid and the samples clarified by centrifugation at 16,000 $\times g$ for 5 min in a microfuge. As was the case with the conventional assay, a 90- μl sample of each supernatant was applied to P81 Whatman paper (2 cm^2), and the papers were washed twice for 10 min in 5% acetic acid (10 ml / cm^2 of paper). The radioactivity bound to the washed papers was determined by liquid scintillation counting.

For the measurement of total membrane PKC (as opposed to that active in membrane preparations), 1 μM TPA was added to the reaction mixture to activate any inactive, membrane-associated enzyme (Chakravarthy et al., 1991, 1992, Durkin et al., 1992). The reaction was conducted as described above for the direct assay. In all cases, PKC activity was expressed as the amount of ^{32}P (cpm) incorporated into the peptide substrate/10 min/8 μg protein.

1.3.3 Assessment of PKC isozyme levels and distribution in CTLL-2 cells

Protein kinase C isozymes present in CTLL-2 cells were determined by Western blot analysis using isozyme specific polyclonal antibodies to PKC (GIBCO BRL, Grand Island, NY, USA). Briefly, CTLL-2 cell membrane and cytosolic fractions were prepared by hypotonic lysis

and centrifugation as described in 1.3.1. Isolated cell membranes were subsequently suspended in direct assay buffer (Appendix A). Equal volumes of SDS solubilization buffer (10% glycerol, 5% β -ME, 3% SDS [w/v], 10 mM Tris, pH 6.8, 0.01% [w/v] bromophenol blue) were added to the membrane and cytosol preparations and 10-20 μ g of protein were electrophoresed by 10% SDS-PAGE (Laemmli, 1970). The gels were then equilibrated for 30 min in transfer buffer (192 mM glycine, 25 mM tris-base [pH8.3], 0.1% SDS in 20% [v/v] methanol) and transferred electrophoretically to nitrocellulose using transblotting apparatus (the series transphor electrophoresis unit from Hoeffer Scientific Instrument, San Francisco 94107) for 2 hour at 4°C at constant voltage of 200V. Following transfer, non-specific binding sites were blocked by incubation of the nitrocellulose paper in blocking solution (1% BSA, 25 mM Tris-HCl, pH8.0, 37.5 mM NaCl, 0.05% Tween 20 [v/v]) for two hours at RT. The papers were then incubated for two hours at RT in TBST solution (25 mM Tris-HCl, pH 8.0, 37.5 mM NaCl, 0.05% Tween 20 [v/v]) containing rabbit polyclonal antibodies against the PKC isozymes α , β , γ , δ , ϵ , ζ at a dilution of 1:1000.

The nitrocellulose papers were washed three times for 10 min each in TBST then incubated for 90 min with a secondary antibody (alkaline phosphatase conjugated affinity purified goat anti-rabbit IgG [H+L]) (Sigma, ST. Louis, MO, USA) diluted in 1:5000 with TBST. The papers were washed three times for 10 min each to remove unbound antibody, and transferred to a color development solution (100 mM Tris-HCl, pH9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% [w/v] nitro blue tetrazolium [NBT], 0.02% [w/v] 5-bromo-4-chloro-3-indolylphosphate [BCIP]) for up to 10 min. The color reaction was stopped by rinsing the membrane in deionized water for several min.

The following is a description of the antibodies used in this study (from GIBCO): anti-PKC α anti-peptide antibodies were generated in rabbits using the peptide described by Makowske et al. (1988) (ala-gly-asn-lys-val-ile-ser-pro-ser-glu-asp-arg-arg-gln). This sequence corresponds to amino acids 313-326 of rat PKC α . Anti-PKC β anti-peptide antibodies were generated in rabbits using the peptide gly-pro-lys-thr-pro-glu-glu-lys-thr-ala-asn-thr-ile-ser-lys-phe-asp (Makowske et al. 1988). This sequence corresponds to amino acid 313-329 of rat PKC β . Anti-PKC β raised in this manner will not distinguish between the β I and β II subtypes which differ only in their C-terminal region. Anti-PKC γ anti-peptide antibodies were generated in rabbits using the peptide asn-tyr-pro-leu-glu-leu-tyr-glu-arg-val-arg-thr-gly (Makowske et al. 1988). This sequence corresponds to amino acid 306-318 of rat PKC γ . Anti-PKC δ anti-peptide antibodies were generated in rabbits using a peptide from the C-terminus of PKC δ , corresponding to amino acids 662-673 (ser-phe-val-asn-pro-lys-tyr-glu-gln-phe-leu-glu) (Ono et al., 1988). Anti-PKC ζ anti-peptide antibodies were generated in rabbits using the peptide described by Ono et al. (1989a) (gly-phe-glu-tyr-ile-asn-pro-leu-leu-leu-ser-ala-glu-glu-ser-val). This sequence corresponds to amino acids 577-592 of PKC ζ . Anti-PKC ϵ anti-peptide antibodies were generated in rabbits using a peptide from the C-terminus of PKC ϵ (lys-gly-phe-ser-tyr-phe-gly-glu-asp-leu-met-pro). This sequence corresponds to amino acids 726-737 of PKC ϵ (Ono et al., 1988).

1.3.4 Assessment of cell proliferation

Cell proliferation was assessed by the extent of [3 H]-thymidine incorporation into cellular DNA. For [3 H]-thymidine incorporation, CTLL-2 cells were washed twice, resuspended in either unsupplemented RPMI 1640 medium, or medium containing serum, IL-2, or serum plus

IL-2, as described in the legends of individual experiments. The cells were plated at a density of 1×10^4 cells/ml into 24 well multiculture dishes. At the indicated times, [^3H] thymidine was added to the medium at a final concentration of $4\mu\text{Ci/ml}$ (25 Ci/mmol) for either 1 or 4 hours. The cells were collected on GF/C glass microfiber filters (VWR Scientific of Canada Ltd, Toronto, Ontario), precipitated on the filter with ice cold 5% perchloric acid, and washed 2 times with PBS containing cold thymidine (0.24 g/ml). The filters were counted in a liquid scintillation counter and DNA synthesis expressed as cpm [^3H]-thymidine incorporated per dish (Jones et al., 1986).

1.3.5 Assessment of cell viability

Viable cells were determined microscopically by their ability to exclude the vital stain, trypan blue (Sigma Chemical Co., ST. Louis, MO, USA). Trypan blue (0.4%) was added to cells at a concentration of $1\mu\text{l}/4\mu\text{l}$ cell suspension and the proportion of cells able to exclude the dye was determined microscopically using a haemocytometer. The degree of cell death in cultures was also determined by the level of lactate dehydrogenase (LDH) release into the medium (Martinek 1972) using a commercially available LDH assay kit (Sigma Chemical Co., ST. Louis, MO, USA). The assay measures the LDH-catalyzed conversion of pyruvate to lactate in the presence of NADH. The rate of decrease at 340 nm , due to the conversion of NADH to NAD^+ , is directly proportional to LDH activity in the sample. Briefly, the media from cell cultures were collected by centrifuging the cultures at 600 xg for 5 min. Samples of media ($50\mu\text{l}$) were added to 1.85 ml of 0.1 M potassium phosphate buffer ($\text{pH}7.5$) containing $100\mu\text{l}$ of substrate (0.53 mM NADH, 22.7 mM sodium pyruvate in 0.1 M potassium phosphate buffer [$\text{pH}7.5$]), and the absorbance at 340 Å recorded every 30 second for 3 min. The readings were plotted against time,

and LDH activities were calculated according to the formula described by Wroblewski and LaDue (1955).

1.3.6 Assessment of cytotoxic activity of CTLL-2 cells

The cytotoxic activity of CTLL-2 cells were determined by a 51 chromium release cytotoxicity assay (Tracey et al., 1977). S49.1 lymphoma cells were used as the target cell population since they bear the same H2^d surface antigen as CTLL-2 cells were raised against. S49.1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in 75 cm² culture flasks and used in cytotoxic assays when they reached a density of 1×10^6 cell/ml. 0.1 ml of the S49.1 cell suspension ($\sim 10^7$ cells) was added to 0.2 ml sodium 51 chromate (1mCi/ml, 200-500 mCi/mg Cr; Amersham Canada, Oakville, Ontario) and incubated for 90 min at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The cells were then washed 3 times in 90%RPMI/10%FCS and cell numbers determined using a hemocytometer. The CTLL-2 cytolytic reaction was started by mixing varying concentrations of CTLL-2 cells (100µl) with the 51 Cr-loaded target cells (100µl, 10^4 cells) in a microfuge tube followed by centrifugation at 300 xg for 5 min to ensure direct contact between the target and effector cells. After a 4 hour incubation at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, samples were centrifuged again for 5 min at 300 xg, and both the 51 Cr released into the media and the total 51 Cr in the sample (i.e. media + cells) were determined by scintillation counting. Specific lytic activity was calculated relative to the spontaneous release observed in tubes containing target cells alone using the formula:

$$\% \text{ specific cytolytic activity} = (\text{test cpm} - \text{spontaneous cpm}) / (\text{total cpm}) \times 100$$

Miscellaneous

Protein concentrations were determined by the method of Bradford (1976).

1.4 RESULTS

1.4A. Characterization of the role of IL-2 in CTLL-2 cell proliferation, viability, and cytotoxic activity

1.4A.1 *Interleukin-2 is critical for CTLL-2 cell survival and proliferation*

Several different techniques were used to determine the effects of IL-2 on CTLL-2 cell survival and proliferation. Initially, the effects of IL-2, serum, and IL-2 plus serum on viable cell numbers was studied. As shown in Figure 1.1, CTLL-2 cells grew exponentially in the presence of 10% serum and 100U/ml IL-2 (1U/ml of IL-2 will induce 50% of the maximum proliferation [10,000cpm/3000cells] during 4 hour pulse with 0.5 μ Ci of 3 H-thymidine [20Ci/mM] [Gillis et al., 1982]). By comparison, serum or IL-2 alone were not able to stimulate an increase in viable cell numbers. In the presence of 100U/ml IL-2 alone, viable cell numbers were maintained up to at least 72 hours and the cells looked morphologically normal (data not shown). By contrast in the presence of serum alone, the cells became morphologically ragged and their viability could not be maintained beyond 48 hours. In the absence of both serum and IL-2, viable cell numbers decreased by 50% within the first 24 hours, and most cells died before 48 hour. In the presence of serum factors, IL-2 stimulated CTLL-2 cell proliferation in a dose dependent manner as measured by both viable cell numbers and by [3 H]-thymidine incorporation into DNA (Fig. 1.2). Both methods indicated that half maximal stimulation occurred at ~5-10 U/ml and maximal effects at ~25-50 U/ml IL-2.

In order to determine whether cells maintained in medium supplemented with IL-2 alone were still capable of proliferating, normal CTLL-2 growth medium (serum + IL-2) was added to cells which had been previously maintained in IL-2 medium for varying times. As shown in Fig 1.3, CTLL-2 cells maintained in IL-2 medium for 24 or 48 hours were indeed capable of

proliferating rapidly once serum factors were restored, indicating that the cells' proliferative machinery were fully functional despite the fact that cell numbers were not increased in the absence of serum.

The inability of IL-2 alone to promote an increase in cell number (Fig. 1.1) could have arisen either from a complete shut-down of cell proliferation or from an increase in cell death that matched the rate of cell proliferation. To distinguish between these two possibilities, lactic dehydrogenase (LDH) activity measurements were conducted. LDH is a cytosolic enzyme which is released into the culture medium as a result of a serious loss of cell membrane integrity, and it is often used as an indicator of cell viability or cell death (Wroblewski and LaDuc 1955). As shown in Table 1.1, LDH activity released into the culture medium increased significantly when the cells were incubated for 24 hour in medium alone (i.e. -IL-2) and this was matched with a 50% reduction in cell number. By contrast, in the presence of IL-2 alone (i.e. +IL-2) viable cell numbers did not change significantly when compared to control cultures, nor did a large release of LDH into the medium occur. In fact, the LDH values for cells in IL-2 alone were not significantly different from those measured in cultures actively proliferating in IL-2 and serum containing medium (Table 1.1). These data indicate that CTLL-2 cells do not die in appreciable numbers when incubated in RPMI supplemented with IL-2 and that the "static" cell numbers observed in Fig. 1.1 under these conditions likely arose from a block in cell proliferation. This conclusion was supported by measurements of DNA synthesis in these cells. As shown in Fig. 1.4, only the combination of IL-2 and serum caused a sizable increase in [^3H]-thymidine incorporation into DNA, with neither IL-2 nor serum alone able to support DNA replication. Collectively, these results indicated that in the presence of IL-2 alone, cell proliferation is abated while cell viability is maintained.

Table 1.1 IL-2 is required for CTLL-2 cell viability

	Cell Number per dish	LDH release
- IL-2, -Serum	$3.5 \times 10^4 \pm 3008$ (0.46) ^b	69 Unit/ml (3.0) ^a
+ IL-2 (100U/ml), -Serum	$1.98 \times 10^5 \pm 0$ (1.08)	23.2 Unit/ml (1.01)
+IL-2 (100U/ml) + Serum (10%)	$4.7 \times 10^5 \pm 26698$ (2.6)	23.0 Unit/ml (1.0)

Exponentially growing CTLL-2 cells were washed twice with prewarmed RPMI medium and resuspended in medium alone or in medium supplemented with the indicated concentrations of IL-2 and /or serum at a density of 2.0×10^5 cells /ml. The cells were incubated at 37°C for 24 h, at which time viable cell numbers were determined as described in *Methods*. The values are the means $\pm$ SEM of triplicate determinations. At the same time, the degree of cell lysis was determined by measuring the amount of LDH released into the medium during the 24 h incubation period, as described in *Methods*. Values are representative of three separate experiments.

^aThe change in LDH activity relative to that found in culture with both IL-2 + serum containing medium.

^bNumber in brackets are the fold changes in cell viability related to initial plating densities.

1.4A.2 *The effects of transient withdrawal of serum and IL-2 on CTLL-2 viability.*

The data in Table 1.1 indicated that a substantial loss in viable cell number and a concomitant increase in LDH activity released into the medium was observed 24-hours after withdrawing IL-2 and serum from actively growing CTLL-2. By contrast, a brief withdrawal of IL-2 and serum was, in itself, not lethal and cells could be "rescued" by the timely readdition of these factors to the medium. Thus, the addition of 100 units/ml IL-2 and 10% serum to CTLL-2 cultures deprived of these factors for the previous 7 hours greatly reduced the extent of cell death

measured at 24 hours and permitted a large expansion in cell number (Fig.1.5). Moreover, the addition of IL-2 alone to 7-hr serum/IL-2 deprived cultures reduced both the levels of LDH released into the medium and the loss in cell number (Figure 1.5). These data suggest that while IL-2 was not capable of promoting proliferation of CTLL-2 cells in the absence of additional serum factors (Fig 1.1, 1.5), it was able to prevent cell death in these cultures, a finding consistent with previous reports (Walker et al., 1993). Since depriving CTLL-2 cells of IL-2/serum for periods longer than 7-hours substantially reduced the proportion of viable cells at 24 hours (data not shown), a 6-7 hour deprivation period was used in most experiments to study the effects of IL-2 on cellular PKC activity.

1.4A.3 *IL-2 is required for the cytolytic activity of CTLL-2 cells*

We have established that IL-2 is critical for both CTLL-2 cell survival and proliferation. As mentioned in the General Introduction, CTLL-2 cells are a cytotoxic T-cell line, thus it was important to determine whether the cytotoxic activity of CTLL-2 cells was also dependent on IL-2. To study the cytotoxic activity of CTLL-2 cells, S49.1 lymphoma cells were chosen as target cell because they bear the same H2^d surface antigen that CTLL-2 cells were raised against (Gillis 1977). The cytotoxic activity of CTLL-2 cells was determined using a ⁵¹chromium release cytotoxicity assay (Tracey et al., 1977) in which the ability of CTLL-2 cells to cause the release of ⁵¹Cr from preloaded S49.1 cells was taken as a measure of S49.1 cell lysis. In establishing the experimental conditions it was initially necessary to determine the optimum preloading conditions to maximally load S49.1 cells. It was determined that a 90 minutes incubation with 0.2 mCi ⁵¹Cr (0.2 mCi /10⁷ cells, 1mCi/ml) gave maximal ⁵¹Cr uptake (data not shown). It was then necessary to test different assay times and different ratios of effector (CTLL) to target (S49.1) cells in the reaction. A series of preliminary experiments

indicated that a 4 hour incubation period at an effector/target ratio of 100:1 gave optimal 51 Cr release (data not shown).

To determine the effects of IL-2, serum, and serum plus IL-2 on CTLL-2 lytic activity, exponentially growing CTLL-2 cells were washed once, resuspended in medium supplemented with either 100 U/ml IL-2, 10% serum, serum plus IL-2, or unsupplemented RPMI medium for 18 hours. CTLL-2 cytolytic activity was measured by a 4 hour lytic assay at a ratio of 100:1 of effector to target cells. As shown in Figure 1.6, the withdrawal of serum and IL-2 for 18 hour caused cells to lose almost 90% of their cytolytic activity when compared to cells cultured in the presence of serum and IL-2 for 18 hour. This loss in cytotoxic activity could be largely accounted for by the removal of IL-2 from the medium since serum-containing RPMI without IL-2 could not support CTLL-2 cytotoxic activity (20% of control) whereas cytotoxic activity was only reduced 30% in cells incubated in medium containing IL-2 without serum. The cytolytic activity was found to be dependent on the dose of IL-2 and was maximal at 50 Units/ml (data not shown). These data confirm that the CTLL-2 cell line is indeed a cytotoxic T-cell and that its cytolytic activity is dependent on IL-2 in the medium.

B. Characterization of PKC in CTLL-2 cells

1.4B.1 PKC distribution in CTLL-2 cells

For the determination of PKC distribution within various cellular compartments, CTLL-2 cells were lysed hypotonically and the nuclei and incompletely lysed cells removed by low-speed centrifugation. This crude nuclear preparation was found to contain < 5% phase bright viable cells (data not shown). A highly enriched nuclear fraction, prepared by Triton X-100 treatment of the crude nuclear preparation, was found to contain <1% of the total assayable PKC present in whole cell extracts prepared by sonication and Triton X-100 treatment (Table 1.2).

The majority of cellular PKC activity was found to be relatively equally distributed between the cytosolic and membrane fractions (Table 1.2). However, as shown in Table 1.2, a sizable amount (~30%) of the total cellular PKC was found in Triton X-100 extractable material associated with the crude nuclear fraction. It is likely that the majority of this PKC activity resided in membranes which were associated with pelleted nuclei (and solubilized by detergent treatment), and thus, the percentage of PKC found in the membrane fraction (32%) was probably an underestimate, although other possibilities exist (see discussion).

It is known that membrane-associated PKC can exist in two forms: peripherally bound PKCs, which can be readily stripped off from the membranes by Ca^{2+} -chelating agents such as EGTA, and 'membrane-inserted' PKCs, which are tightly bound to the membrane and cannot be removed by chelation (Bazzi and Nelsestuen 1988, Nelsestuen and Bazzi 1991, Chakravarthy et al., 1994). Using a conventional PKC assay, it was determined that in exponentially growing CTLL-2 cells, 40% of the total membrane PKC was tightly associated with membrane while 60% was associated with membranes in loosely-bound or peripheral bound form which was readily removed from membranes by brief EGTA treatment (Table 1.3).

1.4B.2 *PKC isozyme distribution in CTLL-2 cells*

Molecular cloning and biochemical analysis has revealed that PKC exists as a family of at least 12 isozymes having closely related structures (Nishizuka 1988, 1992, Stable and Parker 1991). In order to determine the isozyme distribution in CTLL-2 cells, western blots were performed on CTLL-2 cell membrane and cytosolic fractions using commercially available polyclonal PKC antibodies (GIBCO BRL, Grand Island, NY, USA). Six PKC antibodies against PKC α , β , γ , ϵ , δ , ζ were used to test the distribution of PKC isozymes in CTLL-2 cells. The levels of PKC α were relatively low in CTLL-2 cells with the majority found in the cytosol

Table 1.2 The distribution of PKC in CTLL-2 cells

Fraction	PKC Activity (cpm / 10^6 cells)
Total	75791 $\pm$ 4254
Nuclei	538 $\pm$ 50 (0.7%)
Membrane	26899 $\pm$ 1312 (36%)
Cytosol	23918 $\pm$ 413 (32%)
Supernatant from crude nuclei	22858 $\pm$ 539 (30.8%)

Exponentially growing CTLL-2 cells, were washed twice with ice-cold PBS and suspended in ice-cold hypotonic lysis medium (Appendix) at 2.7×10^7 cells/ml. For the measurement of total cellular PKC activity, 1 ml of cell suspension was homogenized in a Potter-Elvehjem homogenizer for 10 seconds, Triton X-100 was added to 100 μ l of the homogenized sample to a final concentration of 0.1%, and 5 μ l was used to determine total PKC by the conventional assay as described in *Methods*. For the determination of PKC in the membrane, cytosol, and nuclear fractions, 1 ml of cell suspension was lysed in hypotonic lysis medium by vortexing as described in *Methods*. Nuclei and unlysed cells were separated by low-speed centrifugation. All subsequent steps were carried out at 4°C. Membrane and cytosol fractions were separated by spinning at 100,000 xg for 10 minutes and the isolated membrane suspended in lysis medium. Triton X-100 was added to the isolated membrane and cytosol preparations to a final concentration of 0.1%, and their PKC activity determined by the conventional PKC assay (details in *Method* section). Crude nuclei were further purified according to the method Weaver et al. (Weaver 1993) with minor modifications. Briefly, Triton X-100 was added to the nuclear pellet to a final concentration of 0.1% and after a 10 min incubation at 4°C, the cleaned nuclei were pelleted at 500 xg for 5 min, the supernatant retained for PKC assay, and the pelleted nuclei suspended in PBS containing 0.1% Triton X-100. The nuclei were lysed by sonification and assayed for PKC activity. Values are the means $\pm$ SD of triplicate determinations of PKC activity in 10^6 cells and are representative of three separate experiments.

Table 1.3 The distribution of PKC in CTLL-2 cell membranes

	PKC activity (cpm / 10 ⁵ cells)	
	- EGTA	+ EGTA
Membrane	19,505 ± 1,617 (47.5%)*	8,032 ± 258 (14.4%)
Cytosol	21,575 ± 3,200 (52.5%)	48,412 ± 1,969 (85.7%)

Exponentially growing CTLL-2 cells were washed twice in PBS, resuspended in hypotonic lysis buffer (1 mM NaHCO₃, 5 mM MgCl₂, and 100 µM phenylmethyl sulfonylfluoride), or in the same buffer containing 1 mM EGTA, and lysed by homogenization in a Potter-Elvehjem homogenizer with 20 up-and-down strokes. All subsequent steps were carried out at 4°C. Nuclei and unlysed cells were sedimented at 600 xg for 10 min. Membrane and cytosol fractions were separated by centrifugation at 100,000 xg for 10 min in a Beckman TL-100 ultracentrifuge. The total amounts of EGTA-extractable and nonextractable membrane PKC, and the corresponding levels of cytosolic enzyme, were determined by using the conventional assay described in *Method*.

* Numbers in brackets denote percentage of total cellular PKC.

fraction. By comparison, far more PKC β was present in membranes than that in the cytosolic fraction (Fig. 1.7). The antibody against PKC β was raised against a peptide corresponding to amino acids 313-329 of the rat PKC β central region and consequently it was not able to distinguish between the β I and β II subtypes which differ only in their C-terminal regions. The analyses as performed suggest that there is more PKC β isozyme than that PKC α in CTLL-2 cells. Although, PKC γ has been reported to be present exclusively in neuronal tissues (Huang et al., 1987, Saito et al., 1988), the anti-PKC γ antibody used did react with CTLL-2 cell membrane and cytosol (Fig.1.7). Whether this indicates the presence of PKC γ in the cells or reflects the non-specificity of the antibody is unclear. At the time at which these experiments were conducted, only antibodies against the nPKC isoforms δ and ϵ were available commercially.

While PKC ϵ was not detected in CTLL-2 cells, PKC δ was present in both cytosol and membrane fractions (Fig. 1.7), but at levels far lower than that observed for cPKC isoforms. The only aPKC antibody available, that against PKC ζ , indicated that this isoform was present in CTLL-2 cells at low titer mainly in cytosol fraction (Fig.1.7).

1.4B.3 PKC activity in exponentially growing CTLL-2 cells

As discussed in the General Introduction (Section 4.6), the activity of membrane-associated PKC can be readily measured in intact isolated membranes by the "direct" PKC assay without the need for prior detergent solubilization and reconstitution of the enzyme with exogenous PS, TPA, and excess Ca^{2+} (as needed in conventional assays). Using this method, the level of membrane-associated PKC activity in exponentially growing CTLL-2 cells was determined. As shown in Fig.1.8A, membrane PKC activity increased significantly after cells were fed with complete CTLL-2 growth medium (100 U/ml IL-2, 10% serum in RPMI). There was a rapid 3.6 fold increase in membrane PKC activity within the first 10 min which decreased to basal level by 1 hour after feeding. Such a spike in PKC activity was not observed in cells fed with medium alone (data not shown). Aside from a slight increase in membrane PKC activity at 10 hours enzyme activity was relatively stable up to 48 hours post-feeding. By contrast, cytosolic PKC, measured by the conventional PKC assay, showed a progressive increase in activity over the first 4 hours after feeding. Cytosolic PKC activity then stabilized at this elevated level for the next 30 hours before declining by 48 hours. The cytosolic PKC data indicates that the cytosolic levels of PKC were highly susceptible to media conditions. Both the rapid increase in membrane PKC and the more gradual increase in cytosolic enzyme likely reflected the fact that cells were beginning to "run out" of vital media constituents at the time of feeding (the cells had been last fed 3 days prior to $t = 0$). The cytosolic PKC data also indicates

that the cells found themselves back in this less than optimal condition 30-48 hours after feeding. Thus, for most subsequent studies, cells were typically feed 24 hr prior to beginning an experiment to ensure optimal, stable and reproducible PKC levels from experiment to experiment.

1.4B.4 *The effect of IL-2, and serum deprivation on PKC activity of CTLL-2 cells*

It has been established that the withdrawal of serum and IL-2 from CTLL-2 cells has a profound effect on cell viability, with 50% of the cells dying within 24 hours (Fig. 1.1, 1.5). The effects of serum and IL-2 withdrawal on CTLL-2 cell PKC activity was determined. As shown in Fig. 1.9, the removal of IL-2 and serum from the media of exponentially growing CTLL-2 cells induced a rapid reduction in membrane PKC activity, which fell to ~38% of control values within 4 hours of serum/IL-2 starvation (average $35.3 \pm 1.3\%$, $n=3$). The ability of IL-2 and/or serum re-stimulate PKC activity in IL-2/serum starved cells was then determined. Exponentially growing CTLL-2 cells were "starved" of IL-2 /serum for either 6 or 24 hours and then, challenged with IL-2, serum, or TPA and membrane-associated PKC activity determined by the direct assay. As shown in Fig. 1.10A, after 6 hours of starvation, IL-2, serum+IL-2, and TPA all stimulated membrane PKC activity to varying degrees with TPA having the maximal effect. Thus, ten minutes of IL-2 stimulation (100U/ml) caused a 3 fold increase in membrane PKC activity, serum (10%) and IL-2 (100U/ml) increased membrane PKC activity 5-fold, and ten minutes TPA (1 μ M) stimulation caused a 7 fold increase in membrane PKC activity. Interestingly, serum alone had no ability to stimulate PKC activity in CTLL-2 cells incubated in RPMI for 6 hours, although there seemed to be a synergistic effect between IL-2 and serum in stimulating PKC (Fig. 1.10A). By 24 hours of starvation, the surviving cells were highly responsive to both serum and IL-2 (Fig. 1.10B) with IL-2, serum+IL-2 and TPA stimulating

membrane PKC activity about same extent. These data suggest that membrane PKC activity was initially sensitive to the loss of IL-2 and that a sensitivity to serum deprivation developed in a more gradual manner. Nevertheless, the important point was that CTLL-2 cells deprived of IL-2 responded to the re-addition of the cytokine by a rapid and robust stimulation of membrane PKC activity. Significantly, withdrawal of serum/IL-2 for only 2 hours did not give a robust and reproducible increase in PKC activity (data not shown). In summary, these data are consistent with the notion that a brief withdrawal (6-7 h) of serum and IL-2 does not affect cell viability, and that these cells respond to IL-2 stimulation with a rapid, and significant increase in membrane PKC activity.

Figure 1.1 CTLL-2 cells are dependent on IL-2 for proliferation and survival

CTLL-2 cells growing exponentially in RPMI medium supplemented with 10% fetal bovine serum and 100 U/ml IL-2, were washed twice with prewarmed RPMI medium and resuspended at a density of 1.5×10^4 cells/ml in medium alone (▼), or medium containing 100 U/ml IL-2 (◆), 10% serum (▲), or 100 U/ml IL-2 and 10% serum (●). At the times indicated, the numbers of viable cells were determined by trypan blue exclusion as described in *Methods*. The values are the means $\pm$ SEM of triplicate cultures and are representative of five separate experiments.

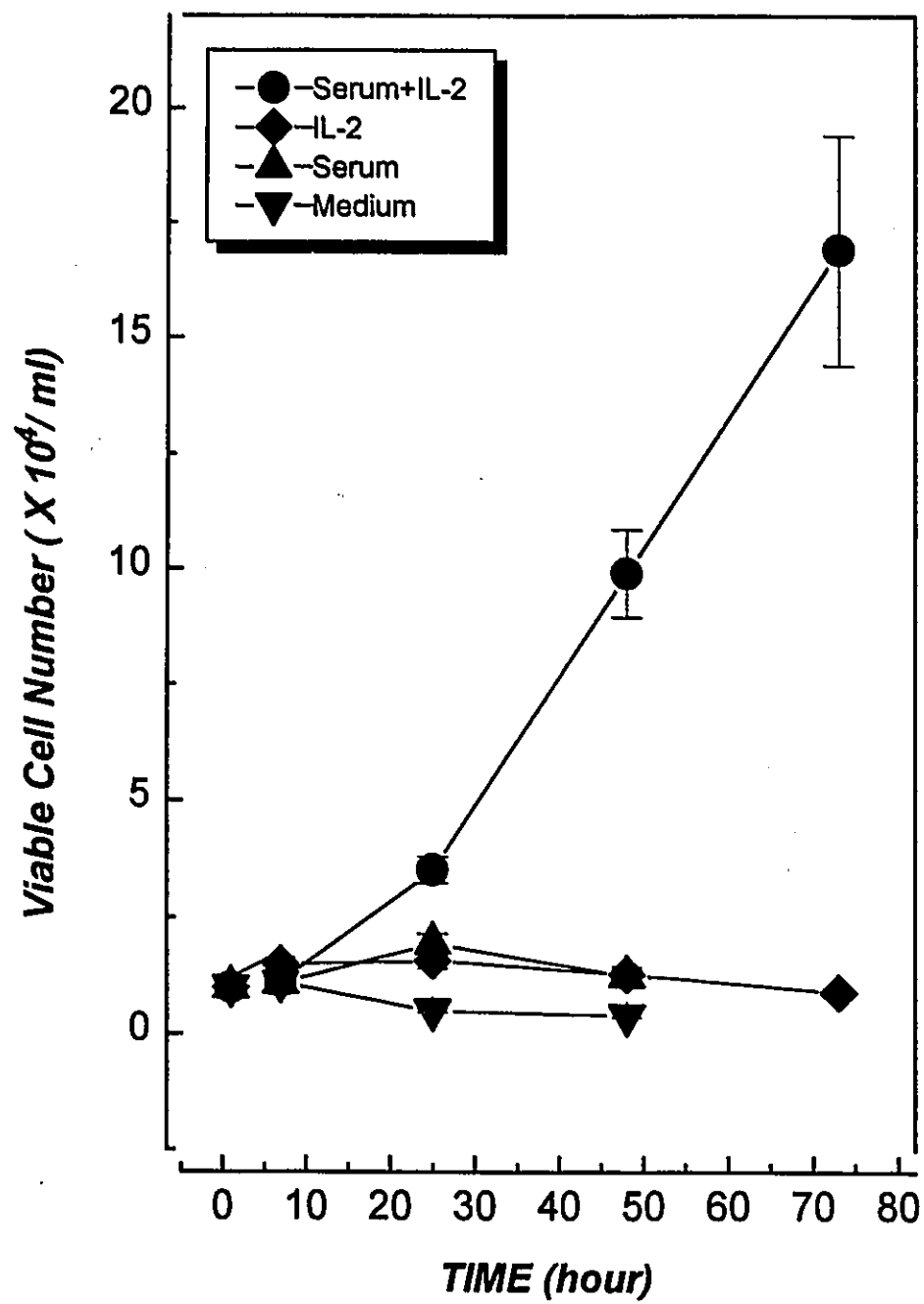


Figure 1.2 IL-2 stimulates CTLL-2 proliferation in a dose-dependent manner

Exponentially growing CTLL-2 cells, were washed twice with prewarmed RPMI, and resuspended at a density of 5×10^5 cells /ml in RPMI medium supplemented with 10% serum and the indicated concentrations of IL-2. After a 24 hour incubation some dishes were used to determine viable cell numbers as described in *Methods*. For other cultures, [^3H]-thymidine ($4\mu\text{Ci}$ /dish, 25 Ci/mmol) was added during the last hour of incubation, and the incorporation of [^3H]-thymidine into DNA determined by the method of Jones et al. as described in *Methods*. The values are means $\pm$ SEM of triplicate cell cultures and are representative of 2 separate experiments.

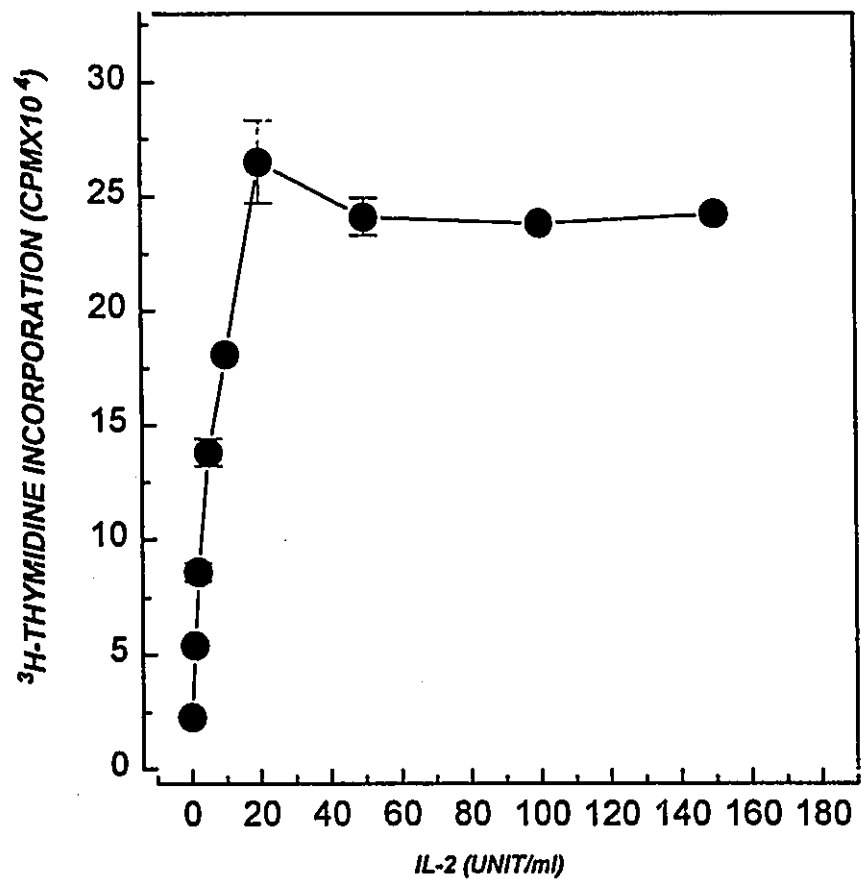
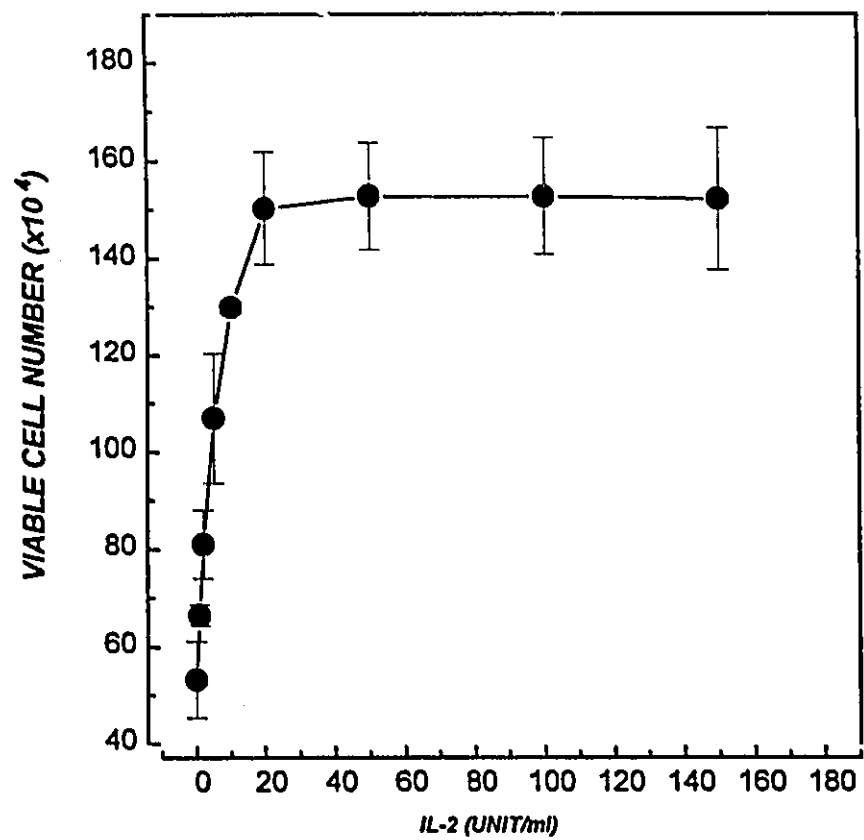


Figure 1.3 IL-2 maintained viable cells can resume proliferation once transferred back to normal growth medium

Exponentially growing CTLL-2 cells were washed twice with prewarmed RPMI and resuspended at 1×10^5 cells /ml in medium containing 100 U/ml IL-2. At 24 (▲) and 48 (▼) hours, serum was added to some cultures to a final concentration of 10%. At 24, 48, 72, and 96 hours, viable cell numbers were determined by the trypan blue exclusion method as described in *Methods*. The values are the means $\pm$ SEM of triplicate samples.

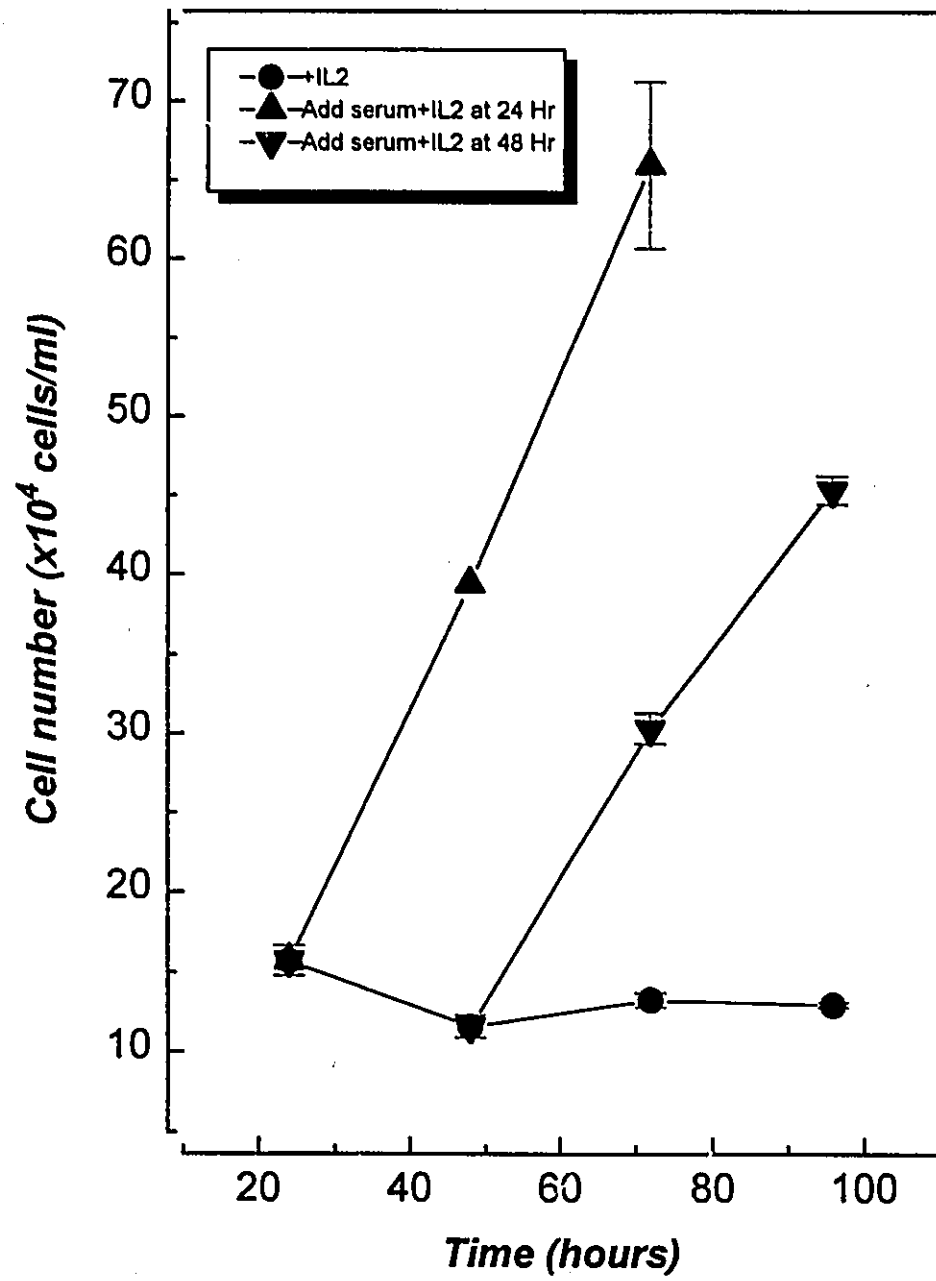


Figure 1.4 Both serum and IL-2 is required for DNA synthesis in CTLL-2 cells

Exponentially growing CTLL-2 cells were washed twice with prewarmed RPMI, resuspended in RPMI containing either 10% serum, 100 U/ml IL-2, or 10% serum plus 100 U/ml IL-2, and incubated at 37°C for 24 h. During the last hour of the incubation, [³H]-thymidine (4 µCi /dish, 25 Ci /mmol) was added to each dish, and the incorporation of [³H]-thymidine into DNA was determined by the method of Jones et al as described in *Methods*. The values are the means ± SEM of triplicate samples and are representative of five separate experiments.

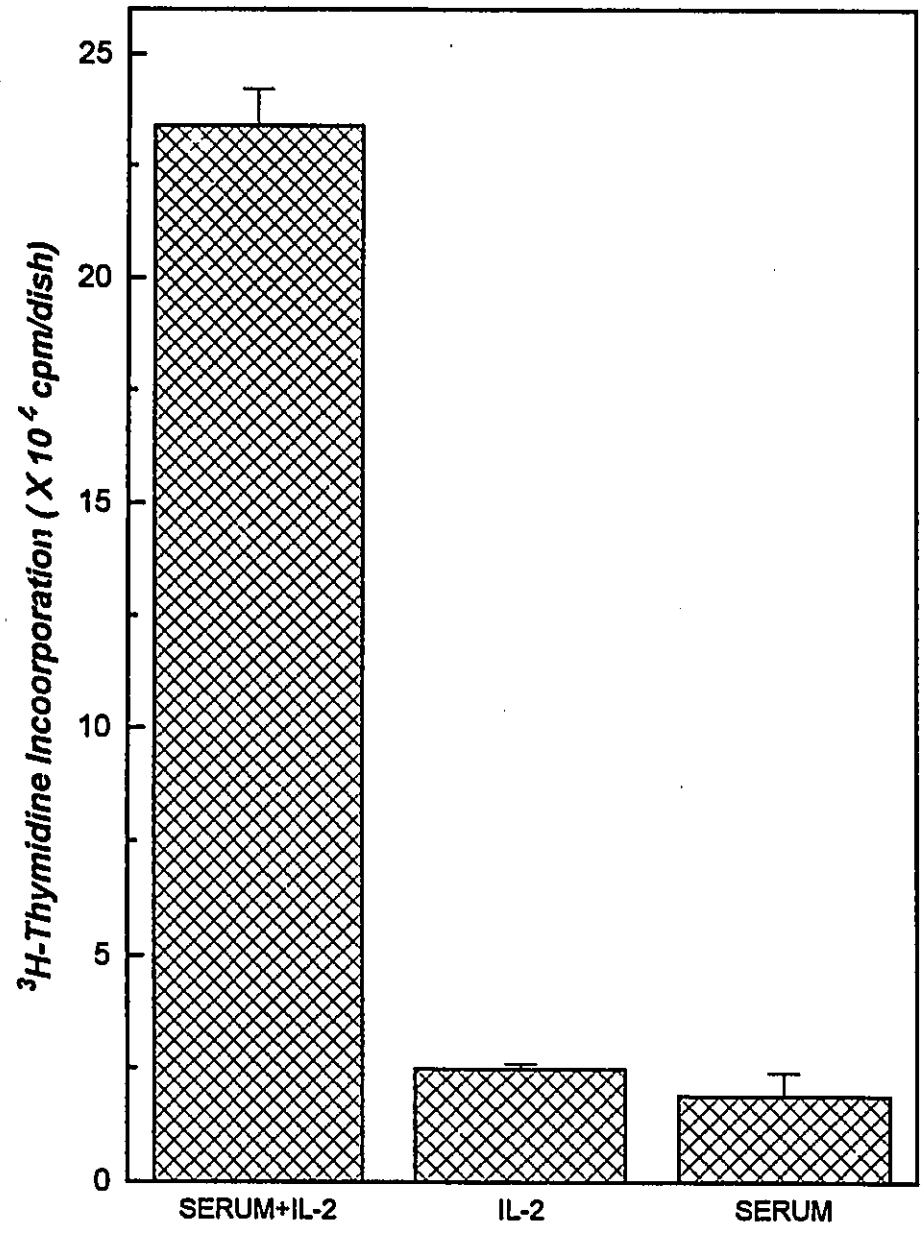


Figure 1.5 IL-2 is required for cell viability

Exponentially growing CTLL-2 cells were washed twice with prewarmed RPMI medium and resuspended in medium without IL-2 and serum (i.e. -IL-2) at a density of 2.5×10^5 cells/ml (i.e. control = 0). The cells were incubated at 37°C for 7 h, at which time 100 U/ml IL-2, or 100 U/ml IL-2 and 10% serum were added to some cultures. The cultures were incubated for an additional 17 h. The percentage of viable cells at $t = 0$, $t = 7$ and following serum and/or IL-2 treatment (i.e. 24 hours) was determined as described in *Methods*. The data points represent the means $\pm$ SEM of triplicate determinations. At the same time, the degree of cell lysis was determined by measuring the amount of LDH released into the medium during the various incubation periods, as described in *Methods*. Values are the units of LDH released into the medium (per dish) above that seen in control cultures. Results are representative of three separate experiments.

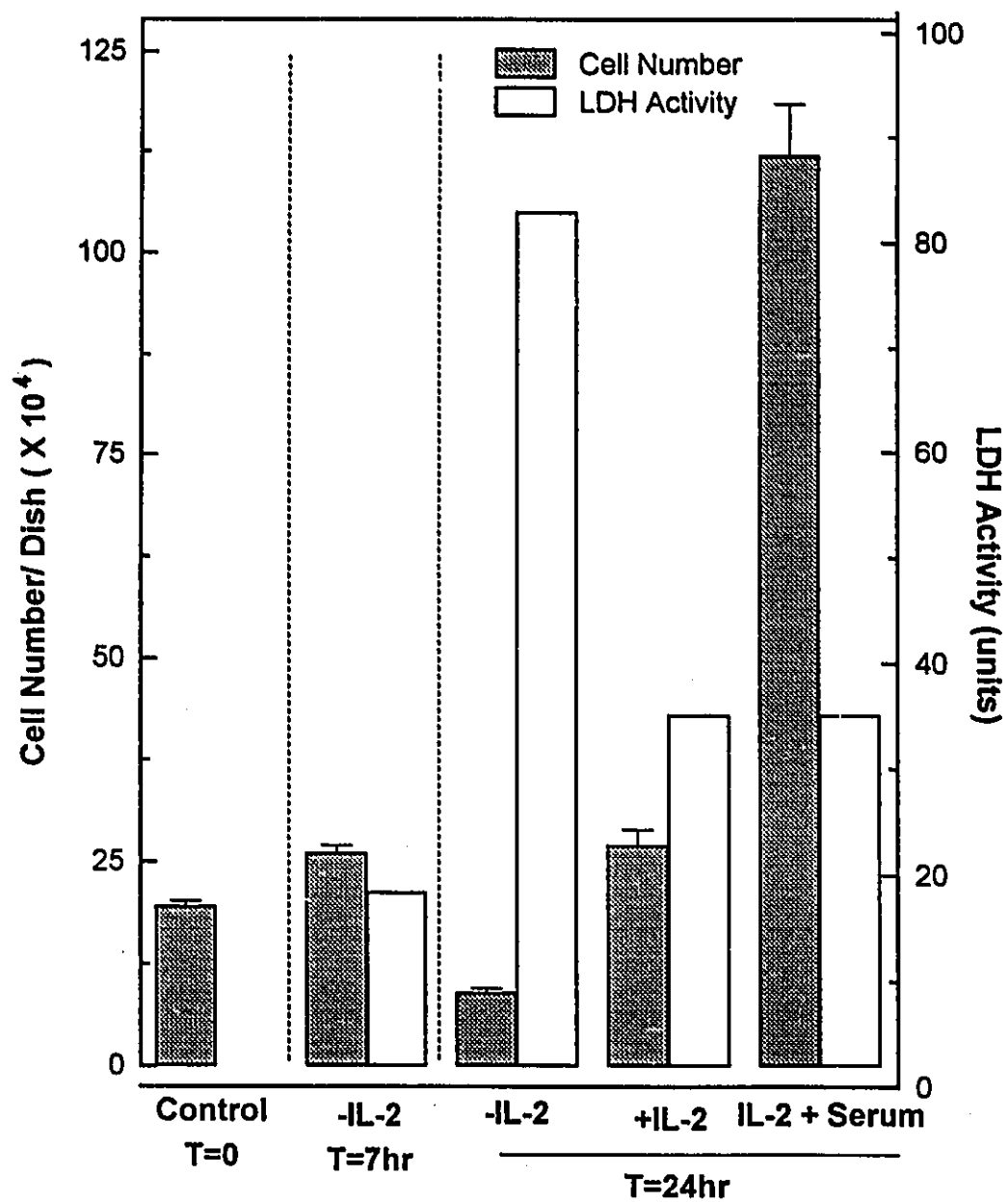


Figure 1.6 The effect of serum and IL-2 on lytic activity of CTLL-2 cells

Exponentially growing CTLL-2 cells were washed twice with prewarmed RPMI medium and incubated for 18 h in RPMI alone, or in RPMI containing either IL-2 (100 U/ml), serum (10%), or IL-2 (100U/ml) plus serum (10%), and viable cell numbers determined. As described in *Methods*, exponentially growing target cells (S49.1, 10^7 cells) were labeled with sodium ^{51}Cr chromate for 90 minutes at 37°C , washed three times in RPMI supplemented with 10% fetal calf serum, and resuspended in the same medium at a density of 1×10^5 cells/ml. Effector and target cells were mixed at a 100:1 ratio in their respective media, incubated at 37°C for 4 hours, and CTLL-2 cytolytic activity determined by the levels of ^{51}Cr released into the medium as described in *Methods*. Values are expressed as the percentage of control (i.e. cells grown in medium containing 10% serum and 100 U/ml IL-2).

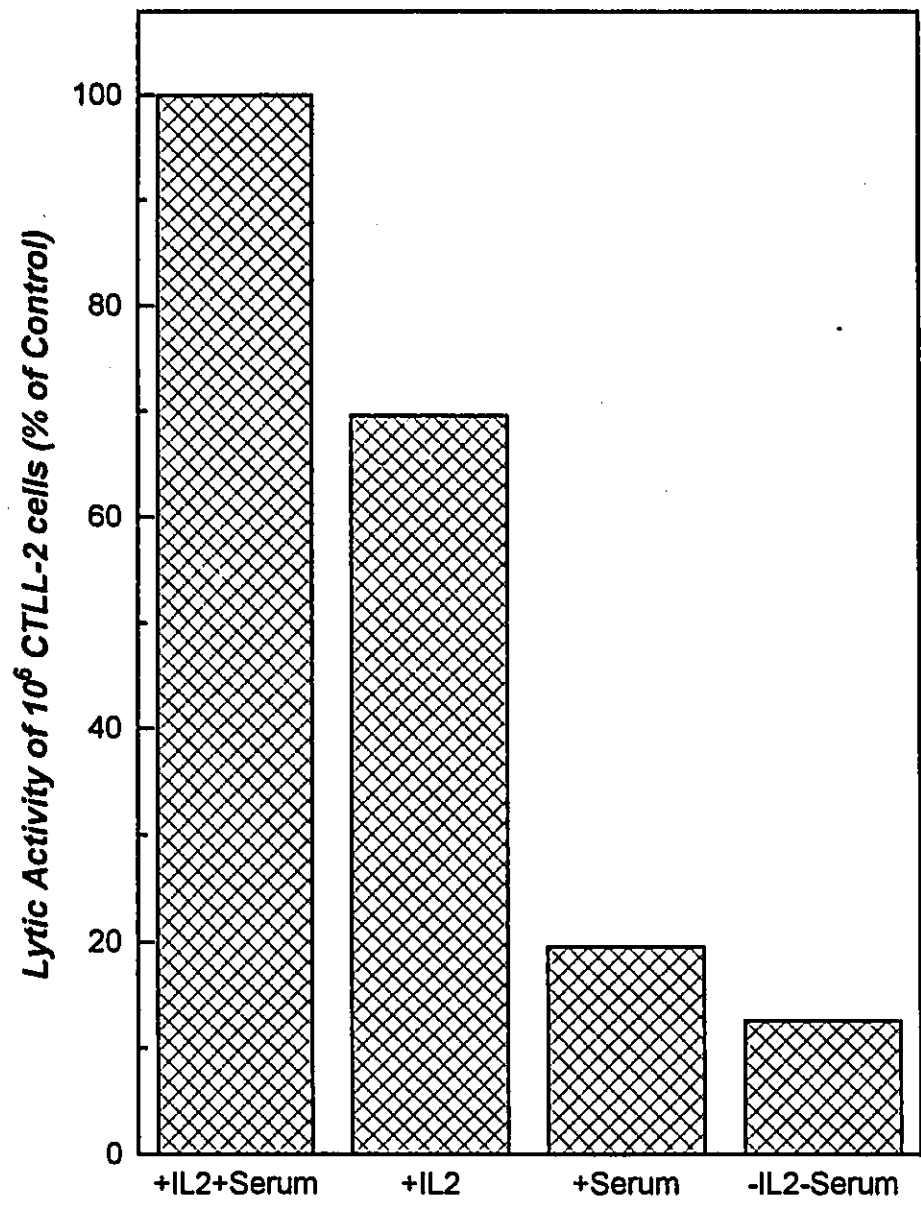


Figure 1.7 PKC isozymes in CTLL-2 cells

Protein kinase C isozymes presented in CTLL-2 cells were determined by Western blot using isozyme specific polyclonal antibodies to PKC isozymes. CTLL-2 cell membrane (20 μ g protein) and cytosolic (20 μ g protein) fractions were solubilized, electrophoresed on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose paper as described in *Methods*. Nitrocellulose papers were probed with different polyclonal antibodies to different PKC isoforms as indicated in the figure. Data were representative of 7 separate experiments (c: cytosol, m: membrane).

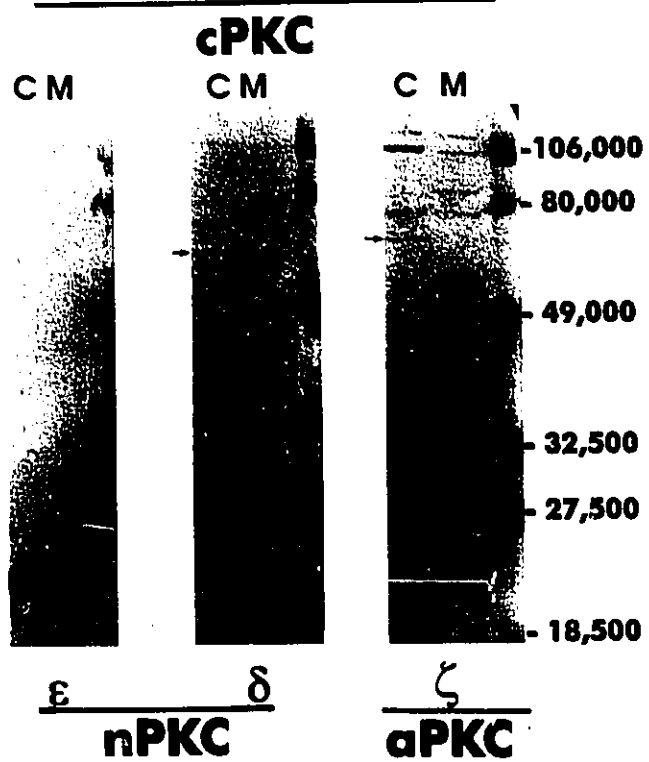
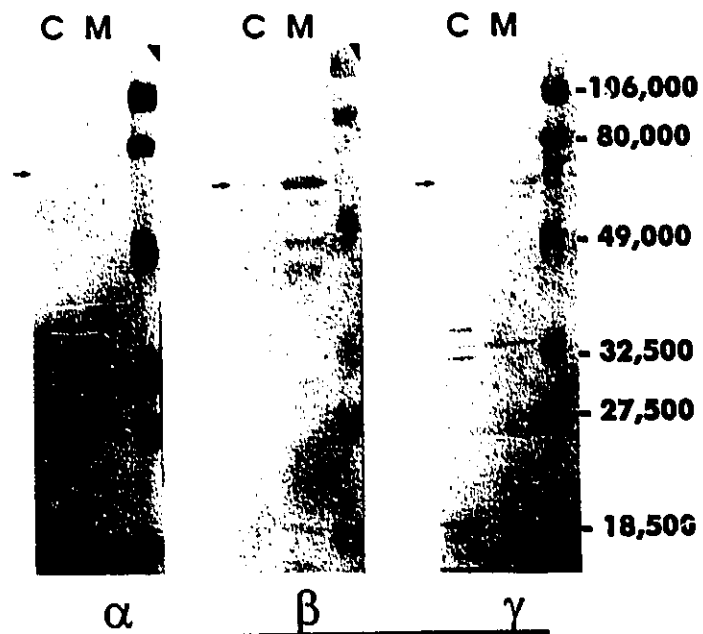


Figure 1.8 Steady state PKC activity in CTLL-2 cells

Exponentially growing CTLL-2 cells were resuspended in RPMI supplemented with 10% serum and 100 U/ml of IL-2 at a density of 1.0×10^6 cells /ml, and incubated at 37°C. At the indicated times, 20×10^6 cells were removed, washed twice with ice-cold PBS, and lysed in hypotonic lysis buffer as described in *Methods*. Nuclei and unlyzed cells were sedimented by low-speed centrifugation, and membrane, cytosol fractions were separated as described in *Methods*. Samples were frozen and stored at -84°C. PKC activity on the thawed membrane and cytosol fractions were determined by the direct and conventional PKC assays, respectively, as described in *Methods*. Data points represent the means $\pm$ SEM of triplicate determinations.

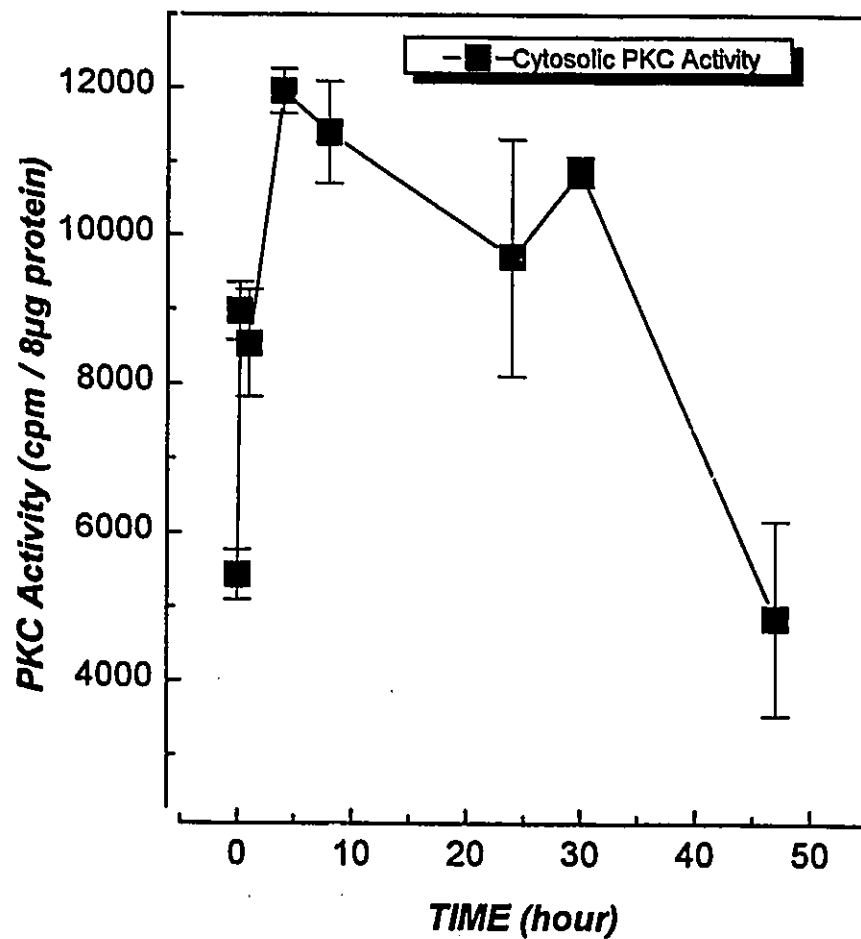
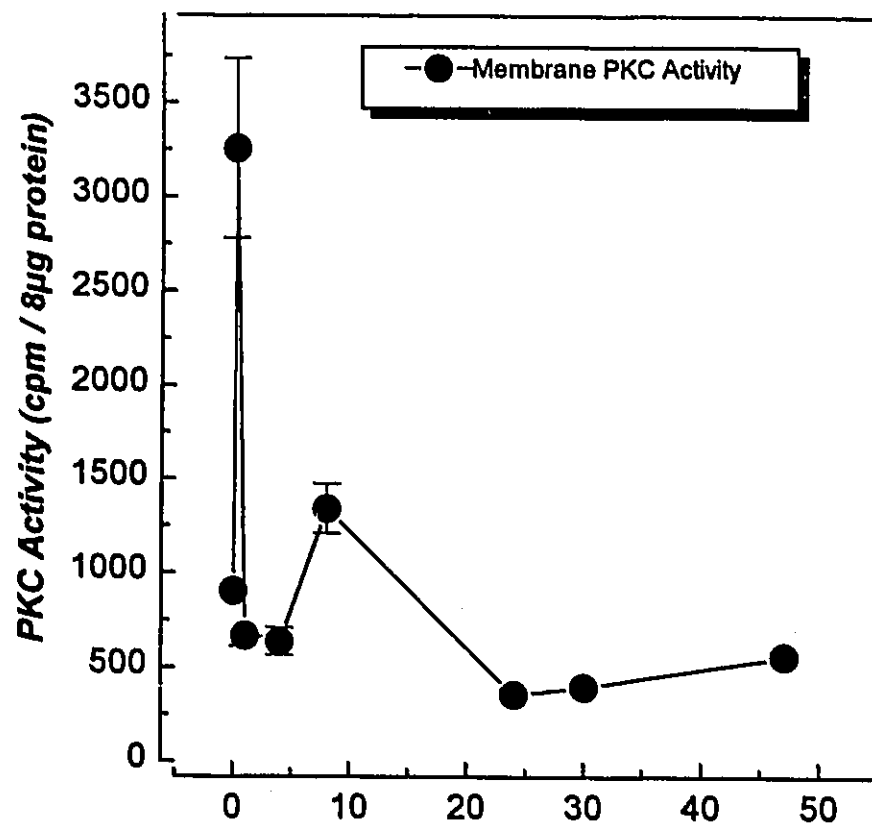


Figure 1.9 The effect of serum and IL-2 deprivation on membrane PKC activity

Exponentially growing CTLL-2 cells were washed twice in prewarmed RPMI medium, suspended in RPMI medium at a density of 1.0×10^6 cells/ml, and incubated for various time at 37°C. At the indicated times, 20×10^6 cells were removed, lysed hypotonically in lysis buffer (Appendix), and nuclei and unlysed cells were sedimented by low-speed centrifugation, membrane and cytosolic fractions prepared as described in *Methods*. Samples were frozen and stored at -84°C. Membrane PKC activity was determined in the thawed samples by the direct assay as described in *Methods*. Values are the means $\pm$ SEM of triplicate determination, and are representative of three separate experiments.

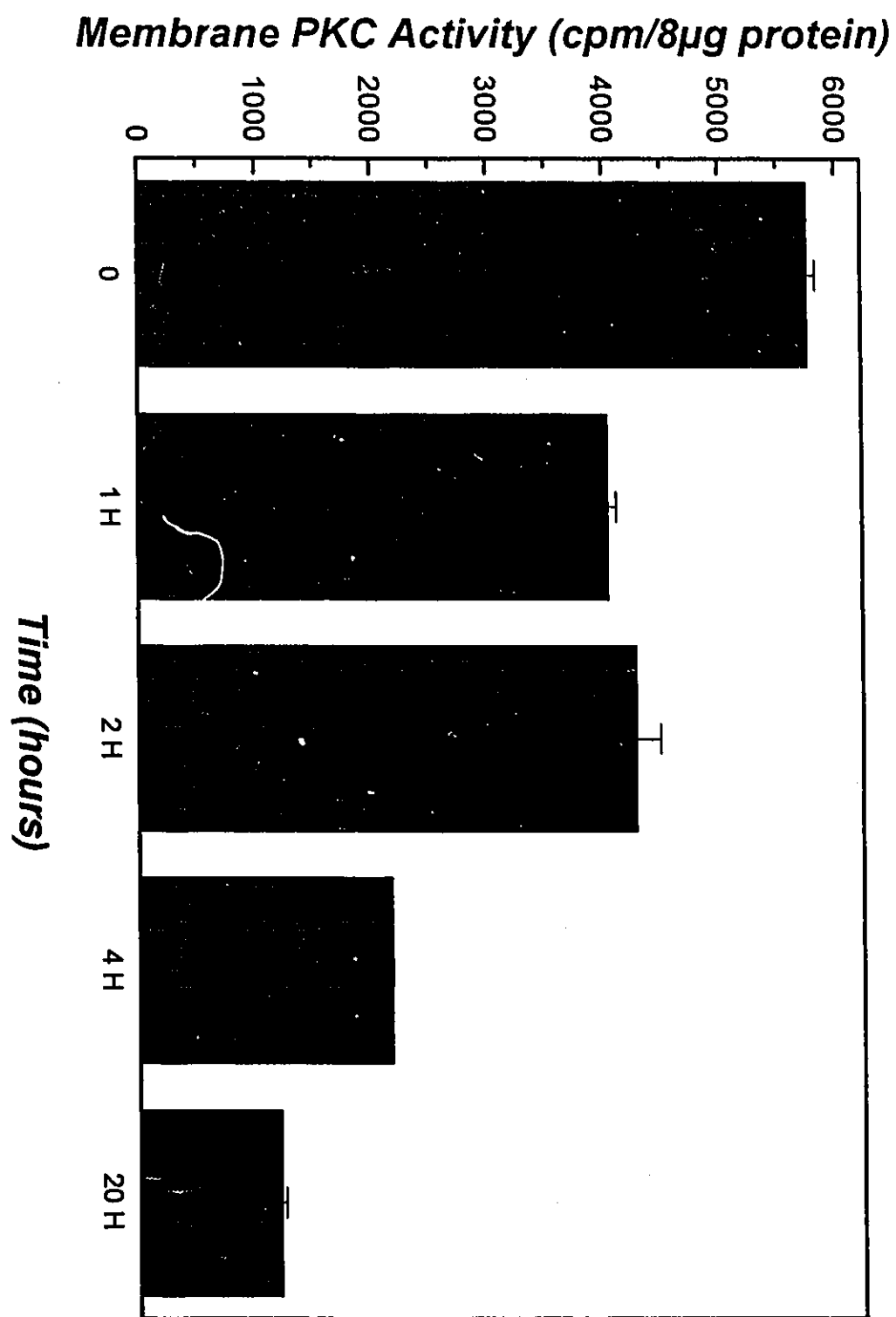
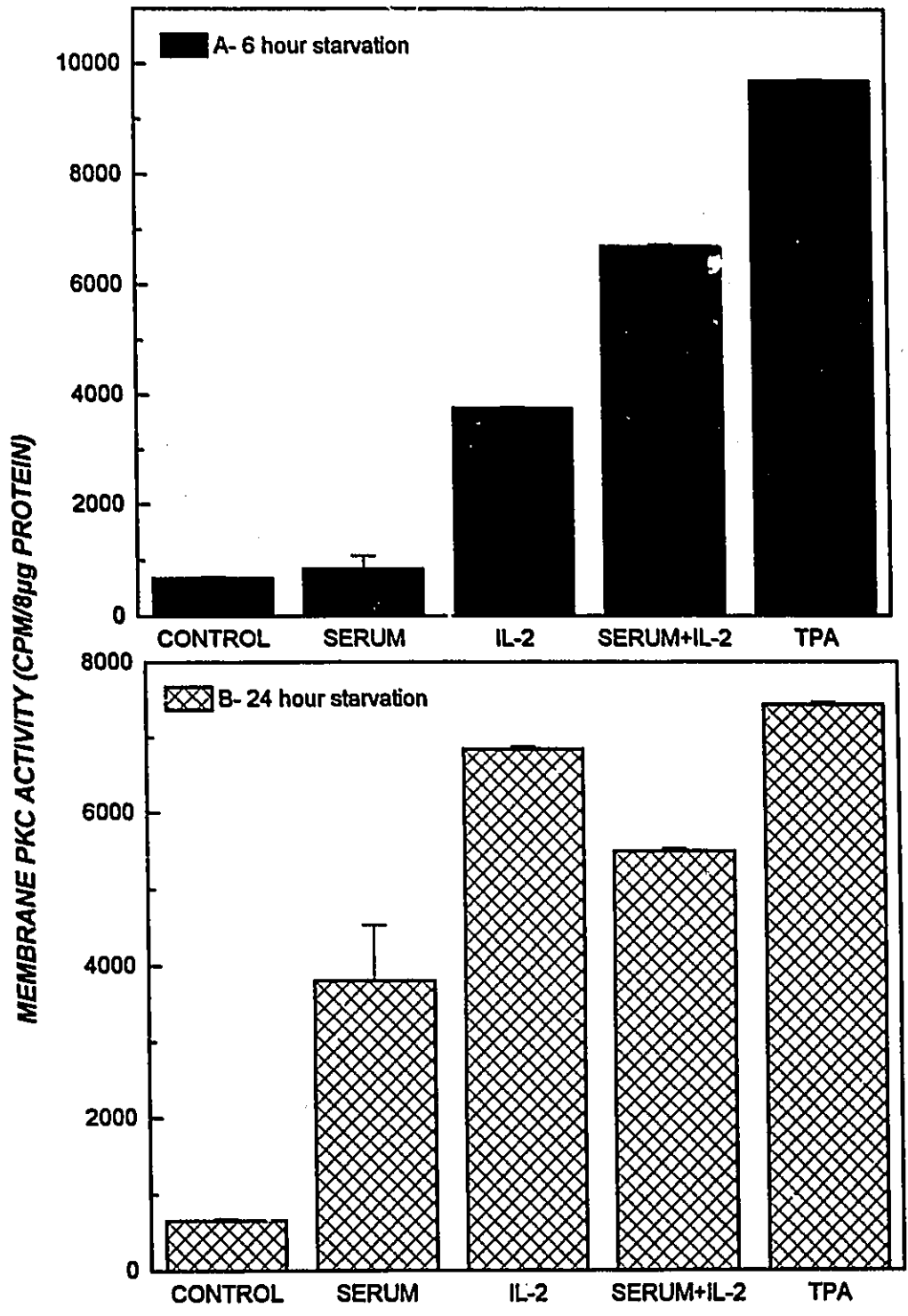


Figure 1.10. The effect of serum and IL-2 readdition on the PKC response in "starved" CTLL-2 cells

Exponentially growing CTLL-2 cells were washed twice, resuspended in RPMI medium at a density of 1.0×10^6 cells/ml, and incubated for either 6 or 24 hours at 37°C. At the indicated time, 20×10^6 cells were removed and stimulated with either IL-2 (100U/ml), serum (10%), serum plus IL-2, or TPA (1 μ M) for 10 min. The reactions were stopped by adding ice-cold PBS, and the cells were lysed hypotonically in lysis buffer (Appendix). Nuclei and unlyzed cells were sedimented by centrifugation, and the membrane and cytosol fractions were prepared as described in *Methods*. Samples were frozen and stored at -84°C. PKC activity on thawed membranes was determined by the direct PKC assay as described in *Methods*. Values are the means $\pm$ SEM of triplicate determinations, and are representative of two separate experiments.



1.5 DISCUSSION

1.5.1 *IL-2 is critical for CTLL-2 cell survival, proliferation, and cytolytic activity*

In this chapter, I have used different experimental approaches to establish that IL-2 is critical for both CTLL-2 cell survival and proliferation (Figs 1.1, 1.2, 1.3, 1.4, 1.5 and Table 1.1). These data indicate that the CTLL-2 cell line is a good *in vitro* model to study the various mechanisms associated with IL-2 signaling. Having reached this conclusion it was important to characterize the serum and IL-2 requirements of these cells in order to establish reproducible and relevant experimental conditions under which to study the role of PKC in IL-2 signaling. Both serum factors and IL-2 were needed to elicit a proliferative response from CTLL-2 cells (Fig. 1.1, 1.4). It was established that in the presence of serum, CTLL-2 responded maximally to IL-2 concentrations between 20 to 50 U/ml (Fig. 1.2). Importantly, it was found that IL-2 maintained CTLL-2 cell viability in the absence of serum factors for at least 48 hour (Fig.1.1, 1.3), and that these cells despite their quiescence maintained their functional proliferative machinery and were able to resume proliferation without a substantial delay once serum factors were restored (Fig.1.3). The LDH data presented in Table 1 indicated that the "stasis" in viable cell numbers observed during extended serum deprivation in the presence of IL-2 arose mainly from a stoppage in cell proliferation and not from a marked acceleration in the rate of cell death (Fig.1.5). In summary, these data indicated that in the presence of IL-2 alone, cell proliferation is blocked while cell viability is maintained.

The withdrawal of both IL-2 and serum was clearly lethal if it persisted for periods approaching 24 hours (Table 1.1, Fig. 1.5) since a substantial loss in viable cell number and a concomitant increase in LDH activity released into the medium was observed 24-hours after withdrawing IL-2 and serum from actively growing CTLL-2 cells. However, a brief withdrawal

of IL-2 and serum was found not to be lethal and the cells were "rescued" by the timely readdition of these factors to the medium (Fig 1.5). The cells were found to recover completely from IL-2/serum deprivation lasting up to 7 hours (Fig. 1.5), but longer periods caused a progressive inability to recover after IL-2 and serum restoration. For these reasons it was decided that in subsequent experiments (i.e. Chapters 2 and 3) a 6-7 hour "starvation" time would be used to study the effects of IL-2 on cell signalling in CTLL-2 cells. It was also decided to use serum + IL-2 deprivation, rather than IL-2 deprivation alone, in subsequent signalling experiments since the response of cells to IL-2 under these conditions could be studied in isolation without the possibility of synergistic serum effects arising from IL-2 addition.

CTLL-2 cells exhibit cytotoxic activity (Fig. 1.6) which appears to mainly depend on the presence of IL-2 in the medium. The removal of IL-2 from the medium for 18 hours caused a >80% reduction in CTLL-2 cell mediated lysis of S49.1 lymphoma cells whereas the withdrawal of serum alone reduced lytic activity by only 30%. Thus, in CTLL-2 cells cytolytic activity is an IL-2 dependent process and this conclusion allowed us to study the role of PKC in IL-2 mediated cytotoxicity (Chapter 2).

1.5.2 *Characterization of PKC in CTLL-2 cells*

Since the main focus of this thesis was to elucidate the role that PKC plays in IL-2 signaling, it was very important to understand and characterize the nature of the enzyme as it exists in CTLL-2 cells. It was determined that four PKC isozymes, α , β , δ , ζ , are the isoforms present in either the membranes or the cytosol of CTLL-2 cells or both (Fig.1.7), with α and β being the predominant forms. Even though it has been reported that PKC γ is exclusively present in neuronal tissue (Huang et al., 1987, Saito et al., 1988), this isoform was indeed detected in CTLL-2 cells membrane and cytosol fractions. There are only two possibilities to explain this

finding- either there is PKC γ present in CTLL-2 cells, or the antibody used to detect PKC γ has cross-reacted against other isoforms. The PKC polyclonal antibodies were raised against an apparently PKC γ -specific peptide (asn-tyr-pro-leu-glu-leu-tyr-glu-arg-val-arg-thr-gly [Makowske et al., 1988]) and it is difficult to see how other PKC isoforms could cross-react. Moreover, the dogma that PKC γ is brain specific is being challenged, most recently in studies within our Institute that show that both the message and protein for PKC γ are produced in cultured NRK cells and in T51B rat liver cells (M. Sikorska, personal communication). Because of the nature of the immunoblot analysis performed, it would be likely unjustified to use the data in Fig. 1.7 to definitively rank the isoforms of PKC in order of abundance- it is however, clear that four out of six PKC isoforms tested are indeed present in CTLL-2 cells.

PKC distribution studies indicated that there is very little (~1% of total) PKC activity present in the nucleus of CTLL-2 cells. This conclusion is valid even though we didn't use enzyme markers to enumerate the degree of non-nuclear contamination in the preparation- any such contamination would have only inflated the value. Clearly the majority of PKC in CTLL-2 cells is located where it was expected, namely the membrane and cytosolic fractions. It is important to note that the "membrane" fraction is a crude preparation that includes all material in the post-nuclear preparation (PNS) that was pelleted by high speed centrifugation. As such the membranes are undoubtedly contaminated with cytoskeletal elements, mitochondria and other organelles. Nevertheless, the majority of PKC was found in this membrane fraction (i.e. 36% of total, Table 1.2) where nearly two-thirds was associated in an EGTA-sensitive, peripherally-bound state (Table 1.3). The estimate that ~36% of the total cellular PKC was membrane associated was likely an underestimate since almost a third of the total cellular PKC was found in the supernatant of the nuclear fraction following triton X-100 treatment. Since the nuclear

fraction was contaminated with less than 5% unlysed cells, this triton X-100 extractable nuclear PKC likely represented membranes and other particulate material attached to the crude nuclei. If this be true then the value given for PKC in the membrane was indeed an underestimate. Alternatively, the triton X-100 treatment may have extracted PKC from the perinuclear membrane of CTLL-2 nuclei wherein the value given for nuclear PKC was an underestimate.

Steady state PKC activity was found to be highly sensitive to the level of nutrients present in the medium (Figure 1.8). Hence, even though the cells were still proliferating exponentially 3 days after being fed with complete medium, their membrane and cytosolic PKC responded within minutes (i.e., membrane) or hours (i.e., cytosolic) to a full medium change. Whether this response was due to IL-2 or serum factors was not determined. These experiments were important in order to determine the best feeding regime for subsequent experiments related to PKC and IL-2 signaling. Based on experiments such as those shown in Fig. 1.8, it was decided that in order to have stable basal PKC levels we would feed the cultures 24 hours before any experiment, despite the fact that the cells would continue to proliferate quite well for several days without such a feed.

CTLL-2 cells were found to be completely depend on IL-2 and serum for proliferation since the withdrawal of serum and IL-2 caused a substantial loss in viable cell number and a concomitant increase in LDH activity released into the medium (Table 1.1). Upon the withdrawal of serum and IL-2 from the medium of exponentially growing CTLL-2 cells, it took between 2 and 4 hours before a substantial decline in membrane PKC activity was observed (Fig. 1.9). This inactivation of PKC activity could be rapidly reversed upon the restoration of IL-2 to cells deprived of serum\IL-2 for 6 and 24 hours, but not for 2 hours (Fig. 1.10). By contrast, while serum factors may be important for proliferation (Fig. 1.1, 1.3, 1.4, 1.5) and cytolytic

activity (Fig.1.6), they do not stimulate PKC activation in CTLL-2 cells starved for 6 hours (Fig. 1.10). The simplest explanation for these results is that the removal of IL-2 from the medium caused a decline in PKC activity over the ensuing 2-6 hours that was reversed by IL-2 addition. When these results were considered with the fact that restoring IL-2 to cultures deprived of serum and IL-2 for 7 hours rescued the cells from cell death (Fig. 1.5), it was determined that a 6-7 hour serum/IL-2 deprivation period was optimal for the study of PKC changes in IL-2 signaling in CTLL-2 cells.

1.6 CONCLUSION

The overall conclusion of the studies presented in this section is that CTLL-2 cells represent an appropriate *in vitro* model to study the role of protein kinase C in IL-2 signal transduction, since;

1.6.1 IL-2 is critical for CTLL-2 cell survival, cytolytic activity and proliferation.

1.6.2 There are measurable levels of PKC in CTLL-2 cells, and membrane PKC activity responds rapidly to the removal and the readdition of IL-2.

The above points were further characterized and it was determined that

1.6.3 The removal of IL-2/serum from the medium can be tolerated for 6-7 hours, in that the restoration of IL-2 is able to rescue the majority of cells from death over the ensuing 24 hours .

1.6.4 The removal of IL-2/serum from the medium for 6-7 hours causes a loss in membrane PKC activity that is rapidly reversed by IL-2 readdition.

1.6.5 There is relatively equal distribution of PKC between membranes and the cytosol in exponentially growing CTLL-2 cells. With respect to membrane-associated PKC, 40% is membrane inserted, 60% is in a loosely bound, EGTA-extractable form.

1.6.5 At least five PKC isozymes are present in CTLL-2 cells, α , β , δ , ζ , and to a lesser extent PKC γ .

CHAPTER TWO: THE ROLE OF INACTIVE MEMBRANE-ASSOCIATED PKC IN T-LYMPHOCYTE SURVIVAL AND CYTOTOXICITY

2.1 INTRODUCTION

2.1.1 PKC activation mechanisms

As discussed in the General Introduction, it is a long held belief that the activation of PKCs in cells by various extracellular agents is linked to a redistribution of cytosolic enzymes to membranes. By this hypothesis, the majority of inactive PKCs in cells is located in the cytosol while that associated with the particulate fraction is constitutively active. However, studies have indicated that PKC can also exist in membranes in an inactive form, and thus, membrane associated PKC activity may be stimulated by extracellular ligands without a redistribution of enzymes from cytosol to membranes (Halsey et al., 1987, Mukaida et al., 1988, Pelech et al., 1990a, 1990b, Chaikin et al., 1990, Tang and Houslay 1992). Early experiments conducted with purified PKCs and phospholipid vesicles (Bazzi and Nelsestuen 1988) or erythrocyte membranes (Wolf et al., 1985, May et al., 1985) gave the first indication that PKCs might associate with cell membranes in an inactive, but still signal-responsive, state (Bell 1986, Bell and Burns 1991). However, it was not until the development of the direct PKC assay (Chakravarthy et al., 1991, see General Introduction), that inactive membrane associated PKC was actually measured in cells. This was accomplished by comparing the basal activity of PKC in isolated membranes to the increased level of activity observed after membranes were incubated with TPA, DAG, or high Ca^{2+} concentrations. Using these methods it has been reported that inactive membrane-associated PKCs are present in a number of cell lines, including; S40 T-lymphoma, sys-S49 T-lymphoma, WEHI 231 B-lymphoma, murine erythroleukaemia, human erythroleukaemia, HL-60 human

myeloid and 3T3-L1 fibroblasts, as well as in tissues such as rat thymus, rat spleen, rat platelets, and whole rat brain (Chakravarthy et al., 1994). Moreover, inactive membrane PKC could be stimulated in intact cells, without detectable translocation, by a number of growth factors including EGF, FGF, DMSO, differentiation-inhibiting protein (DIP), and PTH (Chakravarthy et al., 1992, 1994, Durkin et al., 1992, Jouishomme et al., 1992). Inactive membrane-associated PKC in intact WEHI B-lymphoma cells and isolated rat platelets was shown to be activated by exogenous DAG (Chakravarthy et al., 1994). The presence of inactive membrane PKC is not ubiquitous since it was not present in membranes isolated from NRK rat kidney cells, rat hippocampus cells, and Balb/MK2 keratinocytes (Chakravarthy et al., 1994).

The activation of a DAG-stimulatable pool of inactive-membrane associated PKCs is not the only way to activate membrane associated PKC. It has been reported that PKC can be activated by a process involving covalent modification of the enzyme (Pelech et al., 1990a). In this study, PKC isolated from 3T3 L1 cells treated briefly with platelet-activating factor (PAF) showed a shift in elution profile from hydroxylapatite columns that appeared to result from a modification within or near the catalytic domain of PKC. More recently it has been reported that a pool of PKC exists in the membranes of murine neuroblastoma cells that is inactive, but not activatable by TPA/DAG added to isolated membrane (Chakravarthy et al., 1995). This pool of PKC was shown to be actively suppressed by a membrane-associated PKC inhibitory protein. Most significantly, the activity of this protein was increased when the cells were induced to differentiate into neuronal-like cells upon serum deprivation, an event that was coincident with a loss of PKC activity shown to be a mandatory step in the differentiation process (Chakravarthy et al., 1995). It has also been reported that membrane-associated PKC activity can be modulated by changes in membrane lipid bilayer structure, in that increasing the levels of

phosphatidylethanolamine or cholesterol, or the level of phosphatidylcholine unsaturation either potentates or attenuates the activity of PKC resident in membranes (Slater et al., 1994). This conclusion was consistent with earlier reports showing that bilayer stabilizing compounds inhibit PKC activity (Epand et al., 1989), and that alcohol and anesthetics directly affect membrane PKC activity with a potency that is dependent on lipid bilayer properties (Slater et al., 1993). Thus, a number of different mechanisms can explain the activation of PKC in the absence of translocation that have been reported in many cell types over the years (Halsey et al., 1987, Mukaida et al., 1988, Pelech et al., 1990a, 1990b, Chaikin et al., 1990, Tang and Houslay 1992, Durkin et al., 1995).

2.1.2 IL-2 and apoptosis

Apoptosis is a physiological suicide mechanism for removing unwanted cells and is characterized by morphological as well as biochemical criteria (Wyllie et al., 1980, Cohen et al., 1992, Majno and Joris 1995). Morphologically, cells undergoing apoptosis appear to shrink and become dense with collapsed chromatin packed into smooth masses applied against the nuclear membrane (Cohen et al., 1992). The formation of “apoptotic bodies” which consist of nuclear and cytoplasmic fragments enveloped in plasma membrane is another morphological hallmark of apoptosis (Cohen et al., 1992). Interestingly, the plasma membrane and most cellular organelles remain morphologically and functionally intact for much of the apoptotic process (Majno and Joris 1995). The key biochemical event of apoptosis is endonucleolysis, which typically results in cleavage of nuclear DNA into oligonucleosome-sized fragments that are multiples of approximately 185 bp, due to specific cleavage between nucleosomes (Cohen et al., 1992). The fragmented DNA can be visualized as a “ladder” on agarose gels during electrophoresis, and this procedure is commonly used for the detection of apoptosis. However, it has been recently

shown that intranucleosomal cleavage of DNA does not occur in all apoptotic processes (Weaver et al., 1996) and that proteolytic degradation of specific proteins may also be a key biochemical event during apoptosis (Weaver et al., 1996).

Apoptosis can occur in a number of regressing tissues following the withdrawal of trophic hormones (reviewed by Tenniswood et al., 1992) and is particularly prevalent in the lymphoid system where it forms the basis of negative selection during development (Cohen and Duke 1992). At one stage of thymocyte development the appearance of the T-cell antigen receptor can be the signal to trigger apoptosis; this process serves to eliminate those thymocytes that recognize self antigens and MHC molecules (Cohen et al., 1992). Apoptosis can be triggered by a variety of exogenous stimuli including glucocorticoids and radiation (Wyllie et al., 1980, Tomei et al., 1988). Glucocorticoid-induced apoptosis is a relatively fast process which can be detected by light microscopy within one or two hours of *in vitro* incubation with the steroid. Exogenous stimuli such as radiation (Tomei et al., 1988, Roy et al., 1992) and a number of antineoplastic drugs (Roy et al., 1992, Kwast-Wefeld et al., 1991) have been shown to induce apoptosis in a variety of tumor cells.

The cellular mechanisms responsible for apoptosis have been the subject of intense study and several key events have now been identified. The expression of the tumor suppressor gene, p53, is increased in response to DNA damage (Majno and Joris 1995) and appears necessary for apoptosis since "cell suicide" is not observed in mice in which p53 has been deleted by homologous recombination (Lane 1993). Moreover, it has been reported that p53 overexpression will cause apoptosis in myeloid and epithelial cell lines (Yonish-Rouach et al., 1991, Shaw et al., 1992). The cytoplasmic oncoprotein Bcl-2 has also been widely demonstrated to inhibit apoptosis when over-expressed in a variety of cells, including factor-deprived haematopoietic cells

(Hockenberry 1990) and neurons (Garcia et al., 1992). However, Bcl-2 cannot inhibit all pathways of induction of apoptosis in that, for example, apoptosis in CTL cells is not inhibited by Bcl-2 (Vaux et al., 1992). Protein kinase C activation has been reported both to prevent (McConkey and Orrenius, 1991) and to induce (Tritton 1991) apoptosis in a variety of cell types. Phorbol esters, tumor promoters which activate PKC, have been reported to induce as well as inhibit apoptosis (Cotter et al., 1990, McConkey and Orrenius, 1991, Suzuki et al., 1991, Tritton 1991, Terai et al., 1991, Forbes et al., 1992). Whether PKC is involved in IL-2's ability to suppress apoptosis has not been explored extensively.

By both morphological and biochemical criteria, IL-2 withdrawal-induced death is clearly apoptotic in nature (Cohen et al., 1985, Duke and Cohen 1986, Cohen et al., 1992). Conversely, IL-2 has a profound impact in repressing cell death signals in IL-2-dependent cells that may be independent from proliferation signals (Nieto et al., 1989, Walker et al., 1993). It has been shown that HT-2 cell lines, which grow continuously in the presence of exogenously added IL-2, begin to undergo macromolecular synthesis-dependent apoptosis as early as 6 hours after the withdrawal of IL-2 (Duke and Cohen 1986). Moreover, a role has been postulated for PKC in the induction of apoptosis in IL-2-dependent cells upon withdrawal of the cytokine (Kizaki et al., 1988, Rodringcz-Tarduchy and Lopez-Rival 1989, Walker et al., 1993), although the nature of that role is controversial (Walker et al, 1993).

2.1.3 Role of PKC in IL-2 mediated cytotoxicity

Cytotoxic T lymphocytes (CTL), natural killer (NK) cells, and lymphokine-activated killer cells contribute to host cell-mediated immunity against viral pathogens, parasites, and neoplastic transformation, as well as to host pathology associated with tissue and bone marrow graft rejection and a variety of autoimmune diseases (reviewed by Sitkorsky and Henkart 1993).

Although it is clear that these effector cells can kill their targets by multiple mechanisms, there is abundant evidence that the lethal hit delivered by cytotoxic cells in these immune responses involves the component of their characteristic electron-dense cytoplasmic granules. In the presence of Ca^{2+} , granules of CTL and NK cells are vectorally secreted into the intercellular space formed by the contact between the effector and target cells (conjugation), providing a mechanism for the delivery of the complement-like lesions in target cell membrane (Dourmashkin et al. 1980, Podack and Dennert 1983). These observations led to the granule-exocytosis model of cellular cytotoxicity (Henkart et al., 1985), which postulates that the lethal interaction between a cytolytic effector cell and its target involves recognition by CTL and NK cells of specific targets, delivery of the lethal hit by means of granule exocytosis, and detachment of effector cells from dying targets. However, the cellular mechanisms involved in the killing of target cells is not very well defined. In particular the role of PKC in cytotoxic activity of T-cells is not explored.

2.2 OBJECTIVES

The overall objective of the studies described in this chapter was to investigate the role of inactive membrane-associated PKC in T-lymphocyte survival and cytotoxicity. The specific objectives were:

- 2.2.1 To establish that IL-2 induced PKC activation in CTLL-2 cells does not arise from the translocation of enzyme from the cytosol to membranes.
- 2.2.2 To establish the presence of inactive membrane associated PKC on CTLL-2 cells.
- 2.2.3 To establish the importance of early PKC activation in the survival of CTLL-2 cells.
- 2.2.4 To establish the role of PKC in IL-2's ability to suppress apoptosis in CTLL-2 cells.
- 2.2.5 To investigate the role of PKC in IL-2 stimulated cytolytic activity.

2.3 METHODS

Cell culture

Stock cultures of CTLL-2 cells were maintained and manipulated as described in the *Methods* section of Chapter I. Viable cells were determined microscopically by their ability to exclude trypan blue (Sigma Chemical Co., ST. Louis, MO, USA) as described in *Methods* section of Chapter I (1.3.5).

2.3.1 Cell lysis and isolation of membrane and cytosolic fractions, and PKC assay

Cell lysis, the preparation of membrane and cytosolic fractions, and conventional and direct PKC assays were performed following the protocols described in the *Methods* section of Chapter I (1.3.1, 1.3.2).

2.3.2 Assessment of cytotoxic activity of CTLL-2 cell

The cytotoxic activity of CTLL-2 cells were determined by using the ^{51}Cr release cytotoxicity assay (Tracey et al., 1977) described in detail in the *Methods* section of Chapter I (1.3.6).

2.3.3 Immunoblots

Membrane and cytosolic fractions isolated from control, IL-2 and TPA treated CTLL-2 cells were solubilized, electrophoresed on 10% SDS-polyacrylamide gels, transferred electrophoretically to nitrocellulose paper and probed with a mixture of antibodies recognizing various PKC isoforms as described in Chapter I (1.3.3).

2.3.4 DNA fragmentation analysis

Exponentially growing CTLL-2 cells (10^7) were harvested, centrifuged, and lysed by resuspension in 400 μl lysis buffer (5 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 0.5% Triton X

100). Cellular debris was removed by centrifugation at 600 xg for 5 min, and the fragmented DNA in the supernatant was precipitated at -20°C by the addition of 40 µl of 2.5 M sodium acetate, pH 5.2, and 800 µl absolute ethyl alcohol. The pellets were resuspended in 400 µl Tris/EDTA buffer (10 mM Tris-HCl, pH7.5, 1 mM EDTA), and 10 µl of 2 mg/ml RNase was added and the mixture incubated for 60 min at 65°C. Twenty-five microliters of proteinase K (20mg/ml) and 40µl 10X proteinase K buffer (0.1 M Tris [pH 7.8], 0.05 M EDTA, 5% SDS) were then added, and the mixture was incubated for an additional 2 h at 37°C. The mixture was extracted twice with an equal volume of phenol chloroform, twice with chloroform:isoamyl alcohol (24:1, v/v), and the DNA was then precipitated with 40µl 2.5 M sodium acetate and 1.0 ml ethanol at -20°C overnight. The precipitated DNA was resuspended in 50 to 100 µl Tris/EDTA buffer and loaded onto agarose gels. Conventional agarose gel electrophoresis was conducted at 30V for 18 h on 0.8% agarose gels. DNA ladders were detected by using a solution containing 0.4% µg/ml ethidium bromide, 0.04 M Tris-acetate buffer, pH 8.5, and 2 mM EDTA. Gel were photographed under UV light with a Polaroid camera using positive-negative type 55 film.

2.3.5 Detection of MARCKS protein phosphorylation

CTLL-2 cells (6.5×10^6 cells/ ml) were initially incubated in serum and IL-2 free medium at 37°C for 2 hour before ^{32}P -orthophosphate (330 µCi/ml, 9000 Ci/mmol) was added for additional 2 hours in order to label the intracellular ATP pool. Following the labelling period, 6×10^6 cells were incubated with IL-2 (100U/ml), TPA (1µM) or left untreated for 10 min at 37°C. The cells were then rapidly sedimented by centrifugation at 600 xg for 5 min, and solubilized in 100 µl lysis buffer (50 mM Tris /HCl, pH 7.5, 0.1% Triton X-100, 100 µM

sodium vanadate, 100 μ M sodium pyrophosphate, 1 mM sodium fluoride and 100 μ M PMSF). The cell lysate was clarified in a microfuge and proteins (40 μ g) were separated on an 10% SDS-polyacrylamide gel as described in Chapter I (1.3.3). Phosphorylation of the PKC-specific substrate, the 85 kDa MARCKS protein, was visualized by autoradiography using Kodak XAR film.

Miscellaneous

Protein concentrations were determined by the method of Bradford (1976).

2.4 RESULTS

2.4.1 *Membrane PKC activity in CTLL-2 cells is activated by IL-2 without translocating cytosolic enzyme to membranes.*

As discussed in Chapter 1, IL-2 is required for the proliferation, survival and cytotoxic activity of CTLL-2 cells. It was also shown that a 6-7 hour withdrawal of IL-2 and serum from CTLL-2 cells is in itself not lethal and that these "starved" cells respond to the readdition of IL-2 by a rapid increase in membrane PKC activity. The question to whether this increase in membrane PKC activity was caused by the translocation of cytosolic enzyme to membrane after IL-2 stimulation or by some less conventional mechanism was then investigated. Initially, a conventional PKC assay was performed on CTLL-2 cells that had been deprived of IL-2 and serum for 6 h and then challenged with 100 U/ml IL-2. At various times after IL-2 addition, the cells were cooled rapidly and lysed in a hypotonic buffer that was free of chelating agents, so as to prevent loosely bound PKC from being stripped from the membranes. Both the membrane and cytosol fractions were then subjected to a conventional *in vitro* PKC assay (Chakravarthy et al., 1991, 1992, Durkin et al., 1992) to determine the impact of IL-2 restimulation on cellular PKC. As shown in Figure 2.1A, IL-2 neither increased the amount of membrane PKC nor reduced the cytosolic levels of the enzyme, indicating that the translocation of cytosolic PKC to membranes did not occur. The fact that translocation was not stimulated in CTLL-2 cells by IL-2 was confirmed by immunoblot analysis of membrane and cytosol fractions using a pooled mixtures of α -, β -, δ -, ϵ -, and ζ - specific PKC antibodies (Fig. 2.2). The combination of PKC α and β antibodies failed to show a significant difference between immunodetectable PKC in control and IL-2-stimulated cultures, although TPA-treated CTLL-2 cells exhibited an expected increase in

membrane PKC activity and the amount that most probably arose from the translocation of cytosolic enzyme to membranes (Fig 2.2). Immunoblots probed with a mixture of PKC δ -, ϵ -, and ζ -antibodies gave similar results in that no evidence of PKC translocation after IL-2 treatment was observed (Fig.2.2). The conclusion that IL-2 did not stimulate PKC translocation in CTLL-2 cells was in agreement with the previous study of Mukaida et al. who were also unable to demonstrate PKC translocation in this cell line using a conventional PKC assay with histone S-III serving as substrate (Mukaida et al., 1988).

Despite the inability of IL-2 to induce PKC translocation, the data presented in Chapter I (Fig.1.10) indicated that the direct assay was able to measure an increase in the levels of active membrane PKC following IL-2 addition. For comparative purposes, the direct assay was performed on membranes isolated from the same cultures as those used in the conventional assay shown in Fig.2.1. When the PKC activity in intact membranes isolated from IL-2-stimulated CTLL-2 cells was measured by the direct method, a transient, 2.3-fold increase in enzyme activity was observed, which peaked by 5 min and then rapidly declined to basal levels (Fig. 2.1B). The averaged values from several similar experiments ($n = 7$) indicated that IL-2 induced a 2.4 ± 0.2 fold increase in membrane PKC activity within 5 to 10 min after adding the cytokine. This two-fold plus stimulation of membrane PKC activity by IL-2 represented the activation of a significant proportion of the total amount of the enzyme in the cell, because as shown in Chapter I (Table 1.2) approximately 40% of the total PKC present in CTLL-2 cells is associated with membranes. The activation of membrane PKC was dependent on the dose of IL-2 (Fig. 2.3A) and reached a maximum value at concentrations greater than 50 U/ml IL-2. By contrast, when cytosolic enzyme activity was measured in these same cultures by the conventional PKC assay (Fig.2.3B), no detectable decrease in cytosolic PKC activity was observed at concentrations of

IL-2 as high as 200 U/ml, which confirmed the data in Fig.2.1A that IL-2 did not cause PKC translocation from the cytosol to membranes. Thus, these results indicate that a rapid and transient activation of membrane PKC activity occurs in CTLL-2 cells in response to IL-2 and that this activation cannot be accounted for by the traditional translocation mechanism.

2.4.2 *IL-2 activates inactive membrane-associated PKC in CTLL-2 cells*

The principle advantage of the direct PKC assay is that it measures enzyme activity directly in isolated membranes, without prior detergent solubilization and reconstitution of the enzyme with exogenous PS, TPA, and excess Ca^{2+} . It therefore gives an estimate of what is truly important to cell function -namely, the amount of active PKC present in cell membrane pools. Another major property of the direct assay is its ability to indirectly measure the levels of inactive membrane PKC in cells following the addition of either TPA, DAG, or excess Ca^{2+} to the reaction mixture (Chakravarthy et al., 1991). Under these conditions any inactive, but activatable, PKC in membranes is maximally stimulated. The difference between this "total" membrane PKC and the levels of active PKC measured by the direct assay gives a value of the inactive PKC present in CTLL-2 cell membranes (Chakravarthy et al., 1991). Experimentally, the measurement of total membrane PKC (inactive + active) is typically performed by the addition of 1 μM TPA to the reaction mixture of the direct assay (Chakravarthy et al., 1991, 1994). As shown in Fig. 2.4A, the addition of 1 μM TPA to the reaction mixture increased basal PKC activity approximately 2.5-fold in membranes isolated from unstimulated, IL-2-deprived cells, but it did not increase enzyme activity in membranes isolated from IL-2-stimulated cells. These results indicate that IL-2 activated a pool of membrane PKC in intact CTLL-2 cells that, once stimulated, was no longer available for activation by TPA added to isolated membranes. By contrast, the addition of TPA to intact IL-2-deprived cells caused an increase in membrane PKC

activity well beyond that seen in membranes isolated from unstimulated and IL-2-treated cells exposed to TPA in the assay reaction (Fig. 2.4A). Under these conditions, it was likely that TPA, in contrast to IL-2, caused both the activation of inactive membrane PKC as well as the translocation of cytosolic enzyme when added to intact cells. As such, the addition of TPA to the reaction mixture had no further effect on the PKC activity in membranes isolated from TPA-stimulated cells (Fig. 2.4A), because all membrane-associated enzyme (either pre-existing or translocated) was maximally activated by the phorbol ester *in situ*. Taken collectively, these observations are consistent with CTLL-2 cells having about 50-60% of its membrane PKC in an inactive state, and that this inactive pool is specifically activated by IL-2 in the absence of detectable translocation. This conclusion was confirmed in experiments in which the direct and conventional methods were directly compared (Fig. 2.4B). A variation (Kikkawa et al., 1982) of the conventional method used in Figure 2.1A was utilized in these experiments, in which cytosolic and detergent-extracted membrane PKC were subjected to partial purification by DE-52 ion-exchange chromatography before being assayed in the presence of TPA, PS and Ca^{2+} . The results presented in Figure 2.4B indicate that 1) TPA, but not IL-2, induced the translocation of cytosolic PKC to membranes, and that 2) IL-2 activated a pool of membrane-associated PKC that was detectable by the direct assay, but not by conventional methods for measuring PKC.

The pool of inactive PKC that was stimulated by IL-2 in IL-2/serum deprived CTLL-2 cells was generated by the removal of these factors from the medium. As shown in Fig. 2.5, there was a profound increase in the levels of inactive PKC within the first few hours after removal of IL-2 and serum from exponentially growing CTLL-2 cells. This increase occurred in the absence of any changes in "total" PKC in the membranes, indicating that a conversion from active to

inactive PKC resulted from cytokine starvation. It was most likely that this conversion resulted from IL-2 (rather than serum) withdrawal since 1) IL-2 addition rapidly caused the reactivation of the enzyme (Fig.2.4) and 2) serum itself was unable to induce an increase in membrane PKC in IL-2/serum deprived cultures (Chapter I, Fig.1.10).

It was possible that the ~2 fold increase in membrane PKC activity effected by IL-2 could be detected *in vivo* as an increase in the phosphorylation of the endogenous PKC substrate, the MARCKS protein. However, as shown in Fig.2.6, IL-2 consistently failed to induce MARCKS phosphorylation *in situ*, although TPA-induced translocation of the enzyme gave a robust stimulation in the phosphorylation of MARCKS. These results are of interest in that they suggest that the PKC pool stimulated in CTLL-2 cells by IL-2 exhibits a degree of substrate specificity (due perhaps to isozyme specific activation, or PKC compartmentalization) relative to the broad spectrum activation of PKC effected by powerful PKC activators, such as TPA. It is known that MARCKS associates with discrete membrane structures. MARCKS also binds to calmodulin in a Ca^{2+} -dependent manner which prevents its phosphorylation by PKC.

2.4.3 Establishing the importance of PKC stimulation in the IL-2-mediated survival of CTLL-2 cells

The physiological significance of the activation of inactive membrane PKC by IL-2 in CTLL-2 cells was investigated using PKC specific inhibitors. Many PKC inhibitors are now commercially available which act at a variety of sites on the enzyme and which exhibit a broad range of specificity for PKC relative to other cellular kinases (see General Introduction for details). A variety of PKC inhibitors were available in our laboratory, including isoquinoline (H-7) (Hidaka et al., 1984), bisindolylmaleimide (BIS) (Toullec et al., 1991), 1-o-alkyl-2-o-methyglycerol (AMG) (van Blitterswijk 1987a), staurosporine (Tamaoki et al., 1986, Gross et

al., 1990, Herbert et al., 1990), calphostin-C (Kobayashi et al., 1989a, b, c), and sphingosine (Hannun et al., 1986). Of this group, BIS, AMG, and calphostin-C are among the most potent and selective for PKC (see General Introduction). Thus, these three PKC inhibitors were examined for their ability to block the IL-2-induced activation of membrane PKC when added to CTLL-2 cells at the time of IL-2 stimulation. As shown in Fig. 2.7, the three compounds potently inhibited PKC activity (measured by the direct assay) when added to the culture medium immediately prior to IL-2 stimulation. The levels of inhibitors shown in Fig. 2.7 represent the lowest concentrations that gave maximal PKC inhibition, and are within the range reported effective in inhibiting PKC activity in other studies (Tamaoki et al., 1986, Kobayashi et al., 1989a, b, c, Gross et al., 1990, Herbert et al., 1990, Toullec et al., 1991). BIS is a structural 'upgrade' of staurosporine which acts specifically at the ATP binding site of PKC (Toullec et al., 1991), and AMG is a DAG analog that blocks the DAG binding site of the enzyme (van Blitterswijk 1987a). For these reasons, BIS and AMG were chosen as appropriate PKC inhibitors to study the impact of manipulating PKC activity on the ability of IL-2 to induce behavioral /functional changes in CTLL-2 cells.

Since IL-2 is required for maintaining CTLL-2 cell viability, the effect of PKC inhibitors on IL-2's ability to maintain cell viability was investigated. As shown in Fig. 2.8A, the ability of IL-2 to rescue CTLL-2 cells deprived of IL-2 and serum for 7 hours was substantially reduced when the cells were preincubated with concentrations of BIS and AMG effective in blocking PKC activity in these cells (see Fig. 2.7). These concentrations of AMG and BIS were not in themselves toxic to cells when added to exponentially growing CTLL-2 cells (data not shown). The fact that both AMG and BIS, acting at different sites on PKC, were effective in blocking the IL-2 induced rescue of CTLL-2 cells is consistent with a requirement for PKC in this IL-2

mediated process. While the data in Fig.2.8A suggest that blocking PKC activity during the 17 hour incubation period had an adverse effect on IL-2 induced rescue of the cells, it does not indicate whether the early activation of inactive membrane PKC was the critical PKC event. Evidence that an early PKC event was indeed critical to the IL-2-induced rescue of CTLL-2 cells is shown in Fig.2.8B. The ability of IL-2 to rescue CTLL-2 cells deprived of IL-2 and serum for 7 hour was substantially reduced when the cells were preincubated with BIS or AMG for 30 min prior to stimulation (Fig.2.8A). By contrast, both BIS (Fig.2.8B) and AMG (data not shown) were far less effective in blocking IL-2-induced rescue of cells when added to the medium 2 hour after the cultures were stimulated with IL-2. These findings indicate that the PKC inhibitors blocked a cellular PKC event(s) that was an important early step in the mechanism by which IL-2 suppressed the loss of cell number in CTLL-2 cells. It was most unlikely that the effects of the inhibitors were due to some nonspecific toxicity because, regardless of the time of BIS or AMG addition to the cultures relative to IL-2, all cells were exposed to the inhibitors for a total of 24 hours (Fig.2.8B). It was also unlikely that the BIS- or AMG-mediated reduction in cell number in IL-2-stimulated cells was caused by a direct inhibition of DNA replication because, as was shown in Chapter 1 Fig.1.3, IL-2 itself was unable to support DNA replication in the absence of serum factors. BIS has been shown previously not to directly affect DNA replication in 3T3 cells (Toullec et al., 1991). These data strongly suggest that the inhibitors blocked an early PKC dependent event necessary for the IL-2 induced suppression of cell death in these cells.

2.4.4 PKC plays a role in the ability of IL-2 to suppress apoptosis in CTLL-2 cells.

The mechanism by which PKC blocks IL-2-mediated cell death was examined. As discussed in the Introduction to this chapter, IL-2 receptor stimulation constitutes a powerful signal which suppresses cell death signals (apoptosis) in a variety of IL-2 dependent cells.

Moreover, a role has been postulated for PKC in the induction or suppression of apoptosis in a variety of hematopoietic and nonhematopoietic cells (McConkey and Orreniu 1991, Tritton 1991). As shown in Fig.2.9, DNA laddering, a hallmark of apoptosis, indicated that a significant degradation of DNA occurred by 12 hours, but not 7 hours, after withdrawal IL-2 and serum from actively growing CTLL-2 cells. The readdition of IL-2 after 7 hours of 'starvation' prevented the induction of DNA laddering in these cells. These data are consistent with the results of Fig.1.5, and Fig.2.8A showing that IL-2 restoration at these times can effect the rescue of CTLL-2 cells from IL-2 starvation. Most significantly, preincubating cells with BIS at concentrations sufficient to inhibit PKC activity blocked the ability of IL-2 to suppress DNA degradation over the ensuing 5 hour (Fig. 2.9, lane d and e). These observations indicate that an early, PKC dependent event is likely part of the mechanism by which IL-2 affects the rescue of CTLL-2 cells, and that the rapid activation of inactive membrane-associated PKC by IL-2 may be an important step in the mechanism by which the cytokine suppress apoptotic cell death.

2.4.5 The role of PKC in the cytolytic activity of CTLL-2 cells

As shown in Chapter I, CTLL-2 cells are cytotoxic T-cells which kill antigen-bearing target cells in an IL-2-dependent manner (Chapter 1 Fig. 1.6). The role of PKC in CTLL-2-mediated cytotoxicity was examined. Concentrations of BIS (10 μ M) which effectively inhibited PKC activity and IL-2 mediated cell rescue had no effect on cytotoxic activity in CTLL-2 cells when present during the 4 hour cytotoxic assay (Fig.2.10). Preincubating cells with BIS for 4 hours prior to the assay also had no effect (data not shown). Moreover, the stimulation of PKC activity in CTLL-2 cells by the addition of 1 μ M TPA to the assay had no measurable effects on cytotoxic activity (Fig. 2.10). These results indicate that while PKC may play a role in the IL-2

mediated suppression of apoptosis in CTLL-2 cells, the enzyme is unlikely linked to the mechanism by which IL-2 initiates or maintaining cytotoxic activity in these cells.

Figure 2.1 IL-2 rapidly activates membrane-associated PKC activity in CTLL-2 cells by a mechanism not involving the translocation of cytosolic enzyme.

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium at 5×10^6 cells/ml, and incubated at 37°C for 4 hour. Cells were then stimulated with 100 U/ml IL-2 for the indicated times before being lysed hypotonically and the membrane and cytosolic fractions isolated as described in *Methods*. A) Membrane (8µg membrane protein) and cytosolic PKC were subjected to a conventional assay, as described in *Methods*, in which detergent-solubilized membrane PKC (■) and cytosolic enzyme (○) activity were measured in the presence of 2 mM CaCl_2 , 10 µg phosphatidylserine, and 1 µM TPA to ensure maximal activation of all enzyme. B) The amount of active membrane PKC was determined by the "direct" assay, in which enzyme activity was measured in isolated, intact membranes without detergent extraction and reconstitution with exogenous PS/TPA, as described in *Methods*. Values are the means $\pm$ SEM of triplicate determinations and are representative of five separate determinations.

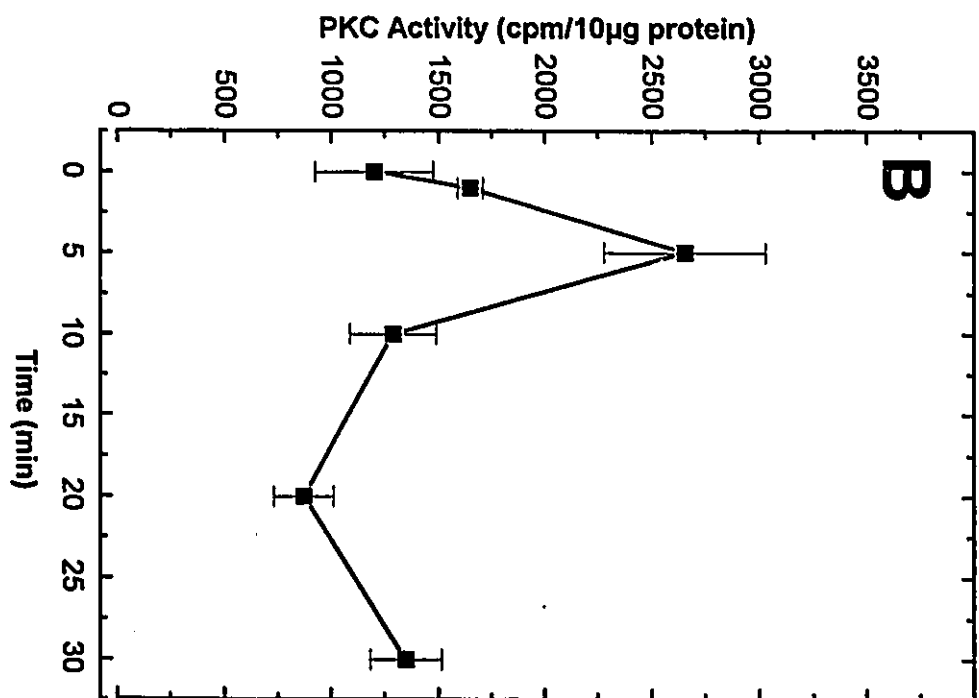
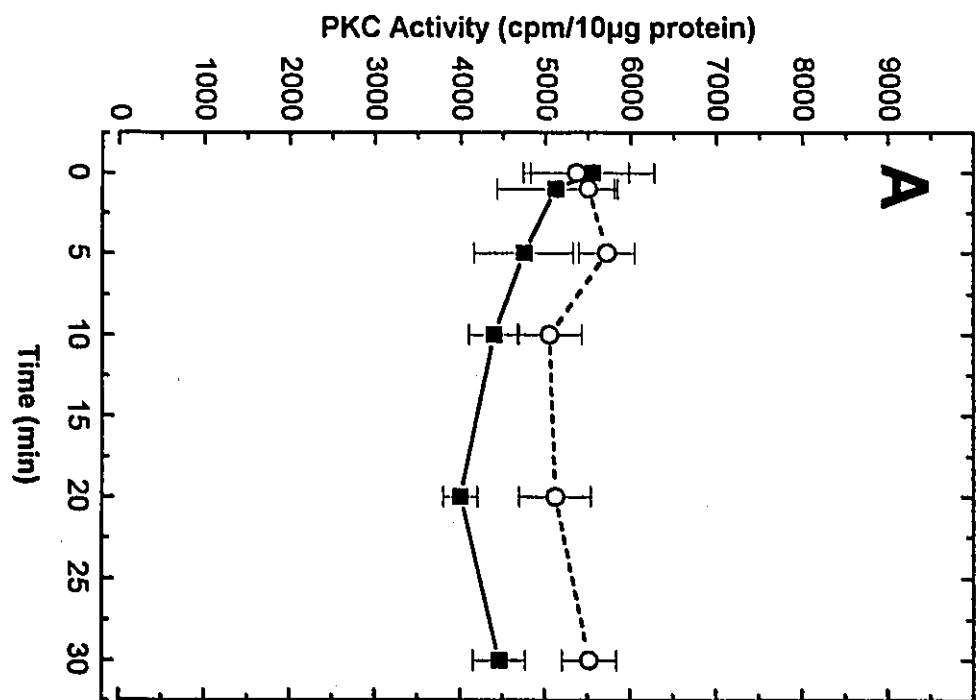


Figure 2.2 TPA, but not IL-2, stimulates PKC translocation in CTLL-2 cells

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium at 5×10^6 cells/ml, and incubated at 37°C for 4 hour. Cells were then left untreated or stimulated with 100 U/ml IL-2, or 1 μ M TPA, for 10 min at 37°C before being lysed hypotonically and the membrane and cytosolic fractions isolated as described in *Methods*. A) Membrane and cytosolic PKC were subjected to direct and conventional assays respectively, as described in *Methods*. Values are the means $\pm$ SEM of triplicate determinations. B) Solubilized membrane and cytosol (20 μ g) from untreated, IL-2 treated, and TPA treated were subjected to immunoblotting with mixed PKC antibodies as indicated in the figure (M: M.W. marker, B: rat brain PKC, the line at left represent 80KDa). The results are representative of two separate experiments.

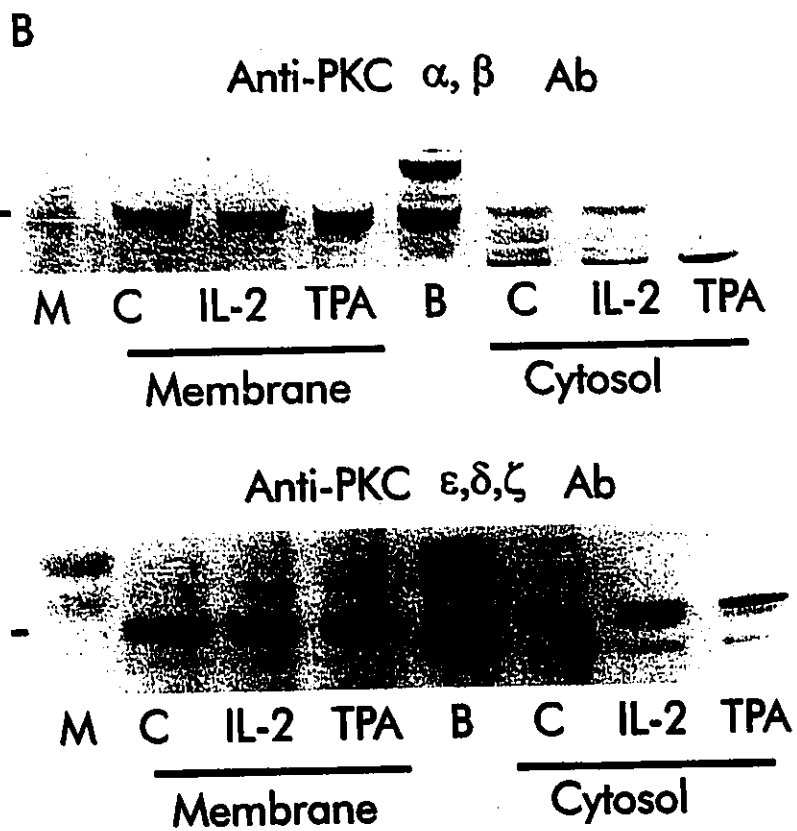
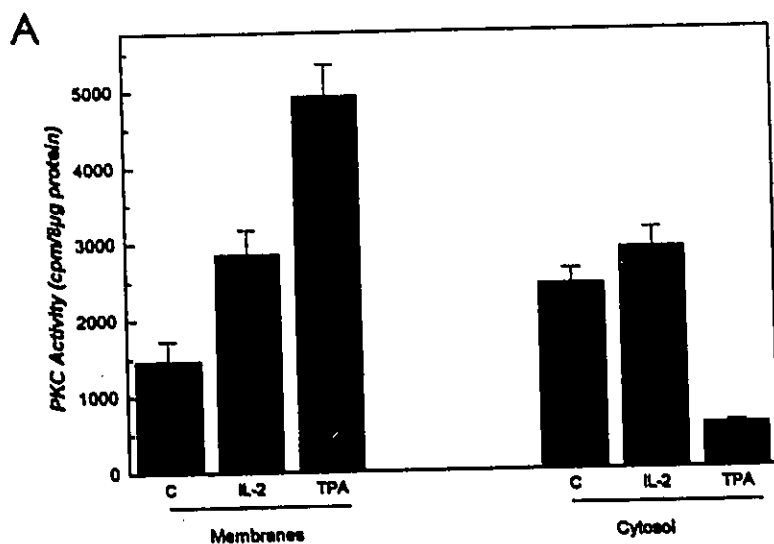


Figure 2.3 IL-2 activates inactive membrane PKC in a dose-dependent manner

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium at a density of 1.0×10^6 cells /ml and incubated for 4 hour at 37°C. The indicated concentrations of IL-2 were added to 20×10^6 cells and incubated for 10 min at 37°C. The reaction was stopped by adding ice-cold PBS. Cells were lysed in hypotonic lysis buffer as described in *Methods*. Nuclei and unlyzed cells were sedimented by centrifugation, and the membrane and cytosol fractions were prepared as described in *Methods*. Membrane and cytosolic PKC activity was determined by the direct and conventional assays, respectively, as described in *Methods*. Values are the means $\pm$ SEM of triplicate determinations, and are representative of two separate experiments.

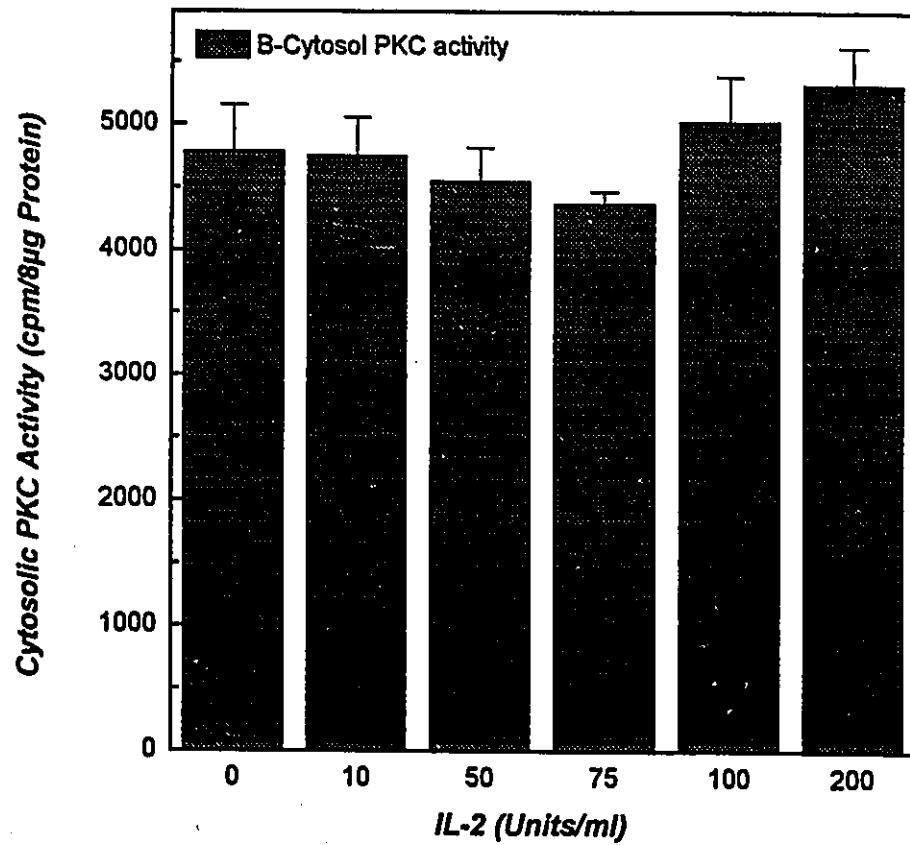
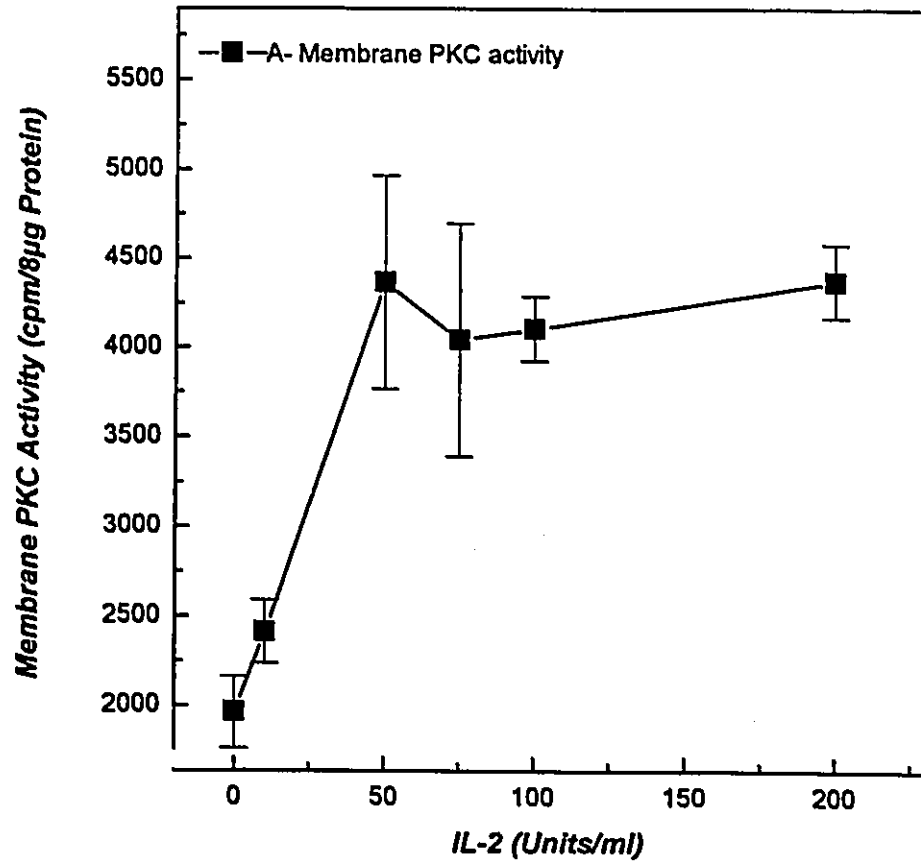


Figure 2.4 IL-2 selectively activates an inactive pool of membrane PKC existing in unstimulated CTLL-2 cells.

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium at 5×10^6 cells/ml, and incubated at 37°C for 4 h. A) Cultures were either left unstimulated (control), or stimulated with 100 U/ml IL-2 or 1 μ M TPA for 5 min. The cells were then lysed hypotonically, the membrane and cytosolic fractions isolated as described in *Methods*, and the PKC activity present in intact membranes was determined by the direct assay in the absence (square) and presence (dotted square) of 1 μ M TPA in the reaction mixture to give a measure of active and total PKC, respectively. Values are the means $\pm$ SEM of four separate determinations. B) Cultures were either left unstimulated (square), or stimulated with 100 U/ml IL-2 (dotted square) or 1 μ M TPA (hatched square) for 5 min before the cells were lysed and membrane and cytosolic fractions isolated, as described in *Methods*. A portion of each membrane preparation was used in the direct assay to determine the levels of active enzyme. In addition, cytosols and detergent-solubilized membrane extracts were first partially purified by DE-52 ion-exchange chromatography, and then subjected to a conventional assay in the presence of PS/TPA/ Ca^{2+} , as described in *Methods*. Values are the means $\pm$ SEM of four separate determinations.

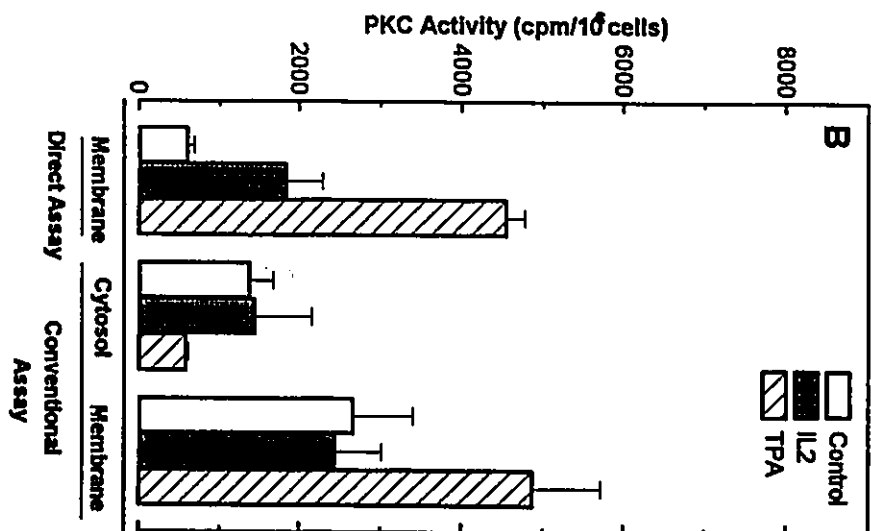
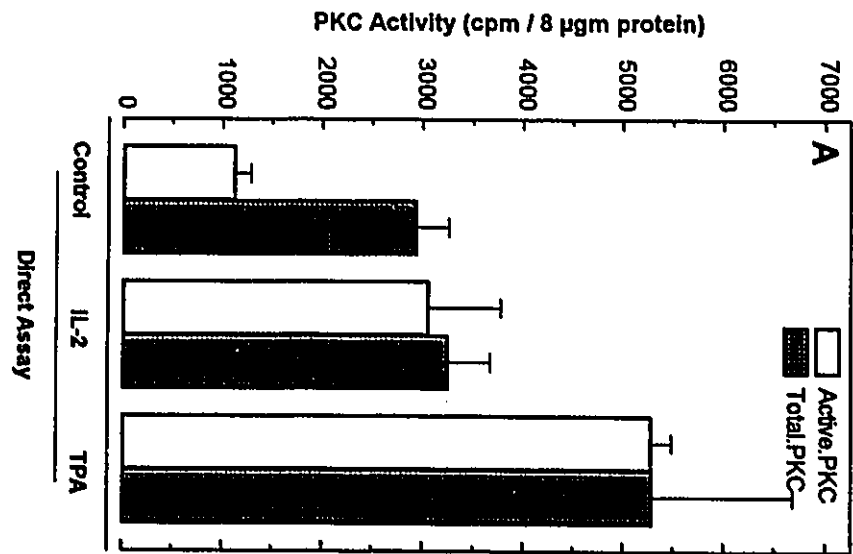


Figure 2.5 The generation of inactive membrane associated PKC in serum/IL-2 deprived CTLL-2 cells

Exponentially growing CTLL-2 cells were washed twice with prewarmed RPMI medium, resuspended in unsupplemented RPMI medium at a density of 5×10^6 cells/ml, and incubated at 37°C. At the indicated times, the cells were lysed and membrane and cytosolic fractions were prepared as described in *Methods*. The amounts of total (■) and inactive (○) PKC in isolated membranes were determined by the direct assay, as described in *Methods*. Inactive PKC was calculated as the difference between the values of total membrane PKC (i.e. with 1 μ M TPA in the reaction mixture) and active membrane PKC (i.e. no TPA) as described above. The values are the means $\pm$ SEM of triplicate determinations and are representative of three separate determinations.

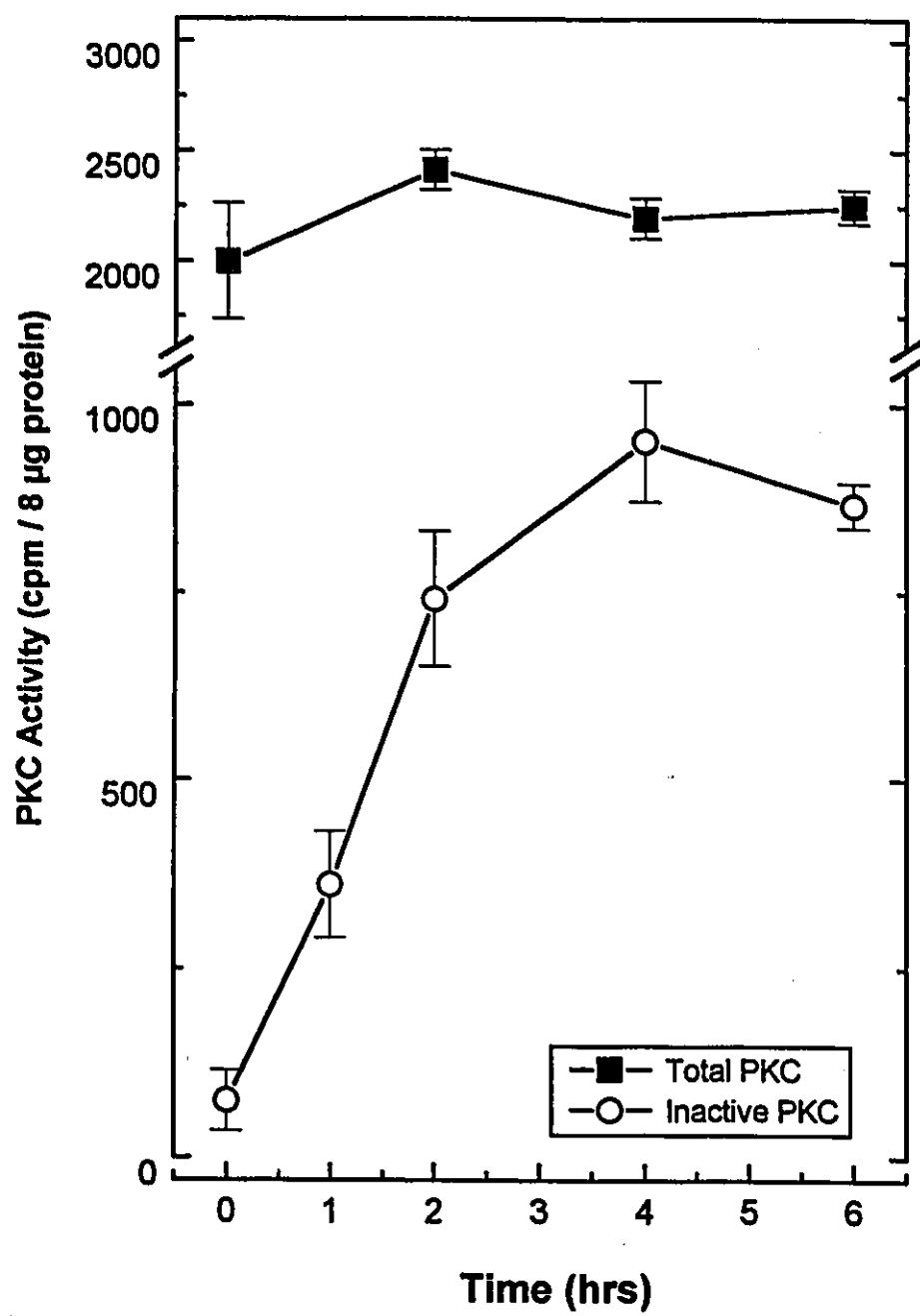
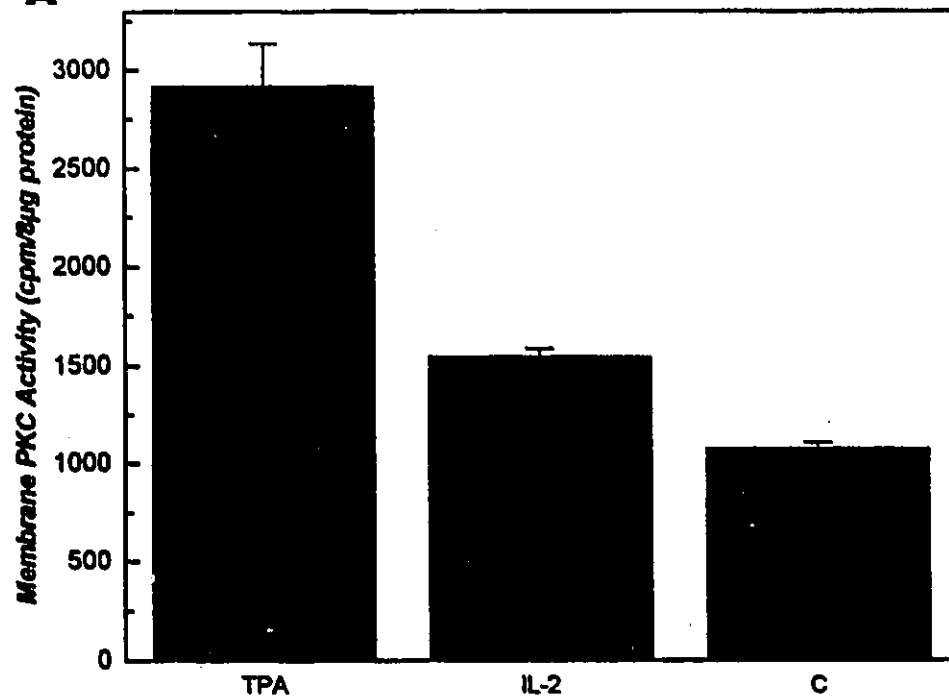


Figure 2.6 TPA, but not IL-2, causes MARCKS protein phosphorylation

Exponentially growing CTLL-2 cells were washed twice in unsupplemented RPMI medium, resuspended in the same medium, and incubated for either 4 h (A), or 2 h (B). A). The serum and IL-2 deprived cells were treated with 100 U/ml IL-2 or 1 μ M TPA at 37°C for 10 min. The reactions were stopped by adding ice-cold PBS, the cells were lysed hypotonically, and membrane and cytosol fractions were prepared as described in *Methods*. Membrane PKC activity was determined by the direct assay as described in *Methods*. Values are the means $\pm$ SEM and are representative of two separate experiments. B). After 2 hrs of incubation, the serum and IL-2 deprived cells were labeled with 32 P_i for two hours as described in *Methods*. The labeled cells (6×10^6 /ml) were then either left untreated or treated with 100 U/ml IL-2 or 1 μ M TPA for 10 min at 37°C. The cells were then rapidly sedimented, solubilized, and the protein content subjected to 10% SDS- PAGE as described in *Methods*. 85 kDa MARCKS phosphorylation was visualized by autoradiography as described in *Methods*.

A



B

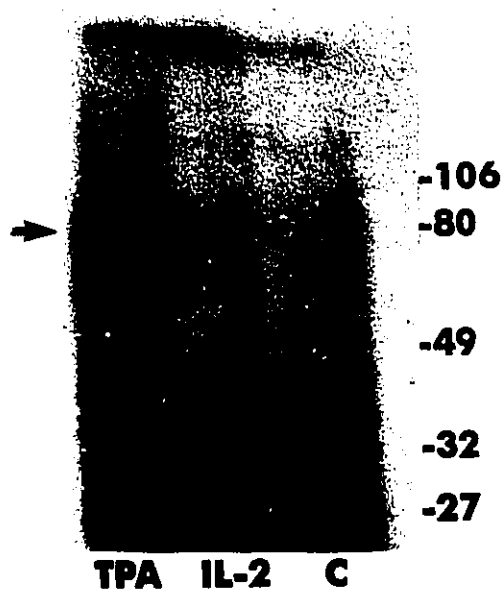


Figure 2.7 The effect of PKC inhibitors on PKC activity

Exponentially growing CTLL-2 cells, were washed twice in unsupplemented RPMI medium, resuspended in same medium at a density of 1.0×10^6 cells /ml and incubated for 4 h at 37°C. The indicated concentrations of different PKC inhibitors (Cal: calphostin C, AMG, Bis: BIS) were added to 20×10^6 cells, incubated for 30 min at 37°C before adding 100 U/ml IL-2, or 1 μ M TPA for 10 min. The reactions were stopped by adding ice-cold PBS and the cells then lysed in hypotonic lysis buffer and the nuclei and unlyzed cells were separated as described in *Methods*. Membrane and cytosol fractions were separated by centrifugation as described in *Methods*. Membrane PKC activity was determined by the direct PKC assay as described in *Methods*. Values are expressed as the percentage of control of triplicate determinations, and are representative of two separate experiments.

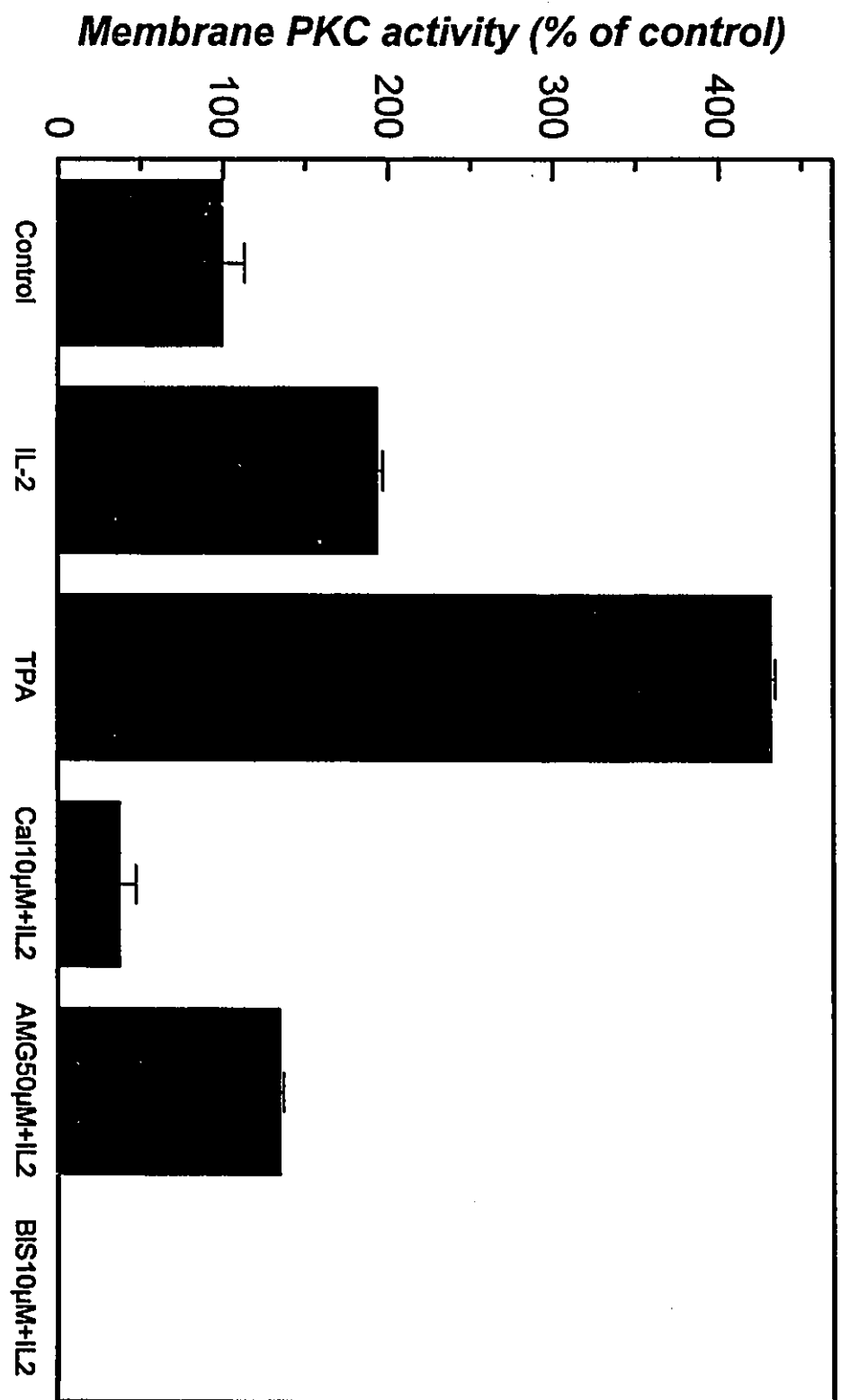


Figure 2.8 Evidence that early PKC-dependent events are associated with the ability of IL-2 to prevent apoptosis in CTLL-2 cells.

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium at 1.5×10^5 cells/ml, and incubated at 37°C for 7 h. A) BIS GF 109203X or AMG were added to some cultures at the indicated concentrations 30 min before the cells were stimulated with 100 U/ml IL-2 for 10 min. Other cultures were left unstimulated (-IL-2). Cultures were incubated an additional 17 h, and then the number of viable cells was measured, as described in *Methods*. The values are the means $\pm$ SEM of three separate determinations. B) The indicated concentrations of BIS were added either 30 min before IL-2 addition (■), or 2 hrs (●) or 4 hrs (○) h after IL-2 addition. The ability of the PKC inhibitor to block the IL-2-induced protection of CTLL-2 cells from cell death was determined 17 hrs later by measuring viable cell numbers, as described in *Methods*. The results are presented as the percent inhibition of the extent of protection afforded CTLL-2 cells by 100 U/ml IL-2. The values are the means $\pm$ SEM of three separate determinations.

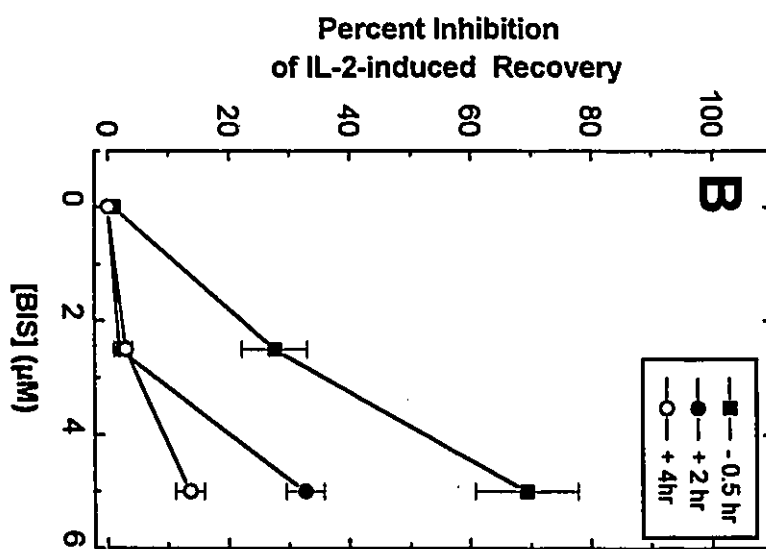
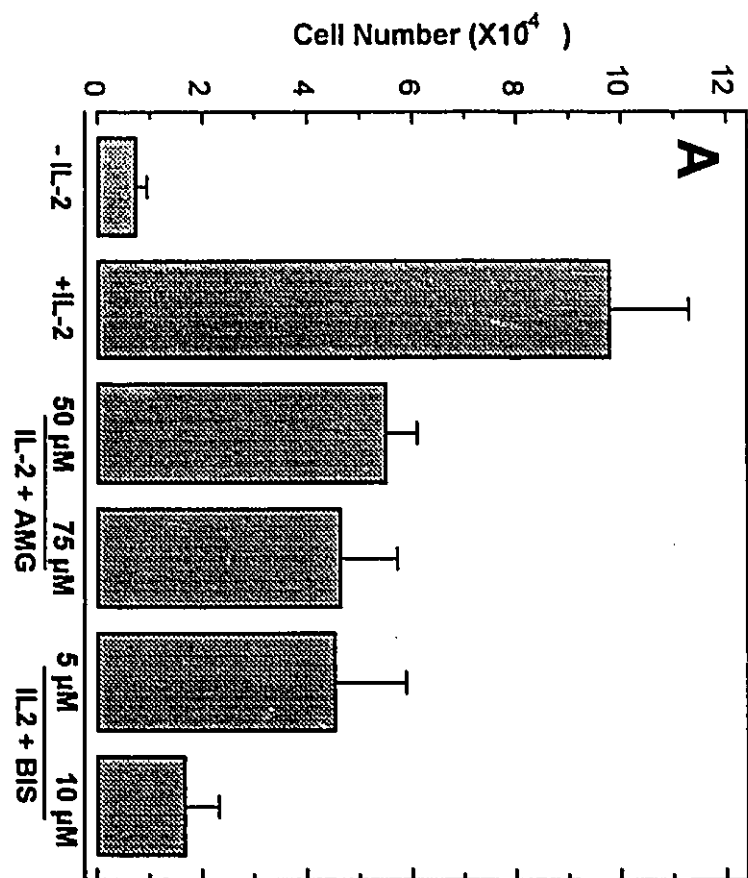


Figure 2.9 The inhibition of PKC by BIS blocks the ability of IL-2 to suppress apoptosis in CTLL-2 cells.

Exponentially growing CTLL-2 cells were washed twice with unsupplemented RPMI medium, resuspended in the same medium at 2×10^5 cells/ml, and incubated at 37°C for 7 h. The extent of DNA fragmentation, assessed by agarose gel electrophoresis as described in *Methods*, was determined immediately (a), or after the cells had been incubated for an additional 5 h with no further additions (b), or with either 100 U/ml IL-2 (c), 10 μM BIS added 30 min before 100 U/ml IL-2 (d), or 5 μM BIS added 30 min before 100 U/ml IL-2 (e). Lane f: m.w. markers (123-bp ladder). The results are representative of two separate experiments.

abcdef

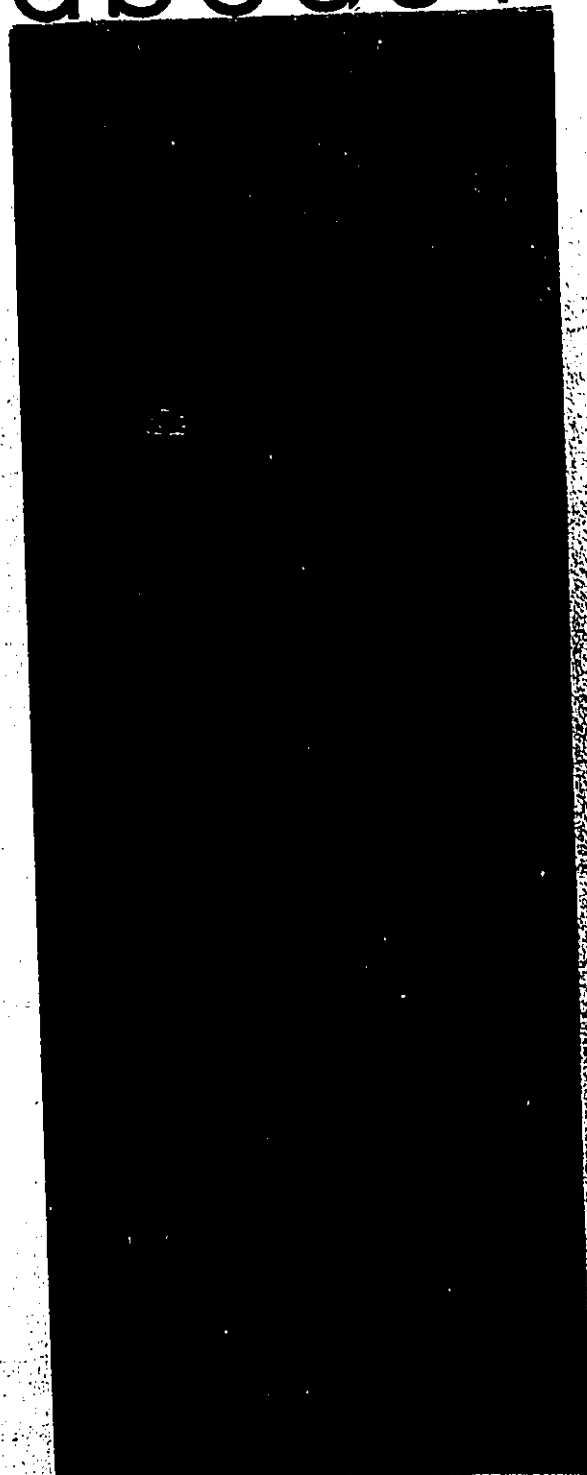
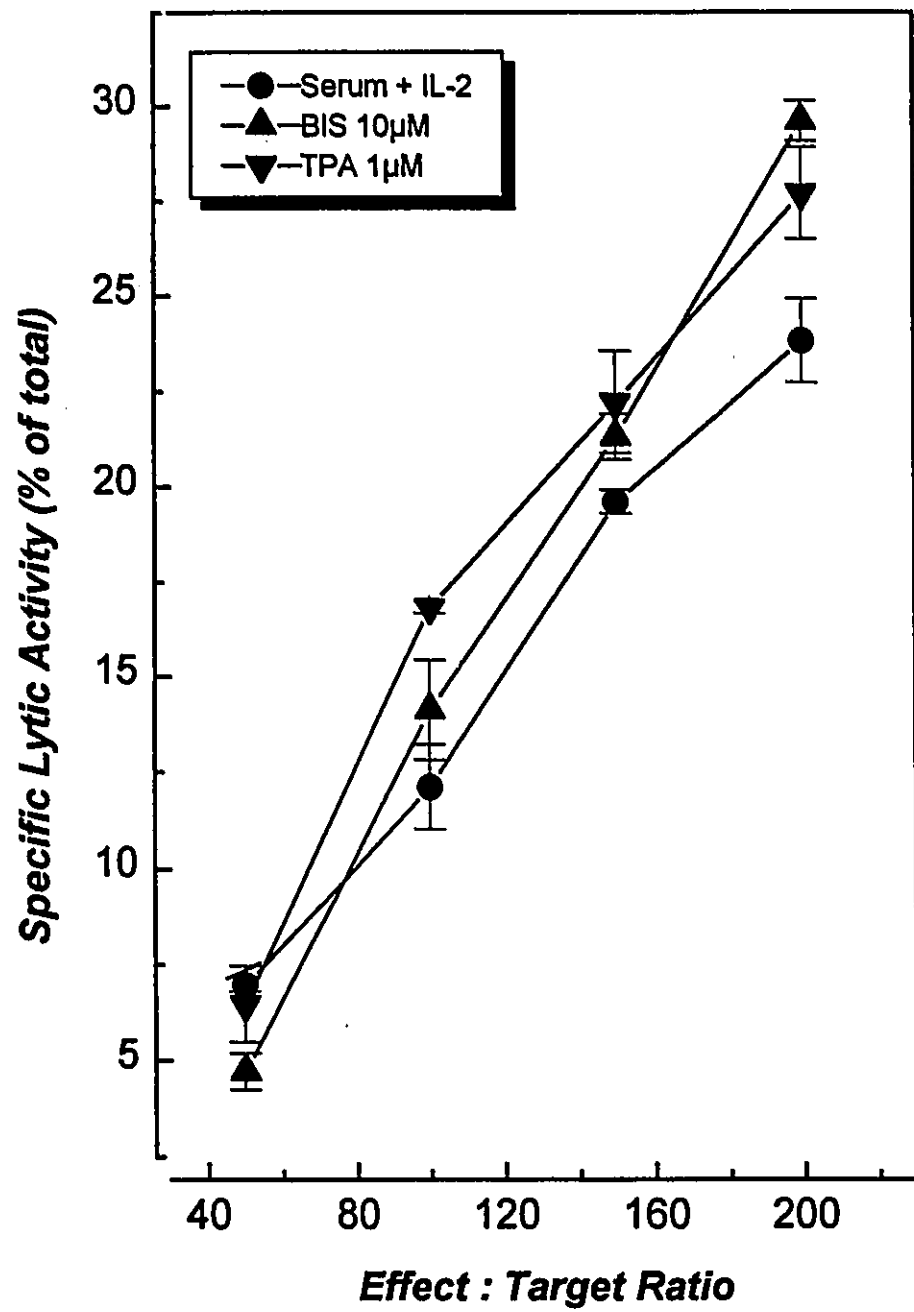


Figure 2.10 The effect of PKC inhibition or activation on the cytolytic activity of CTLL-2 cells

Exponentially growing CTLL-2 cells, in fresh RPMI medium supplemented with IL-2 (100 U/ml) and serum (10%) were added to 100 μ l of S49.1 cells (prelabelled with ^{51}Cr as described in Method section) mixed at the ratio indicated in the figure at a final volume of 200 μ l. The target ratio is defined as the concentration of effector cells (i.e. CTLL-2) to target cells (i.e. S49.1). To some cultures the PKC inhibitor BIS ($\blacktriangle$) or the PKC activator TPA ($\blacktriangledown$) was added at 10 μM and 1 μM respectively. Other cultures were left untreated ($\bullet$). After a 4 hr incubation at 37°C, CTLL-2 cytolytic activity was determined as described in *Methods*. Values are the mean $\pm$ SEM of the cellular ^{51}Cr released by S49.1 cells, performed in triplicate cell cultures and are representative of three different experiments.



2.5 DISCUSSION

2.5.1 *IL-2 stimulates PKC in CTLL-2 cells by activating a pool of inactive membrane associated PKC*

Both the conventional assay and immunoblot analysis (Fig.2.1A, Fig.2.2) indicated that IL-2 failed to stimulate the translocation of PKC from the cytosol to membranes in CTLL-2 cells. This finding was in agreement with the previous report of Mukaida et al. (1988) who were also unable to demonstrate IL-2-mediated PKC translocation in these cells. However, the present study has significantly extended these early finding by showing that while translocation does not occur in these cells in response to IL-2, there is indeed IL-2-mediated activation of cellular PKC- an activation that occurs in, and appears restricted to, an existing pool of PKC resident in CTLL-2 membranes. This pool of IL-2 stimutable PKC, identified by the direct PKC assay, exists in CTLL-2 cell membranes in an inactive, but primed state where it can be readily activated by the DAG analog, TPA. This conclusion was based on experiments (Fig.2.4) showing that TPA, which reproducibly stimulated PKC activity in membrane preparations isolated from IL-2 deprived cells, was unable to do so in membranes from IL-2 treated cells because the cytokine had already activated the TPA-stimulatable pool prior to membrane isolation. A similar pool of TPA-sensitive membrane PKC has been identified in a number of cell types which can be readily activated by a variety of extracellular stimuli (Chakravarthy et al., 1994). By contrast, the characteristics of the IL-2 stimutable PKC in CTLL-2 cells is clearly different from that pool of membrane PKC rendered inactive by the action of a recently identified endogenous PKC inhibitory protein present in neuroblastoma cells and in rat brain (Chakravarty et al., 1995).

Interestingly, TPA, but not IL-2, was found to stimulate the phosphorylation of the endogenous PKC selective substrate, the MARCKS protein, in intact CTLL-2 cells (Fig.2.6). A possible explanation for this finding is that IL-2 selectively stimulated a pool of PKC not physically or functionally linked to the MARCKS protein in these cells, whereas the nonselective activation of virtually all cellular PKC by TPA resulted in the PKC catalyzed phosphorylation of the MARCKS protein. Moreover, since the data presented in Fig.2.4 indicated that IL-2 added to intact cells stimulated the same *membrane* PKC pool as did TPA added to isolated membranes, the increased MARCKS protein phosphorylation effected by TPA was likely due to PKC activated by the translocation of cytosolic enzyme to membranes. Collectively, these results clearly indicate that translocation is not the sole mechanism for PKC activation in CTLL-2 cells, and that translocation perhaps may not be an important part of the mechanism by which physiologically relevant extracellular stimuli activate PKC. This conclusion is supported by previous studies showing that IL-3, PDGF, FGF, and EGF all stimulate PKC *via* the activation of inactive membrane associated PKC without translocation (Chakravarthy et al., 1994), and that the activation of inactive membrane associated PKC is responsible for DMSO induced erythroleukemia cell differentiation (Chakravarthy et al., 1992).

2.5.2 Activation of inactive PKC is responsible for IL-2 to suppress apoptosis

Using the specific PKC inhibitors, BIS and AMG, it was demonstrated that inhibiting PKC activity blocked IL-2's ability to maintain CTLL-2 cell viability (Fig.2.8A). The early activation of PKC by IL-2 appeared to be an important aspect of IL-2 mediated suppression of cell death, since the ability of BIS to block IL-2 mediated cell rescue (when added *before* IL-2) was progressively lost when the inhibitor was added 2-4 hours after IL-2 (Fig.2.8B). These data strongly suggest that the inhibitors blocked an early PKC-dependent event necessary for the IL-

2-induced suppression of apoptosis in these cells. This conclusion was confirmed by studies in which the effects of PKC inhibition on DNA laddering, a marker of apoptosis, were monitored. The laddering of DNA which was apparent in CTLL-2 cells after 12 hours of IL-2 and serum deprivation, was almost completely suppressed by the addition of 100 U/ml IL-2 at 7 hour. However, preincubating cells with BIS at concentrations sufficient to inhibit PKC activity blocked the ability of IL-2 to suppress DNA degradation over the ensuing 5 hours (Fig. 2.9). These observations indicate that an early, PKC-dependent event is likely part of the mechanism by which IL-2 affects the rescue of CTLL-2 cells, and that the rapid activation of inactive membrane-associated PKC by IL-2 may be an important step in the mechanism by which IL-2 suppresses apoptotic cell death. This conclusion is supported by the study of Walker et al. which demonstrated that in the absence of IL-2, TPA can suppress the onset of apoptosis in these cells (Walker et al., 1993). Nevertheless, it is important to note that while the data presented indicates that an early PKC dependent event is likely necessary for IL-2 mediated cell survival, it does not constitute direct proof that the *increase* in PKC by IL-2 is absolutely needed. Moreover, one cannot completely rule out the possibility that the inhibitor-induced effects on apoptosis arose from inhibition of other uncharacterized protein kinases with PKC-like functions.

Previous studies have observed that phorbol esters can either protect (Rodriguez-Tarduchy and Lopez-Rivas 1989, Walker et al., 1993) or exacerbate (Kizaki et al., 1988) apoptosis in lymphocytes upon IL-2 withdrawal. However, interpreting TPA effects on cellular behavior can be difficult because, aside from questions related to TPA specificity, phorbol esters have a complex effect on cellular PKC, in that they initially activate the enzyme, but this is followed shortly by a profound and persistent loss in enzyme activity (i.e., down regulation).

2.5.3 *The role of PKC in cytolytic activity*

As shown in Chapter 1 (Fig. 1.6) IL-2 appears to be intimately involved in the cytolytic activity of CTLL-2 cells. However, the results presented in Fig.2.10 clearly indicate that neither the activation nor the inhibition of PKC had much of an effect on CTLL-2 cytolytic activity, suggesting that PKC may not be linked to this IL-2 mediated process (Fig.2.10). It has been reported that p56^{lck}-deficient CTLL-2 cells display a profound reduction in T-cell antigen receptor-dependent cytolytic activity (Karnitz et al., 1992), and that the cytolytic defects were completely reversed by the transfection of these cells with a wild-type *lck* expression vector. Hence, the available data suggests that tyrosine kinase may play a more important role in IL-2 mediated cytolytic activity in CTLL-2 cells than does PKC. On the other hand, there is evidence that PKC is involved in the IL-2-dependent cytolytic activity exhibited by NK cells (Ortaldo et al., 1989, Ting et al., 1992) suggesting that the killing mechanisms of CTLL-2 cells and NK cells are fundamentally different, at least at the signalling level. More importantly, the PKC data presented is consistent with the notion that IL-2 elicits different cellular responses in CTLL-2 cells (i.e., cell survival and cytolytic activity) by activating different cellular signalling pathways.

2.6 CONCLUSION

The general conclusion of the studies described in this chapter is that the activation of inactive membrane associated PKC is part of the mechanism responsible for the ability of IL-2 to suppress apoptotic cell death, but not in IL-2 mediated cytolytic activity. The specific conclusions are:

- 2.6.1 IL-2 does not stimulate PKC translocation from cytosol to membranes.
- 2.6.2 IL-2 does stimulate PKC in CTLL-2 cells, by activating a pool of membrane-associated inactive PKC. This pool is generated by the conversion of active PKC to an inactive state during IL-2 deprivation.
- 2.6.3 The early and transient activation of PKC by IL-2 appears to be important to the IL-2-induced suppression of apoptosis in CTLL-2 cells.
- 2.6.4 PKC appears not to play a critical role in the IL-2 mediated cytolytic activity of CTLL-2.

CHAPTER THREE: MECHANISMS OF IL-2 INDUCED PKC ACTIVATION IN CTLL-2 CELLS

3.1 INTRODUCTION

As described in the General Introduction, PKC is a ubiquitous and central cell signaling enzyme that is coupled to biological responses evolved by a plethora of extracellular stimuli. Some mechanisms responsible for PKC activation are now well understood (i.e. PI pathway) while others are relatively poorly defined.

3.1.1 Phosphatidylinositol phospholipase C pathway

The receptor-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) is now recognized as a widespread signal transduction pathway, the major elements of which are conserved throughout eukaryotes from yeast to man. This pathway is utilised by a variety of hormones and neurotransmitters (Downes and Michell 1985; Berridge 1987) as well as mitogenic growth factors (Berridge 1987, Whitman and Cantley 1988) and is known to regulate a large array of cellular processes including metabolism, secretion, contraction, neural activity and cell proliferation (Berridge and Irvine 1984, Berridge 1987, Nishizuka 1988).

The two products of PIP₂ hydrolysis are both second messengers; inositol-1,4,5-trisphosphate (IP₃) binds to a specific intracellular receptor and releases sequestered calcium from a sub-population of the endoplasmic reticulum, while sn-1,2-diacylglycerol (DAG) activates PKC. However, DAG has functions other than activating PKC. One is to serve as a source of arachidonic acid for eicosanoid production. Other possible actions include the translocation of diacylglycerol kinase to membrane-bound compartments (Besterman et al., 1986), and the activation of phospholipase A₂ (Kolesnick and Paley 1987), cytidylyltransferase (Pelech and Vance 1984), and phospholipase D (Billah et al., 1989, Kiss and Anderson 1989).

Several distinct inositol phospholipid-specific phospholipase Cs (PI-PLCs) have been purified from a variety of mammalian tissues (reviewed by Rhee et al., 1989), and a total of 16 amino acid sequences (14 mammalian enzymes and 2 *drosophila* enzymes) have been deduced from the nucleotide sequences of their corresponding cDNAs (Rhee and Choi 1992). Comparison of deduced amino acid sequences has indicated that the PI-PLCs can be divided into three types (PLC- β , PLC- γ , and PLC- δ) and that each type contains more than one subtype. PI-PLC isozymes exhibit distinct (albeit subtle) differences in their catalytic properties, their cell type-specific expression and their mode of activation (Rhee and Choi 1992). The catalytic activities of all three types of PLC are dependent on Ca^{2+} . All three types of PLC catalyze the hydrolysis of the three common inositol-containing phospholipids: phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP), and phosphatidylinositol 4,5 bisphosphate (PIP_2). PIP and PIP_2 are the preferred substrates for these enzymes. Different members of the PI-PLC family are regulated by different mechanisms and the activation processes of two isozymes, PLC- $\beta 1$ and PLC $\gamma 1$, are particularly well understood and involve interactions with stimulatory guanine nucleotide-binding (G) proteins and tyrosine kinases, respectively. By comparison PLC- $\beta 1$ is activated by members of the G_q class of pertussis toxin-insensitive G proteins (Rhee 1992). PLC- $\gamma 1$ is activated upon the phosphorylation of specific tyrosine residues (Tyr 783 and Tyr 1254) by receptor tyrosine kinases as well as by cytoplasmic, non-receptor tyrosine kinases (Rhee and Choi 1992).

Receptor-coupled PLC activity in certain types of cells is regulated by PKC or PKA activation, thus providing a negative feedback signal to limit the magnitude and duration of receptor signaling (Huckle et al., 1990). The major mechanism by which PKC attenuates the EGF-triggered accumulation of inositol phosphates has been elucidated. Phosphorylation of Thr-

654 of the EGF receptor by PKC reduces the capacity of the receptor tyrosine kinase to phosphorylate PLC- γ 1 thereby preventing PLC- γ 1 activation (Huckle et al., 1990). A decrease in the extent of tyrosine phosphorylation of PLC- γ 1 has also been linked to the mechanism by which TPA and cAMP attenuate PIP₂ hydrolysis induced by the activation of TCR (Park et al., 1991). In this instance, it appears that the target of both PKC and PKA is the Ser-1248 of PLC- γ 1, and that the phosphorylation of this residue alters the interaction of PLC- γ 1 with the non-receptor PTK or with a protein tyrosine phosphatase. PLC- β 1 also appears to be a target for regulation by PKC in certain cells (Ryu et al., 1990).

3.1.2 Phosphatidylcholine (PC)-specific phospholipase C and D pathways

The signal-activated formation of DAG is often biphasic: it consists of an early peak, which is rapid and transient (and parallels the increase in IP₃ and intracellular Ca²⁺ concentration), followed by a later phase which is slow in onset but is sustained (Nishizuka 1992, Liscovitch 1992). The source of the 'early' DAG is most likely phosphoinositides, whereas the 'late' DAG is probable derived from the hydrolysis of other phospholipids (reviewed by Exton 1990, Billah and Anthes 1990). Several lines of evidence indicate that PIP₂ hydrolysis is not the only source of PKC-activating DAG's: (1) in bombesin-stimulated Swiss 3T3 cells, PKC-mediated increases in pH_i, S6 kinase activity, and MARCKS protein phosphorylation persists long after the IP₃-stimulated [Ca²⁺]_i elevation has declined (Biermant et al., 1990, Takuwa et al., 1991); and (2) a number of growth factors such as CSF-1 (Imamura et al., 1990) and embryonal carcinoma-derived growth factor (Mahadevan et al., 1987) do not stimulate inositol lipid hydrolysis but are still able to increase cellular DAG content or activate a PKC cascade.

It is now clear that phosphatidylcholine (PC) hydrolysis may be linked to PKC activation (Schutze et al., 1991, Nishizuka 1992). PC is the principal phospholipid present in mammalian cells (White 1973). PC in mammalian tissues normally contains a saturated fatty acid residue at the *c*-1 position and an unsaturated fatty acyl residue at the *c*-2 position of the glycerol moiety. Compared to phosphoinositides, which are relatively enriched in stearic acid and arachidonic acid, PC is relatively deficient in arachidonic acid and contains mostly oleic acid and linoleic acid at the *c*-2 position (Mueller et al., 1984, Ojima-Uchiyama et al., 1988). These characteristics have been used to identify PC as a source of DAGs generated by extracellular stimuli (Mueller et al., 1984, Ojima-Uchiyama et al., 1988).

Two pathways for DAG formation from PC have been proposed (Exton 1990, Billah and Anthes 1990), one involving direct hydrolysis of PC by a PC-PLC to give DAG and phosphorylcholine (ChoP) and a second pathway in which PC hydrolysis is coupled to phospholipase D (PLD) activity. The direct, PC-PLC-mediated generation of DAG has now been suggested to occur in a number of cells, in response to certain cytokines. A recent report by Schutz et al. (Schutze et al., 1991) is among the few studies that provide convincing evidence for the activation of PC-PLC (by tumor necrosis factor α [TNF α]) in the absence of PI-PLC activation, Ca^{2+} mobilization, and PC-PLD activation. Similarly, several other cytokines, including interferon- α , interleukin-1, interleukin 3 and colony-stimulating factor 1, do not induce PI breakdown, but stimulate the production of DAG from PC, *via* a PC-PLC pathway (Rosoff et al., 1988, Durino et al., 1989, Imamura et al., 1990, Pfeffer et al., 1990). However, little information is yet available on the regulation of a PC-specific phospholipase C(s).

An alternative, indirect hydrolysis of a PC-selective phospholipid has been shown to be mediated by the activation of a PLD (Exton 1994). By this process the activation of PLD gives

rise to phosphatidic acid (PtdOH) and choline, and the PtdOH is then dephosphorylated by PtdOH phosphohydrolase (PPH) to yield DAG.

Although phospholipase D was initially thought not to exist in mammalian tissues, Kanfer et al (Kanfer et al., 1980) have provided evidence to the contrary. A phospholipase D active on PC and PE has been partially purified from rat brain (Taki and Kanfer 1979). Membrane-bound phospholipase D activity directed towards PC has also been detected in rat tissues provided that certain detergents are present in the assay (Chalifour and Kanfer 1980, Chalifour and Kanfer 1983, Martin 1988).

The assessment of PLD activity in whole cells can be achieved by either measuring the formation of [32 P]PtdOH in cells labeled with [32 P]PC (Exton 1994) or by the use of primary alcohol to substitute for water in the phosphatidyltransferase reaction of PLD giving rise to the corresponding phosphatidylalcohol (Exton 1994). Moreover, since phosphatidyl-alcohols are poor substrates for PPH (Metz and Dunlop 1991) the use of primary alcohol allows for the intervention in the PLD/PPH pathway leading to DAG formation (Bonser et al., 1989).

A number of growth factors and mitogenic agents have been shown to stimulate PC hydrolysis by a PLD pathway. Phorbol ester-stimulated PLD activity has been reported in a variety of cell types and appears to be an almost ubiquitous phenomenon (reviewed by Billah and Anthes 1990). PDGF is able to stimulate PLD transferase activity in NIH3T3 (Ben-Avi and Liscovitch 1989) and Swiss 3T3 cells (Plevin et al., 1991), and EGF is able to stimulate PC-PLD activity in Swiss 3T3 cells. Despite this, the role of PLD in the sustained formation of DAG is unclear.

3.1.3 Phospholipase A₂ pathway

Phospholipase A₂ (PLA₂) hydrolyzes several phospholipids including PI, PC and phosphatidylethanolamine (PE) to liberate arachidonic acid. Arachidonic acid can be converted *via* the cyclo-oxygenase and lipoxygenase pathways to eicosanoids, including prostaglandins, thromboxanes, prostacyclins, leukotrienes and lipoxins (Needleman et al., 1986). It has been reported that arachidonate and other unsaturated long-chain fatty acids activate PKC in a dose-dependent fashion in human neutrophils (McPhail et al., 1984). It has also been described that a mixture of rat brain PKCs can be activated significantly by oleic and arachidonic acid, and that this activation is independent of phospholipids and Ca²⁺ (Lester 1990, Lester et al., 1991). Arachidonic acid derivatives such as lipoxin A (5,6-15L-trihydroxy-7,9,11,13-eicosatetraenoic acid) activate PKC as well (Piomelli 1993). Sodium oleate preferentially activates soluble PKC and is unable to interact with membrane-bound enzyme (Piomelli 1993). Therefore, unsaturated fatty acids may have a physiological role in activating discrete pools of PKC leading to phosphorylation of specific set of cellular substrates; a set of substrates perhaps distinct from those phosphorylated by phorbol ester/DAG-activated PKC. To complicate the issue further, synergy between oleic acid and DAG in the presence of calcium has been observed with respect to PKC activation (Lester et al., 1990). The physiological significance of these observations is not clear at present.

Recently, the possibility that PLA₂ is directly regulated by G proteins has been explored. These studies may be difficult to interpret because in contrast to inositol phosphates, which are derived solely from PI-PLC mediated hydrolysis of phosphoinositides, arachidonic acid is also derived from the hydrolysis of DAG by DAG lipases (Bell et al., 1979). Because DAG is produced during the hydrolysis of phosphoglycerides by PLC, an increase in arachidonic acid

production may result from the indirect action of PLC followed by DAG lipase rather than from direct stimulation of PLA₂. A further complication is that some PLA₂ is stimulated by Ca²⁺ at physiologically relevant concentrations (Slivka and Insel 1987), allowing PLA₂ to be indirectly stimulated by an IP₃-mediated increase in Ca²⁺. Nevertheless, there is evidence (reviewed by Burch 1989, 1990) suggesting that G proteins might be involved in receptor-stimulated production of arachidonic acid and that separate G proteins mediate the stimulation of PLA₂ and PLC. Additional evidence suggesting that distinct G proteins are coupled to PLC and PLA₂ comes from studies using pertussis toxin (Ui 1990).

3.1.4 Guanine nucleotide regulation of phospholipase activity

Functional G proteins that have been characterized to date include those that stimulate adenylyl cyclase (G_s), inhibit adenylyl cyclase (G_i), stimulate cGMP phosphodiesterase (G_t or transducin), activate ion conductances (G_k), and stimulate PI-PLC-β (G_q) (reviewed by Neer 1995). These functional studies have revealed certain characteristics that are common to all of G proteins. G-proteins are GTPases composed of a α subunit (catalytic) and a βγ subunit (regulatory). In order for a G protein to be activated it must be maintained in the GTP-bound form. In the absence of receptor stimulation the G-protein remains inactive because of its ability to hydrolyse GTP to GDP. However, the interaction of the G-protein with an activated receptor blocks the GTPase activity, and caused the dissociation of the GTP-α subunit from the regulatory βγ subunits (Neer 1995). The liberated α subunit is thought to be the principle cell signaling mediator, although recent evidence indicates that the βγ subunits can propagate cell signals as well (Neer 1995). Inhibitory G proteins (i.e. G_{iαs}) can be specifically inhibited by the action of pertussis toxin (PTX), which causes the irreversible ADP-ribosylation of G_{iα} (Ui

1990). Thus, G-protein coupled cell signaling can be grossly subdivided into PTX-sensitive and PTX-insensitive processes.

The possibility of G-protein regulation of PC hydrolysis by both a PC-specific PLC and PLD has been examined. A role for a guanine nucleotide-binding protein in regulating PLD activity has been suggested by reports of stimulation of PLD transferase activity by non-hydrolysable GTP analogues in homogenates from HL-60 cells (Anthes et al., 1989) and by the stimulated release of Cho from hepatocytes (Bocckno et al., 1987). Similar observations have been made in cell-free preparations from rat brain (Kobayashi and Kanfer 1987) and in permeabilised endothelial cells (Martin and Michaelis 1989). In rat liver plasma membrane, the addition of GTP analogues causes the hydrolysis of PC with the release of choline, P-choline, DAG, and PA (Bocckino et al., 1987, Irving et al., 1987). These findings indicate that the stimulation of both phospholipase C and D can be G-protein mediated. The involvement of G-proteins in these processes is also supported by the observation that the stimulation of PLC and PLD is effected at submicromolar concentrations of non-hydrolyzable GTP analogues, but not other nucleotides, requires millimolar Mg^{2+} , and is inhibited by a stable analogue of GDP (Bocckino et al., 1987, Irving et al., 1987). As discussed previously, it is also possible that PLA_2 is regulated by G-proteins. However, the exact mechanism of G-protein interaction with these enzymes has not yet been well defined.

3.1.5 Protein tyrosine kinase in PKC activation

The mechanism by which growth factors of the receptor tyrosine kinase (RTK) family, e.g. epidermal growth factor (EGF) and PDGF, stimulate PIP_2 hydrolysis is well established. Upon ligand-induced activation of their RTK cytoplasmic domains both the EGF and PDGF receptors physically recruit and phosphorylate a distinct isoform of PLC, $PLC\gamma_1$, and it has been

confirmed that tyrosine phosphorylation of PLC γ 1 increases its catalytic activity (reviewed by Rhee and Choi 1992). PLC γ subsequently stimulates PIP $_2$ breakdown, generating DAG and IP $_3$ which is responsible for PKC activation and mobilization of intracellular Ca $^{2+}$, respectively (Nishibe et al., 1990). PI-PLC γ 1 can also be activated by nonreceptor tyrosine kinases (Rhee and Choi 1992).

3.1.6 The mechanism of PKC activation in IL-2 signaling

As discussed in detail in the General Introduction, the mechanisms of PKC activation in IL-2 signal remains controversial. First of all, whether PIP $_2$ breakdown occurs after IL-2 stimulation is not clear. Phosphoinositide hydrolysis, increases in intracellular Ca $^{2+}$, and the activation of PKC have all been reported (Farrar and Anderson 1985, Farrar et al., 1986, Evans et al., 1987b) suggesting that the activation of PKC may occur *via* the PI-PLC pathway. However, other studies have failed to observe the same PI-PLC pathway linkage to PKC activation (Mills et al., 1985a, 1986, Gelfand et al., 1987, Redondo et al., 1988, Valge et al., 1988, Paetkau and Mills 1989). Furthermore, as discussed above, it is now well recognized that multiple pathways exist in cells to generate DAGs. Whether any of these "alternate" pathways (i.e. PC-PLC, PC-PLD) are involved in PKC activation after IL-2 stimulation has not been explored at all. Moreover, the products of the PLA $_2$ pathway (AA, unsaturated long-chain fatty acids, lipoxin) have been reported to activate PKC by themselves or in synergy with other lipids (McPhail et al., 1984, Lester 1990, Lester et al., 1991). The role of PLA $_2$ in PKC activation after IL-2 stimulation has yet to be addressed. Finally, it was reported that P56^{lck} is physically associated with IL-2R β , and its activity is regulated by IL-2 (Hatakeyama et al., 1991, Horak et al., 1991). Whether this tyrosine kinase is responsible for PKC activation is unknown. Many of

these issues have been addressed in the study of IL-2 mediated PKC activation in CTLL-2 cells as presented in Chapter 3 of this document.

3.2 OBJECTIVES

The overall objectives of the studies described in this chapter were to elucidate the mechanisms of PKC activation in CTLL-2 cells after IL-2 stimulation. The specific objectives were:

- 3.2.1 To establish the role of protein tyrosine kinases in IL-2 stimulated PKC activation.**
- 3.2.2 To establish the role of G-proteins in IL-2 stimulated PKC activation.**
- 3.2.3 To investigate the sources of DAG after IL-2 stimulation in CTLL-2 cells.**
- 3.2.4 To study the mechanism(s) responsible for IL-2 induced DAG production**
- 3.2.5 To study the role of arachidonic acid on IL-2 stimulated PKC activation.**
- 3.2.6 To establish the role of a PKC stimulating cytosolic component(s) (factor X) in the mechanism by which IL-2 stimulates PKC activation in CTLL-2 cells.**
 - 3.2.6.1 To characterize PKC activation by factor X.**
 - 3.2.6.2 To effect a preliminary characterization of factor X.**
 - 3.2.6.3 To study the mechanism of factor X stimulated PKC activity**

3.3 METHODS

3.3.1 Cell culture, cell proliferation and viability

Stock cultures of CTLL-2 cells were maintained and manipulated as described in the *Methods* section of Chapter I. Viable cells were determined microscopically by their ability to exclude the dye, trypan blue (Sigma Chemical Co., ST. Louis, MO, USA) as described in the *Methods* section of Chapter I (1.3.5). Cell proliferation was assessed by [³H]-thymidine incorporation following the protocol described in the *Methods* section of Chapter I (1.3.5).

3.3.2 The assay of PKC activity

Cell lysis, the preparation of membrane and cytosolic fractions, and conventional and direct PKC assays were performed following the protocols described in the *Methods* section of Chapter I (1.3.1, 1.3.2).

3.3.3 The assay of protein tyrosine kinase activity

Protein tyrosine kinase activity was measured using a commercial assay kit purchased from GIBCO BRL (Grand Island, NY, USA). Briefly, CTLL-2 cells were lysed in hypotonic lysis medium (Appendix) as described in the *Methods* section of Chapter I (1.3.1). Nuclei and unlysed cells were separated by centrifugation, and the amount of protein in the resultant post-nuclear fractions was determined by the method of Bradford (1976). Five to ten micrograms of protein from the post-nucleus supernatants in control buffer (30 mM HEPES, 10 mM MgCl₂, 20 μM EDTA, 0.1 mM DTT, 25 μg/ml BSA, 0.15% [v/v] nonidet P-40, 70 μM sodium orthovanadate, 60 μM ATP) was incubated with the substrate peptide (containing 0.5 mM RR-SRC peptide substrate in control buffer) in the presence of [³²P] ATP (1 μCi/tube, 6,000Ci/mmol) at 30°C for 10 minutes (total volume of reaction mixture 20 μl). The reaction was stopped by adding 20 μl of cold 10% TCA. Samples were centrifuged to remove precipitated

protein, the peptide substrate remained in solution. Twenty microliter of supernatant from each tube was removed and spotted onto separate phosphocellulose discs. The discs were washed twice in 250 ml 1% acetic acid for 5 minutes, and then in 250 ml water for 5 minutes. The radioactivity bound to the washed discs was determined by liquid scintillation counting using Formula-989 scintillation cocktail.

The substrate for tyrosine kinase, RR-SRC, is derived from the amino acid sequence surrounding the phosphorylation site of pp60^{src} and is specific for tyrosine kinase (Pike et al., 1982). The peptide sequence is: Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly includes two arginine residues at the amino terminus of RR-SRC that enables it to bind to phosphocellulose paper.

3.3.4 Inositol phosphates measurements

To measure inositol phosphate formation, CTLL-2 cells were prelabeled with [³H] myo-inositol (5.0 µCi/ml) for 24 h in inositol-free RPMI-1640 in the presence of serum and IL-2. The cells were then changed from the serum and IL-2 containing medium to unsupplemented inositol-free RPMI medium containing [³H]myo-inositol and incubated for an additional 4 h. The cells were washed thoroughly 3 times with RPMI medium, and LiCl (20 mM in M199) was added to the cells and incubate for 10 min at 37°C in order to inhibit inositol phosphate metabolism (Berridge et al., 1982). The cells were then treated with the indicated test compounds and incubated for an additional 10 min. The cells were rapidly pelleted by centrifugation, and 10% TCA then added to each cell pellet. The samples were vortexed, and allowed to sit on ice for 5-15 min. Following TCA precipitation the supernatants were collected by centrifugation and subjected to lipid extraction. Briefly, the supernatants following TCA precipitation were washed 3 times with 3 mls of anhydrous diethyl ether, and the last traces of ether were evaporated from

the aqueous phase under nitrogen flow. The acidic pH of the aqueous phase was neutralized by the addition of 6.25 M sodium tetraborate and the volume adjusted to 2.0 ml with distilled water. Inositol phosphates (IP₁, IP₂, IP₃) were separated by passing the preparation through a 1 ml DOWEX-AG 1X8-formate form anion exchange column. The columns were washed with 20 volumes of 60 mM ammonium formate/5 mM sodium tetraborate to remove free inositol. Inositol phosphates were subsequently eluted from the columns in a stepwise manner. IP₁ was eluted from the column with 0.2 M ammonium formate/0.1N formic acid; IP₂ was eluted next with 0.4 M ammonium formate/ 0.1 N formic acid; and finally IP₃ was eluted with 1 M ammonium formate /0.1 N formic acid. The levels of inositol phosphates were quantified by scintillation counting and expressed as cpm/mg protein. It should be noted that the IP₃ fraction was a composite of all forms of IP₃. Protein content was determined in the 1M NaOH-digested cellular pellet by the method of Lowry et al. (1951). As a positive control for these experiments, confluent rat type I astrocytes grown in 60 mm Petri dishes were prelabeled with [³H]myo-inositol (2.5 µCi/ml) for 16-18 h in serum-and inositol-free DMEM. Unbound [³H]myo-inositol was washed thoroughly with DMEM. LiCl (20mM) was added to the cells for 10 min at 37°C. Endothelin-1 were added to astrocytes in the presence of 20 mM LiCl for 10 min. The reaction was stopped by replacing the medium with cold 0.3 M TCA, and the cells were scraped, briefly sonicated, and sedimented by centrifugation. Lipids were extracted and inositol phosphates were separated and measured as described above.

3.3.5 Total lipid extraction and thin layer chromatography

Total cellular lipids were extracted using the procedure of Folch et al. (Folch et al., 1957, Chakravarthy 1985). Briefly, CTLL-2 cells (10 X10⁶ cells), incubated with various ligands as described in the legends to the appropriate figures, were collected by centrifugation

and lysed in 0.5 ml water. Chloroform: methanol (2:1, v/v) was added to cell lysate at a ratio (8:1, v/v) and usually left overnight at 4°C. The lipid extract was partitioned into an aqueous upper-phase and an organic lower-phase by adding 0.1N KCl (sample: KCl ratio was 5:1) and centrifuging at 500 xg for 5 min. The two phases were separated and the lower organic phase was washed once with equal volume of ideal upper phase [chloroform : methanol : 0.1N KCl (3:48:47, v/v)].

The organic phase was dried at 37°C under a stream of nitrogen. The lipid extract was dissolved in 25 µl chloroform : methanol (19:1, v/v) and aliquots were taken for radioactivity measurements of total lipids. Individual lipid classes were separated by directly subjecting the lipid extract to thin layer chromatography. Briefly, individual classes of neutral lipids and phospholipids were resolved on silica-gel G 60 plates. Phospholipid classes were separated using chloroform : ethanol : water : triethylamine (4:5:1:4, by vol.) as the developing system. Neutral lipid classes were separated using petroleum ether : diethyl ether : acetic acid (70:30:1, by vol.). Individual lipids were identified by comparison with authentic standards added to each sample. Phospholipids and neutral lipids were visualized after exposure to iodine vapor. After identification by comparison to internal standards, gel samples containing individual lipids were scraped and used directly for radioactivity determination.

3.3.6 Intracellular Calcium Measurements

To determine $[Ca^{2+}]_i$, CTLL-2 cells were cultured in 175cm² culture flasks and used for experiments when the cultures reached a density of 5×10^5 cells/ml. CTLL-2 cells were then starved for 2 hours in serum and IL-2 free RPMI medium. Intracellular calcium concentrations were determined by measuring the fluorescence signal from the calcium-sensitive indicator fura-2. CTLL-2 cells were loaded with fura-2 by incubation for 30 min at 37°C in a normal buffer

solution (NBS: 140 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.1 mM MgCl₂, 2.6 mM dextrose, and 10 mM HEPES), containing 0.00125% (wt/vol) pluronic acid (Molecular Probes Inc., Eugene, OR) and 5.0 μ M fura-2-acetoxymethyl ester (Molecular Probes Inc.). Fura-2-acetoxymethyl ester is a membrane-permeable, calcium-insensitive ester of the calcium fluorophore fura-2, which becomes Ca²⁺-sensitive and remains trapped intracellularly after hydrolysis by nonspecific intracellular esterases (Grybjuewcz G. et al. 1985). After 3 washes in fura-free NBS (Appendix), the cells were post-incubated for 5-30 min in NBS at room temperature to ensure full hydrolysis of the fura-2 ester. This was verified by running excitation spectra between 320 nm and 420 nm at various times during the post-incubation period. They were monophasic and peaked around 365 nm. Experiments were conducted at room temperature on 5 X 10⁶ suspended cells in a cuvette placed in a CM3 cation measurement spectrofluorimeter (Spex Inc., Newark, NJ). Measurements were performed using 350-nm and 380-nm excitation wavelengths alternating at a frequency of 1 Hz. The emitted light was passed through a photomultiplier, and its intensity was recorded by a photon counter (Spex Inc.) detector. Background fluorescence was subtracted from the cuvette. Dye leakage, as determined by loss of fluorescence over a period of 10 min, was undetectable at both excitation wavelengths with the excitation monochromator slits set at 0.5 nm.

The concentration of intracellular ionized calcium, [Ca²⁺]_i, was estimated from the ratio of the intensities of fura-2 emission at 505 nm induced by the alternating excitation wavelengths (350 nm and 380 nm) according to the following formula (Grybjuewcz G. et al., 1985):

$$[\text{Ca}^{2+}]_i = K_d \times (F_{\min}/F_{\max}) \times (R - R_{\min}) / (R_{\max} - R),$$

where R , $R_{\min}$ and $R_{\max}$ are the fluorescence ratios recorded during the experiment (R) and during calibration tests on unlysed cells using 4 μ M ionomycin in NBS ($R_{\max}$) followed by 10 mM EGTA added at pH 8.2 ($R_{\min}$). $F_{\max}$ and $F_{\min}$ are the corresponding fluorescence intensities for the 380-nm excitation, and K_d is the Fura-2 dissociation constant at room temperature (135 nm).

3.3.7 Isolation of IL-2 treated cytosol

Exponentially growing CTLL-2 cells were incubated in unsupplemented RPMI medium for 4 hour at 37°C. The cells were then stimulated with IL-2 (50-100 U/ml) for 10 min at 37°C or left untreated, and the reaction stopped by adding ice cold PBS as described previously. The cells were lysed with hypotonic lysis buffer (Appendix) at 1/4 of the normal volume (i.e., 20 X 10⁶ cells per ml lysis buffer) exactly as described in the *Methods* section of Chapter 1 (1.3.1). The membranes and cytosol were separated by centrifugation as described in Chapter 1 *Methods* section 1.3.1, and the cytosolic fractions (designated IL-2 cytosol and control cytosol) were collected and stored at - 80°C until used. To test the ability of IL-2 and control cytosols to activate control membrane PKC, CTLL-2 cell membrane were prepared from 5-10X10⁶ cells deprived of IL-2 and serum for 4 hour as described previously, and incubated with either IL-2 cytosol or control cytosol for 10 minute at 27°C with gentle shaking. The amounts of cytosol and membranes in these assays are indicated in each individual experiment. The reaction was stopped by placing the samples on the ice for 5 minutes and the membrane and cytosol fractions were then separated by ultracentrifugation as described in the *Methods* section of Chapter I. The membrane PKC activity was measured by the direct PKC assay as described in the *Methods* section of Chapter I (1.3.2).

Miscellaneous

Protein concentration was determined by the method of Bradford (1976).

3.4 RESULTS

3.4.1 *The role of protein tyrosine kinases in IL-2 stimulated PKC activity.*

Given the importance of tyrosine kinase activity in IL-2 signaling and the fact that in many systems there is a complex relationship between PKC and tyrosine kinases, it was appropriate to test the linkage, if any, between IL-2 induced tyrosine kinases and the activation of inactive membrane PKC. As shown in Fig. 3.1, 100 U/ml IL-2 stimulated a transient activation of tyrosine kinase activity in CTLL-2 cells. The sizable stimulation of tyrosine kinase activity occurred with 5 minutes of IL-2 application, peaked at 10 minutes, and returned to near basal levels within 35 minutes. Given the fact that tyrosine kinase activity was rapidly increased in CTLL-2 cells in response to IL-2, the effects of two tyrosine kinase inhibitors, herbimycin A and genistein, on IL-2 stimulated PKC activity was determined. Herbimycin A is a benzoquinoid antibiotic isolated from *Streptomyces hygroscopicus* which is a powerful and relatively specific inhibitor of protein tyrosine kinases (Uehara et al., 1986, 1989a, 1989b). Genistein (4', 5, 7-trihydroxyisoflavone) is a potent specific tyrosine kinase inhibitor that competes for the ATP binding site on the kinase with a reported IC_{50} ranging from 2.6- 20 μ M (Akiyama et al., 1987, Dean et al., 1989). Genistein is one of only a few tyrosine kinase inhibitors which has been demonstrated to inhibit tyrosine kinases other than EGF-R, such as pp60^{v-src} and pp110^{src-fcs} (Akiyama et al., 1987). Neither herbimycin A (875 nM) nor genistein (14.8 μ M) had a significant effect on IL-2 stimulated PKC activity when added to intact CTLL-2 cells 30 minutes before IL-2 stimulation (Figure 3.2). Moreover, neither inhibitor had a marked effect on basal PKC activity in unstimulated control cultures (data not shown). These concentrations of inhibitors were chosen because they have been shown to effectively block tyrosine kinase activity in other systems (Uehara et al., 1986, 1989a, 1989b, Akiyama et al., 1987, Dean et al., 1989).

To ensure that these compounds were indeed effective in CTLL-2 cells, the effects of herbimycin A and genistein on IL-2 /serum induced cell proliferation was assessed. As shown in Fig.3.3 and 3.4, doses of herbimycin A and genistein that had no effect on IL-2-induced PKC activity (875 nM, and 14.8 μ M, respectively) had a profound effect on [3 H]-thymidine incorporation and cell proliferation in CTLL-2 cells. These observations are consistent with previous reports that tyrosine kinase activity is involved in the proliferation (Mills et al., 1991) of IL-2 dependent cells. These results suggest that the inhibition of tyrosine kinase activity did not affect the ability of IL-2 to stimulate PKC in CTLL-2 cells.

3.4.2 *The role of G-proteins in IL-2 stimulated PKC activation*

As detailed in the Introduction, it is well established that DAG is the principle co-factor responsible for PKC activation in cells after receptor stimulation. However, it is far less clear how these receptor signals are coupled to the production of DAG. It is now apparent that several DAG-generating pathways exist (Billah and Anthes 1990, Exton 1994), and that G-proteins are an important element of at least one of these pathways (Rhee et al., 1992). The role of G-proteins in cell signaling has been widely studied and much is now understood with respect to their importance in receptor-mediated signal transduction coupled to enzymes such as adenylate cyclase and phospholipase C- β (Casey and Gilman 1988, Rhee et al., 1992). Nonhydrolysable GTP analogues (e.g. GTP γ S) and pertussis toxin, which catalyses the ADP-ribosylation of G_i and G_o -like proteins, have been used extensively to study the role of G-proteins in the activation of phospholipases (Ui 1990).

Pertussis toxin (PTX) was used to investigate whether the activation of inactive membrane PKC by IL-2 was a G_i protein-mediated event in CTLL-2 cells. CTLL-2 cells were starved of IL-2 and serum in the presence of 75 ng/ml PTX for 4 hours before being stimulated

by IL-2. As shown in Fig. 3.5, PKC activation by IL-2 was almost completely blocked by the pretreatment of cells with pertussis toxin, while pertussis toxin itself had no effect on basal PKC activity. Since we had previously determined that PKC activation was linked to IL-2's ability to suppress cell death, the question as to whether PTX could impact the IL-2 induced rescue of cells was addressed. The pretreatment of cells with 100 ng/ml PTX for 7 hours effected a 44% reduction in IL-2's ability to suppress apoptotic cell death in IL-2 "starved" CTLL-2 cells (Fig. 3.6). This PTX-induced partial loss in IL-2's ability to suppress cell death in IL-2/serum starved CTLL-2 cells was confirmed in three independent experiments. These results suggest that a pertussis toxin-sensitive G-protein is likely involved in the mechanism by which IL-2 causes PKC activation in CTLL-2 cells, and that this activation of PKC was at least partially responsible for the IL-2 induced rescue of cells.

3.4.3 The production of DAG is responsible for IL-2 stimulated PKC activation in CTLL-2 cells

Results presented in Chapter 2 (see Fig. 2.4) indicated that the IL-2 induced increase in PKC activity resulted from the activation of a pool of inactive membrane PKC that was readily activated by the DAG analog, TPA. These observations suggested that the formation of DAG in response to IL2 stimulation was the likely route by which this inactive membrane pool of PKC underwent activation. For this to be true, the addition of IL-2 to "starved" CTLL-2 cells should evoke a measurable increase in cellular DAG content. To measure DAG production, uniform labeling of the phospholipid pool was accomplished by an 18-hour incubation of CTLL-2 cell cultures with [^3H]-arachidonic acid which labels phospholipids mainly in the C-2 position (Chakravorthy, 1985). As shown in Fig. 3.7, the addition of 100U/ml IL-2 to cells previously starved of IL-2 and serum for 4 hours, evoked a rapid and sustained increase in cellular DAG

content. Thus, a 40% increase in ^3H -DAG was detected by thin layer chromatography (TLC) in cells stimulated with IL-2 for as little as 5 min (Fig. 3.7). By contrast, very little stimulation in DAG was observed 2.5 min after IL-2 application, indicating that relative to other growth factor/cell systems (Nishizuka 1992, Liscovitch 1992), the rate of DAG production by IL-2R stimulation was rather slow in CTLL-2 cells. Nevertheless, the production of DAG was temporally coupled to the activation of inactive PKC in IL-2 treated cells (see Chapter II, Fig.2.1) suggesting a linkage between both events.

If the production of endogenous DAG by IL-2R stimulation was directly responsible for the activation of inactive membrane PKC, then the application of exogenous DAG to either intact cells or to isolated CTLL-2 cell membranes should evoke PKC activation. The data presented in Fig.3.8 indicated that a brief (10 min) incubation of IL-2- and serum-deprived CTLL-2 cells with DAG caused a dose-dependent increase in membrane PKC activity that plateaued at DAG concentrations greater than 30 μM . However, this experiment was unable to determine whether the increase in PKC arose from the activation of inactive membrane-associated PKC or from the translocation of cytosolic enzyme to membranes. To distinguish between these two possibilities, DAG was added directly to CTLL-2 cell membranes isolated from IL-2 and serum starved cells. As shown in Fig.3.9, DAG concentrations as low as 10 μM completely activated the inactive PKC pool in these membranes. The addition of TPA to membranes stimulated with 10 μM DAG gave no further increase in PKC activity (data not shown) indicating that DAG was activating the same pool of PKC as was TPA. It is worthwhile to note that the concentration of DAG needed to fully activate inactive membrane PKC in isolated membranes (10 μM) was substantially less than the 30 μM needed to maximally stimulate PKC activity in intact cells. This observation is consistent with the results of Chakravarthy et al (1994) who showed that the

activation of inactive membrane PKC in WEHI cells occurred at DAG concentrations significantly less than that needed to evoke translocation.

3.4.4 *Mechanisms of DAG generation in IL-2 stimulated CTLL-2 cells.*

Since it was found that IL-2 did stimulate DAG production in CTLL-2 cells, and that exogenously applied DAG caused the activation of inactive membrane PKC, attention focused next on which of the several possible mechanisms of DAG generation were responsible for the increase.

The most obvious place to start was the hydrolysis of PIP₂ by PI-PLC, since this is a major source of receptor-linked DAG in many cells systems (Cook and Wakelam 1992). The measurement of inositol phosphates (i.e. IP₁, IP₂, IP₃) was made possible by a 24 hour prelabeling of the CTLL-2 cell phosphoinositol pool with [³H]-myo-inositol. These labeled cells were then "starved" of IL-2 and serum for 4 hours before being challenged with IL-2 for 10 min. Inositol phosphate levels were stabilized by adding LiCl to the medium before IL-2 in order to prevent the breakdown of inositol phosphate (Berridge et al., 1982). IP₁, IP₂, and IP₃ in TCA-precipitated cell lysates were then separated by DOWEX-AG anion exchange chromatography. The data presented in Fig.3.10A indicated that neither 25 U/ml nor 100 U/ml IL-2 induced detectable increases in any of the principle products of PIP₂ hydrolysis. By contrast, experiments conducted in parallel with those shown in Fig 3.10A indicated that a robust 3-5 fold increase in IP₃ formation was effected by the addition of endothelin-I to cultured astrocytes (Fig.3.10 B), a finding consistent with previous reports (Stanimirovic et al., 1995). Thus, the assay was clearly sensitive enough to measure PIP₂ hydrolysis in CTLL-2 cells if it had occurred, and these results strongly suggest that IL-2 does not stimulate detectable levels of PIP₂ hydrolysis in these cells. This conclusion was supported by experiments in which the effects of IL-2 on intracellular Ca²⁺ (

$[Ca^{2+}]_{in}$) were measured. It is well understood that IP_3 production in cells will induce the release of Ca^{2+} from intracellular stores (Nishizuka 1992), and that this increase can be readily detected in cells labelled with the Ca^{2+} -indicator, fura-2 (Grybjuewcz et al., 1985). In control experiments, fura-loaded CTLL-2 cells were found to respond to the addition of the Ca^{2+} ionophore, ionomycin, with a rapid increase in $[Ca^{2+}]_{in}$, an increase which was partially reversed by the chelation of extracellular Ca^{2+} by EGTA (Fig.3.11A). Under these same conditions, IL-2 concentrations up to 1000 U/ml had no effect on $[Ca^{2+}]_{in}$ (Fig.3.11 A-D). The results of Fig 3.11 (D-G) indicated that CTLL-2 cells were indeed capable of releasing Ca^{2+} from intracellular stores. Both thapsigargin (TG: Fig 3.11D-F) and cyclopiazonic acid (Fig. 3.11G), potent agents which trigger the release of intracellular Ca^{2+} stores (Seidler et al., 1989, Demaurex et al., 1992, Morley et al., 1992), were able to stimulate measurable, albeit transient, increases in $[Ca^{2+}]_{in}$ in serum and IL-2 starved CTLL-2 cells. TG was able to increase $[Ca^{2+}]_{in}$ even in the absence of extracellular Ca^{2+} (Fig.3.11F), thereby confirming that it was acting on the intracellular Ca^{2+} reservoirs of these cells, - a reservoir not affected by IL-2 (Fig. 3.11D-G). Thus, these results clearly demonstrate that while IL-2 and serum-starved CTLL-2 cells react as expected to a series of Ca^{2+} modulators, they did not respond to IL-2 addition with measurable changes in $[Ca^{2+}]_{in}$. Collectively, the results of Fig.3.7, 3.10 and 3.11 indicated that the production of DAG that was temporally linked to PKC activation by IL-2 did not arise from PIP_2 hydrolysis. These data suggest that IL-2 stimulates DAG production from sources other than that from PI-PLC activation, most likely from the stimulation of PC-PLC or PC-PLD activity.

3.4.5 *The role of arachidonic acid in IL-2 stimulated PKC activation*

As described in the Introduction, it is becoming increasingly clear that the production of arachidonic acid (AA) by the action of PLA_2 can also cause the activation of PKC (McPhail et

al., 1984, Lester 1990, Lester et al., 1990), in some cases by acting in concert with DAG (Piomelli 1993). Thus, the role of AA in the IL-2 stimulated activation of PKC in CTLL-2 cells was examined. As was the case with DAG production, AA levels were measured in cells that had been previously labelled to uniformity with (^3H)-arachidonic acid. These labelled cells were then deprived of IL-2 and serum for 4 hours in the absence of the radiolabel before being challenged with IL-2 for 10 min. As shown in Fig.3.12, IL-2 generated a biphasic production of AA in CTLL-2 cells, with a minor peak occurring 5-10 minutes after addition of the cytokine. This relatively rapid, but transient 70% increase in AA levels was reproducibly found in each of 3 independent experiments performed. This initial peak was followed by a slower, but significantly larger increase in AA that occur 30-45 minutes after stimulation. Once again, as was the case with endogenous DAG production, the early increase in AA observed was temporally coupled to the activation of PKC in CTLL-2 cells (see Fig.2.1, Chapter II).

The exogenous application of AA to IL-2 and serum deprived CTLL-2 cells was also a powerful PKC stimulant in that AA concentrations as low as 1 ng/ml gave a robust and near maximal stimulation of PKC activity (Fig. 3.13). Interestingly, the addition of exogenous AA seemed to induce a "biphasic" PKC response, in that the rapid increase in PKC activity by low AA concentrations was followed by a much more gradual increase as AA levels increased further. A similar situation was observed when AA was directly applied to isolated CTLL-2 membranes (Fig.3.14). AA concentrations as low as 0.25 ng/ml caused maximal stimulation of inactive PKC in isolated membranes, a stimulation that matched that evoked by TPA. The co-application of TPA and AA did not give an additive response (data not shown) suggesting that both agents stimulated the same pool of inactive membrane PKC. The above data suggested that the production of AA in response to IL-2 could have contributed to the stimulation of inactive

PKC by the cytokine. This suggestion was supported by experiments using PLA₂ inhibitors. Oleoyloxyethyl phosphocholine (OOPC) is a site-specific porcine pancreatic PLA₂ inhibitor (IC₅₀ = 6.2 μM) (Magolda et al., 1985), which completely blocks AA-induced myogenic responses in perfused dog renal arcuate arteries at 10 μM (Kauser et al., 1991). The trifluoromethyl ketone analogue of AA, AACOCF₃, is a potent and selective slow-binding inhibitor of human cytosolic (85 kDa) PLA₂ which can readily permeate the cell (Street et al., 1993, Bartoli, et al., 1994). As shown in Fig. 3.15 and 3.16, both PLA₂ inhibitors were effective in reversing the IL-2 induced activation of PKC activity within the concentrations range previously shown to inhibit PLA₂. Thus, 10 μM OOPC completely inhibited the increase in PKC activity after IL-2 stimulation, without reducing PKC activity below basal levels (Fig. 3.15). This observation was even more dramatic in experiments with AACF. Whereas 10 μM AACF completely inhibited the IL-2 stimulated PKC response, concentrations of the inhibitor as high as 100 μM had no further effect on basal PKC levels in these cells. These data suggest that AA production is indeed an intermediate step in the mechanism by which IL-2 induced PKC activation in CTLL-2 cells.

Further evidence was found suggesting that the production of AA was actively involved in IL-2-stimulated PKC production. The Upjohn compound U73122, was initially heralded as being a PI-PLC specific inhibitor (Bleasdale et al., 1989, Smith et al., 1990b). However, more recent studies have indicated that this compound is also a potent blocker of PLA₂ activity (Cass et al., 1991). As shown in Fig. 3.17, the compound inhibited IL-2 stimulated PKC activity in a dose-dependent manner, with complete inhibition observed at 7.5 μM. By contrast, even higher doses (i.e. 10 μM) did not affect basal PKC activity. Since the compound can block both PLC and PLA₂ activity, and since we have shown that IL-2 does not stimulate PLC's, these results

add further support that AA production via PLA₂ activity is an important step in IL-2 stimulated PKC activity.

3.4.6 *The role of a cytosolic component in IL-2 stimulated PKC activation*

3.4.6.1 IL-2 treated cytosol can activate inactive membrane PKC and possibly induce PKC translocation

The data presented to this point indicates that a pool of membrane-resident PKC exists in CTLL-2 cells which can be activated by IL-2R stimulation in intact cells and by DAG and arachidonic acid *in vitro*. Interestingly, during these studies it was observed that the cytosolic fraction isolated from IL-2 treated CTLL-2 cells was in itself able to activate inactive membrane-associated PKC in membranes isolated from unstimulated CTLL-2 cells, and possibly to induce PKC translocation from the cytosol to membranes as well. Thus, cytosol isolated from CTLL-2 cells (i.e. IL-2 cytosol) previously stimulated for 10 minutes with 50 units/ml IL-2 was found to activate PKC activity in membranes isolated from control, unstimulated cells (Fig. 3.18). In these experiments, control CTLL-2 membranes were mixed with IL-2 treated or control cytosol and incubated at 27°C for 10 min with gentle shaking. The membranes and cytosols were subsequently separated by ultracentrifugation, and membrane PKC activity then measured by the direct PKC assay. As shown in the Figure 3.18, IL-2 treated cytosol stimulated a 3 fold increase in membrane PKC activity while the control cytosol had no measurable effect. As a control in these experiments, TPA was added to the assay mixture so as to determine the levels of inactive PKC in the control unstimulated membrane preparation. Surprisingly, the effects of IL2 cytosol on membrane PKC far exceeded that of TPA, indicating that PKC pools other than the "inactive" membrane pool were being stimulated. It was probable that PKC present in the cytosolic fraction may have been translocated to the membranes during the incubation period.

This possibility was confirmed in experiments in which membrane and cytosolic PKC activities were measured by the conventional assay before and after incubation with IL-2 cytosol. As shown in Table 3.1 the addition of IL-2 cytosol increased membrane PKC activity greater than two fold with a compensatory loss in assayable cytosol enzyme, a characteristic feature of translocation.

IL-2 cytosol increased membrane PKC activity in a dose-dependent manner up to concentrations as high as 400 μ l (1mg cytosol protein, Fig. 3.19), whereas control cytosol had no activity over this volume range (data not shown). IL-2 cytosol was also found to stimulate PKC activity in membranes isolated from NIH3T3 fibroblasts (data not shown) indicating that the factor present in IL-2 cytosol (i.e. factor X), was able to stimulate PKC from cell sources other than CTLL-2 cells. Given these results it was clearly important to further define the nature of factor X.

3.6.4.2 Preliminary characterization of factor X

It was apparent that the activation of membrane PKC by cytosol isolated from IL-2 treated cells (i.e., IL-2 cytosol) did not arise from the introduction of stimulatory levels of the known PKC intracellular Ca^{2+} in CTLL-2 cells (see Chapter III, section 3.4.4), the cytosol used in these experiments was diluted by a factor of 100,000 before being used in the direct PKC assay.

It is clear that under these conditions the added cytosol would at best make an inconsequential contribution to the 1 μM Ca^{2+} already present in the assay buffer. The same argument holds for DAG, AA or any other of the known PKC co-factors as being responsible for the observed phenomenon. Thus, these arguments suggest that some cytosolic factor was generated during the brief (i.e., 10 min) exposure of CTLL-2 cells to IL-2 that had the ability to

Table 3.1 IL-2 treated cytosol caused PKC translocation from cytosol to membrane

	Membrane PKC activity (cpm/8µg protein)	Cytosolic PKC activity (cpm/8µg protein)
CONTROL	3956 ± 322	1730 ± 223
+IL-2 CYTOSOL	8962 ± 773 (226%)	542 ± 5

Exponentially growing CTLL-2 cells (20×10^6 cells) were washed twice, resuspended in unsupplemented RPMI medium incubated for 4 hour at 37°C. The cells were then lysed hypotonically and the membrane and cytosolic fractions isolated as described in *Methods*. Membranes were incubated with 200 µl IL-2 stimulated CTLL-2 cell cytosol, which was generated according to the procedures described in the *Methods* section, at 27°C for 10 min. Membrane and cytosol fractions were separated again, and total membrane and cytosolic PKC activity was measured by the conventional PKC assay as described in *Methods*. These values were compared to the levels of PKC activity in the membranes and IL-2 cytosol before being mixed (i.e. control). The PKC activity in control cytosol was 1151 ± 80 . The values are the means $\pm$ SEM of triplicate determinations, and are representative of three experiments. cofactors DAG, Ca^{2+} . Aside from the fact that IL-2 was found not to stimulate increases in

activate PKC in isolated membranes. This possibility was explored in some detail in our effort to understand the mechanism(s) of PKC activation in IL-2 stimulated T-cells.

To determine whether factor X was a protein, IL-2 cytosol and control cytosols were treated with trypsin for 10 minutes at 27°C and then inactivated with a large excess of soybean trypsin inhibitor. The trypsin-treated and untreated cytosols were then assayed for their ability to stimulate membrane PKC activity in unstimulated control membranes. As shown in Fig. 3.20, trypsin treatment completely ablated the PKC stimulatory activity present in IL-2 cytosol. The observation that membranes exposed to trypsin-treated control cytosol exhibited PKC levels indistinguishable from membranes incubated with untreated control cytosol, indicated that the loss in PKC activity noted with trypsin-treated IL-2 cytosol was not due to residual trypsin (if

any) effecting membrane PKC itself. It was a real possibility that the large drop in IL-2 cytosol-induced PKC activity by trypsin was due to the protease digesting the cytosolic PKC destined to translocate to the membrane during incubation. This may have been true, but this fact could not account for the levels of PKC activity in membranes exposed to trypsin-treated IL-2 cytosol being substantially less than that observed in TPA-treated membranes (Fig 3.20). This latter observation indicated that membrane-associated "inactive" PKC was not activated by IL-2 cytosol after it had been subjected to trypsin treatment. The results of these trypsin experiments are therefore consistent with factor X being a protein.

To determine the possible molecular size of factor X, control and IL-2 treated cytosols were fractionated through spin columns of 10 kDa molecular weight cut off. The eluents were collected, incubated with control CTLL-2 cell membranes, and membrane PKC activity subsequently determined. As shown in Fig 3.21, the eluents from fractionated IL-2 cytosol were still able to activate inactive membrane PKC while the control cytosols had little or no stimulatory activity.

It was clear from the results presented in Figure 3.22 that factor X was unlikely a phosphatase since the potent phosphatase 1 and 2A inhibitor okadaic acid (MacKintosh and MacKintosh 1994) was unable to block the PKC-stimulating activity present in IL-2 cytosol or fractionated IL-2 cytosol when it was added directly to the assay at 1 μ M. Moreover, the PKC-stimulating activity of IL-2 cytosol was equally unaffected if 1 μ M okadaic acid was added to the IL-2 cytosol 10 min before it was added to the control membrane preparation (data not shown). The concentration of okadaic acid used in these experiments was 1000 times that used in other studies (MacKintosh and MacKintosh 1994) to effectively inhibit cellular phosphatase activity. Similarly, the potent tyrosine kinase inhibitors herbimycin A and genistein used at

concentrations previously shown to effectively block IL-2 mediated cell proliferation (see Fig. 3.4) were unable to block the PKC stimulatory activity of IL-2 cytosol when added to the preparation for 10 min before assay (data not shown). Collectively, these data suggest that the cytosolic component(s) was unlikely a protein phosphatase or a tyrosine kinase.

3.6.4.3 Studies on the mechanism by which factor X activates PKC

During this study we have shown that the IL-2R-mediated activation of inactive membrane PKC in CTLL-2 cells is a process which likely involves a PTX-sensitive G_i -protein, AA, and DAG. Thus, it was important to determine which step in the process from IL-2R stimulation to PKC activation the cytosolic component (factor X) acted. Did factor X act upstream or downstream of the G-protein step or the steps generating AA and/or DAG? In order to address these questions, exponentially growing CTLL-2 cells were labeled with ^3H -AA for 24 hr, followed by a 4 hour period in serum/IL-2 free medium in the presence or absence of PTX (75ng/ml). The cell membranes were subsequently isolated and stimulated with IL-2 cytosol for 10 min, and the extent of membrane PKC activation as well as the levels of membrane AA and DAG was determined. As shown in Fig. 3.23, factor X stimulated PKC activity equally well in PTX-treated and untreated membranes, suggesting that factor X either acts downstream of the G protein in this pathway, or is activating PKC through a PTX-sensitive G protein independent pathway. When AA and DAG level were compared (Fig.3.24), both AA and DAG level *decreased* when membranes were incubated with IL-2 cytosol. The reasons for IL-2 cytosol effecting a reduction in AA and DAG are unclear, but the fact remains that the levels of these PKC co-factors were indeed less in membranes treated with IL-2 cytosol despite the fact that PKC activity was stimulated under these conditions. These results suggest that some component, likely a protein, is rapidly activated in the cytosol of CTLL-2 cells during a brief exposure to IL-

2 which is capable of activating PKC in isolated membranes, and that its mechanism of action cannot be readily linked to the mechanism by which IL-2 appears to activate inactive membrane PKC in intact CTLL-2 cells.

Figure 3.1 IL-2 stimulated tyrosine kinase activity in CTLL-2 cells

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium at a density of 5×10^6 cells/ml and incubated for 4 hour at 37°C. The cells were stimulated with 100 U/ml IL-2 for indicated times, and the reaction stopped by adding ice cold PBS. The cells were lysed hypotonically and cell nuclei and unlysed cells were sedimented by centrifugation as described in *Methods*. The post-nuclear supernatants were collected and tyrosine kinase activity determined as described in *Methods*. The values are the means $\pm$ SEM of triplicate determinations, and are representative of two separate determinations.

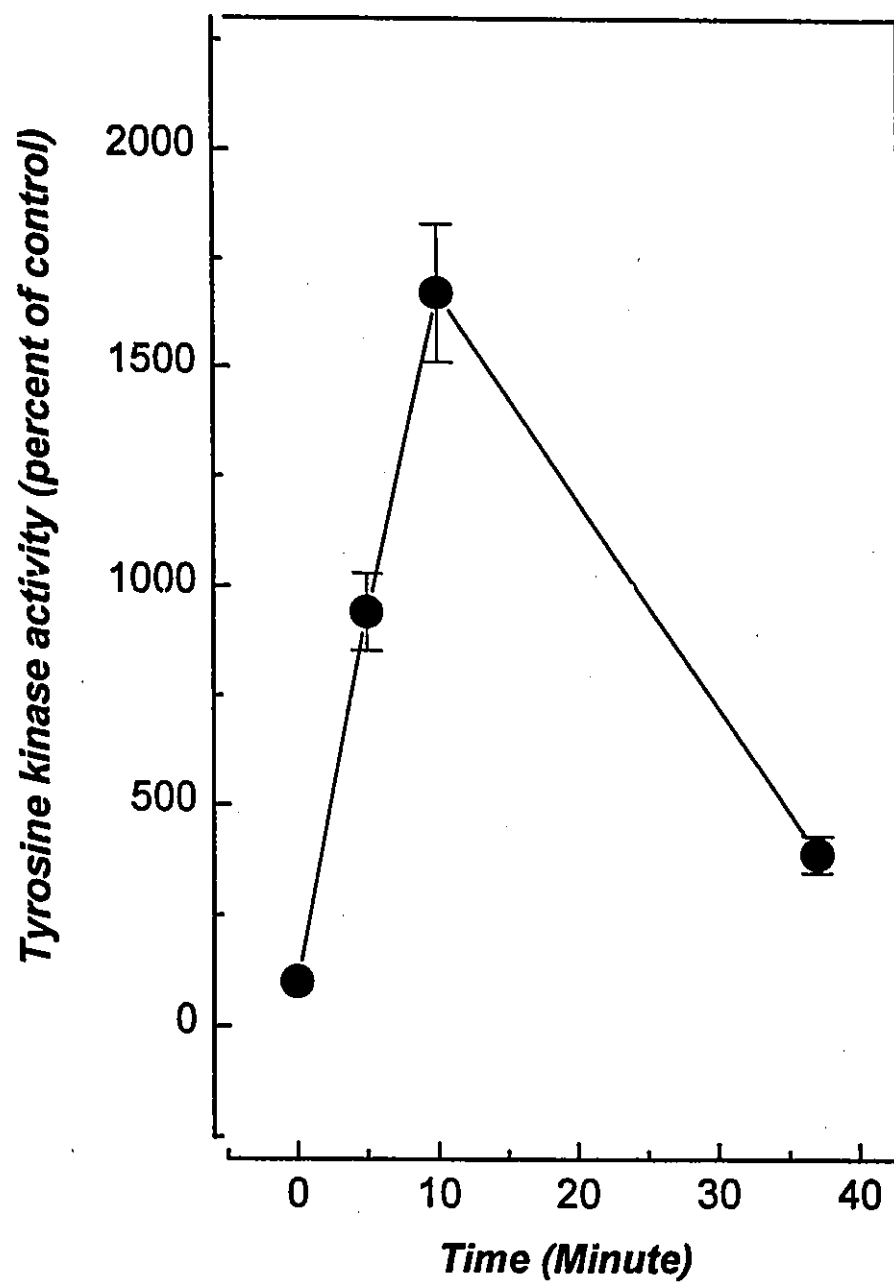


Figure 3.2 Tyrosine kinase activity is not involved in IL-2 stimulated PKC activation

Exponentially growing CTLL-2 cells were washed twice in unsupplemented RPMI medium, resuspended in same medium at a density of 6×10^6 cells/ml, and incubated for 4 hour at 37°C. The cells were then treated with the tyrosine kinase inhibitors, genistein (14.8 μ M) and herbimycin A (875 nM) for 30 min before being challenged with 100 U/ml IL-2 for 10 min at 37°C. The reaction was stopped by adding ice cold PBS, and the cells were lysed hypotonically and the membrane and cytosolic fractions isolated as described in *Methods*. Membrane PKC activity were measured by the direct PKC assay as described in *Methods*. The values are the means $\pm$ SEM of triplicate determinations and are representative of four separate experiments. $P > 0.05$ between IL-2 and herbimycin A (student t-test).

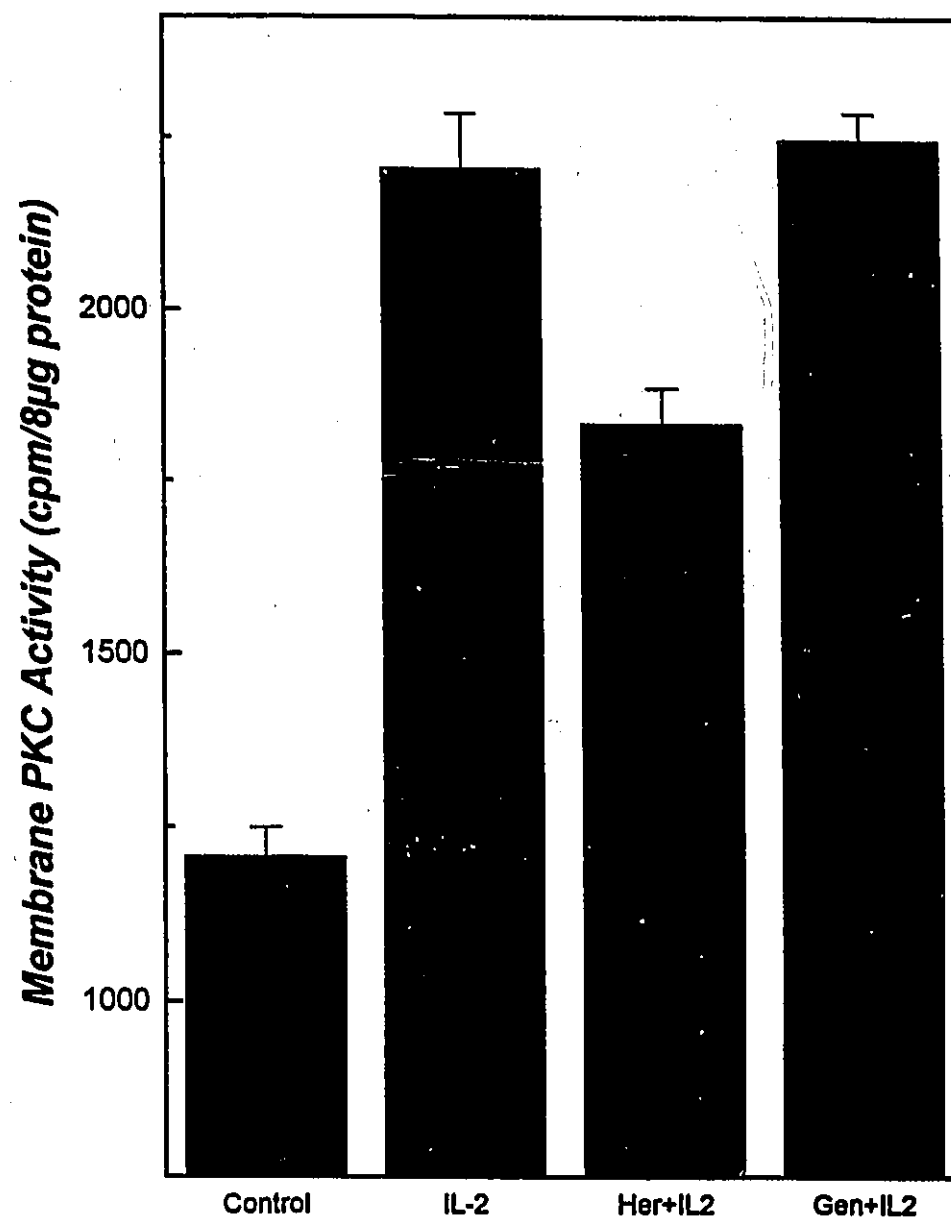


Figure 3.3 Herbimycin A inhibits CTLL-2 cells DNA synthesis and proliferation

Exponentially growing CTLL-2 cells were resuspended in RPMI medium supplemented with 10 % serum and 100 U/ml IL-2 at a density of 1×10^5 cells/ml and the indicated concentrations of herbimycin A were added to culture dishes for 24 hours at 37°C. For some cultures, viable cell numbers were determined by trypan blue exclusion method as described in *Methods*. For others, [³H]-thymidine uptake was determined. Cells were labeled with [³H]-thymidine (3 µl/dish, 25 Ci/mmol) for the last 17 hours of the 24 hour incubation period and the incorporation of [³H]-thymidine into DNA was determined as described in *Methods*. The values are the means $\pm$ SEM of triplicate cell cultures and are representative of two separate experiments.

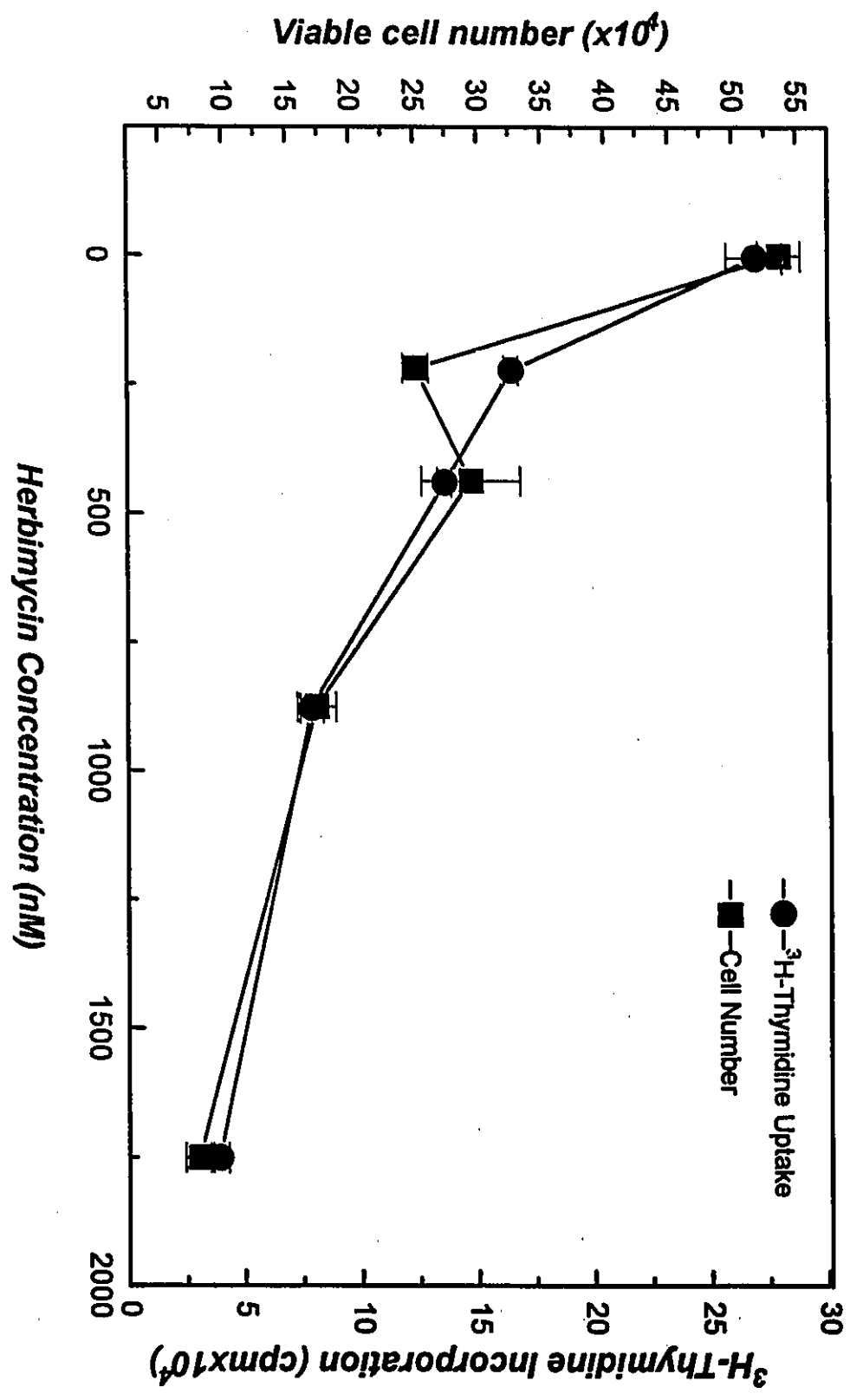


Figure 3.4 Genistein inhibits CTLL-2 cells DNA synthesis and proliferation

Exponentially growing CTLL-2 cells were resuspended in RPMI medium supplemented with 10 % serum and 100 U/ml IL-2 at a density of 1×10^5 cells/ml and the indicated concentrations of genistein were added to culture dishes for 24 hours at 37°C. At that time, viable cell numbers were determined in some cultures by the trypan blue exclusion method as described in *Methods*. For others, [^3H]-thymidine uptake was in a manner detailed in the legend to Fig.3.3.

^3H -Thymidine Incorporation per dish(CPMX10⁴)

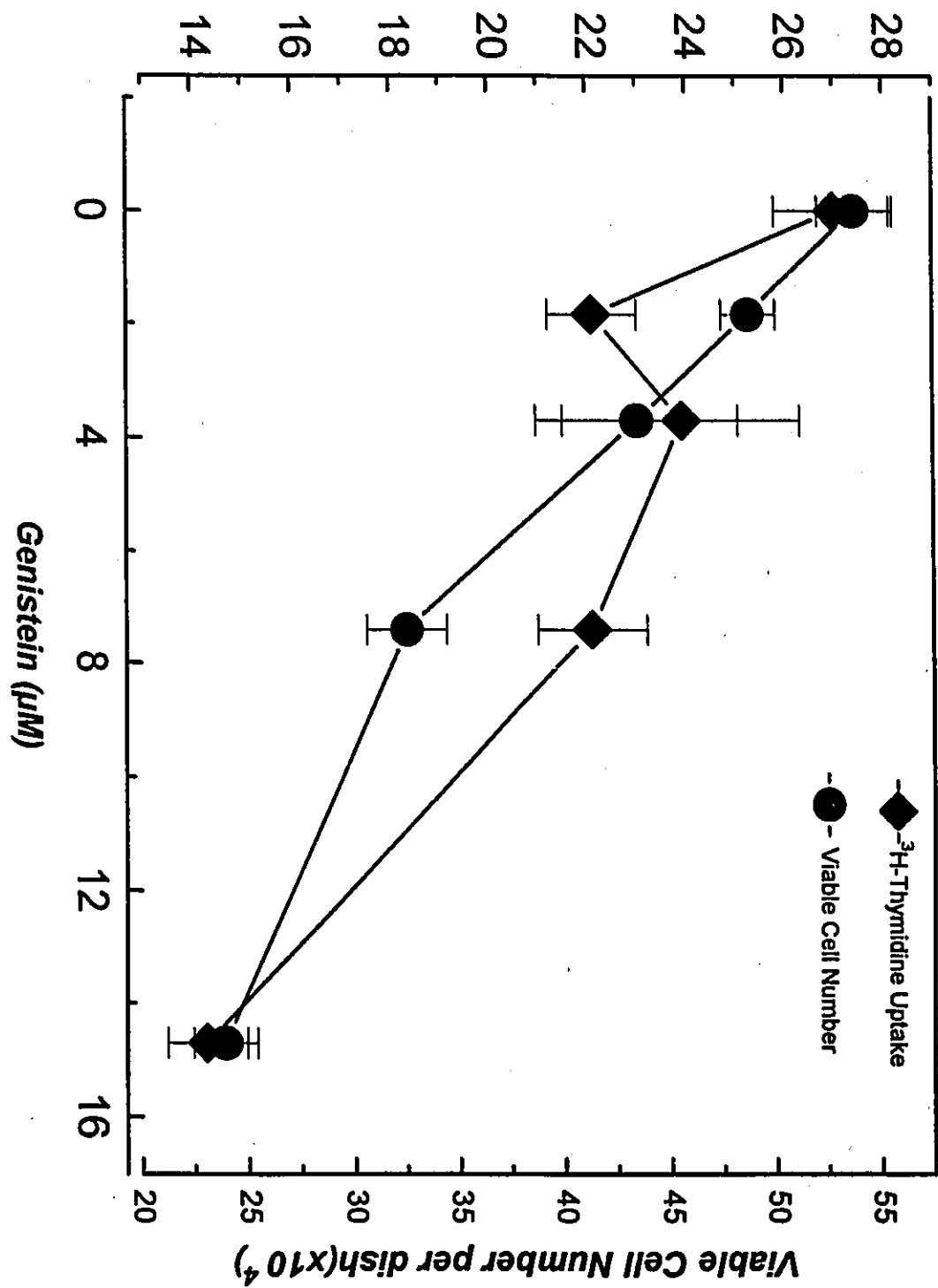


Figure 3.5 The effect of pertussis toxin on IL-2 stimulated PKC activity

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium at a density of 6×10^6 cells/ml either in the presence or absence of pertussis toxin (75 ng/ml) and incubated for 4 hours at 37°C. Cells were then stimulated with 100 U/ml IL-2 for 10 min at 37°C, and the reaction was stopped by adding ice cold PBS. Cells were then lysed hypotonically and the membrane and cytosolic fractions isolated as described in *Methods*. Membrane PKC activity were measured by the direct PKC assay as described in *Methods*. The values are the means $\pm$ SEM of triplicate determinations and are representative of three separate experiments.

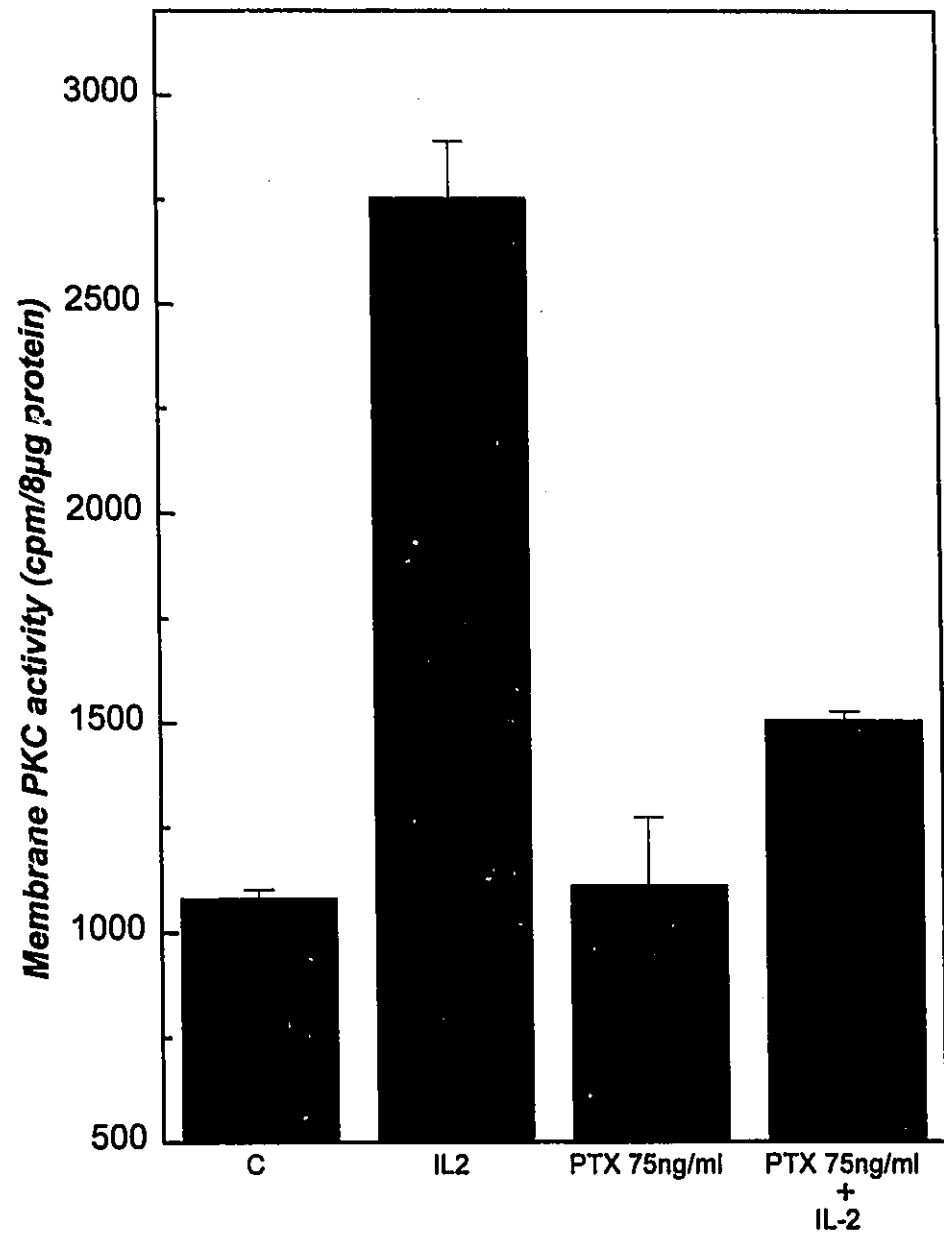


Figure 3.6 The effect of pertussis toxin on IL-2's ability to maintain cell viability

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium at a density of 2×10^5 cells/ml and incubated for 7 hour at 37°C either in the presence or absence of pertussis toxin (100 ng/ml). Then 100 U/ml IL-2 was added to some culture dishes and incubated for an additional 19 hour. Viable cell numbers were determined as described in *Method*. The values are the means $\pm$ SEM of triplicate cell cultures and are representative of three separate experiments. * $P < 0.001$ between IL-2 and PTX plus IL-2 (student t-test).

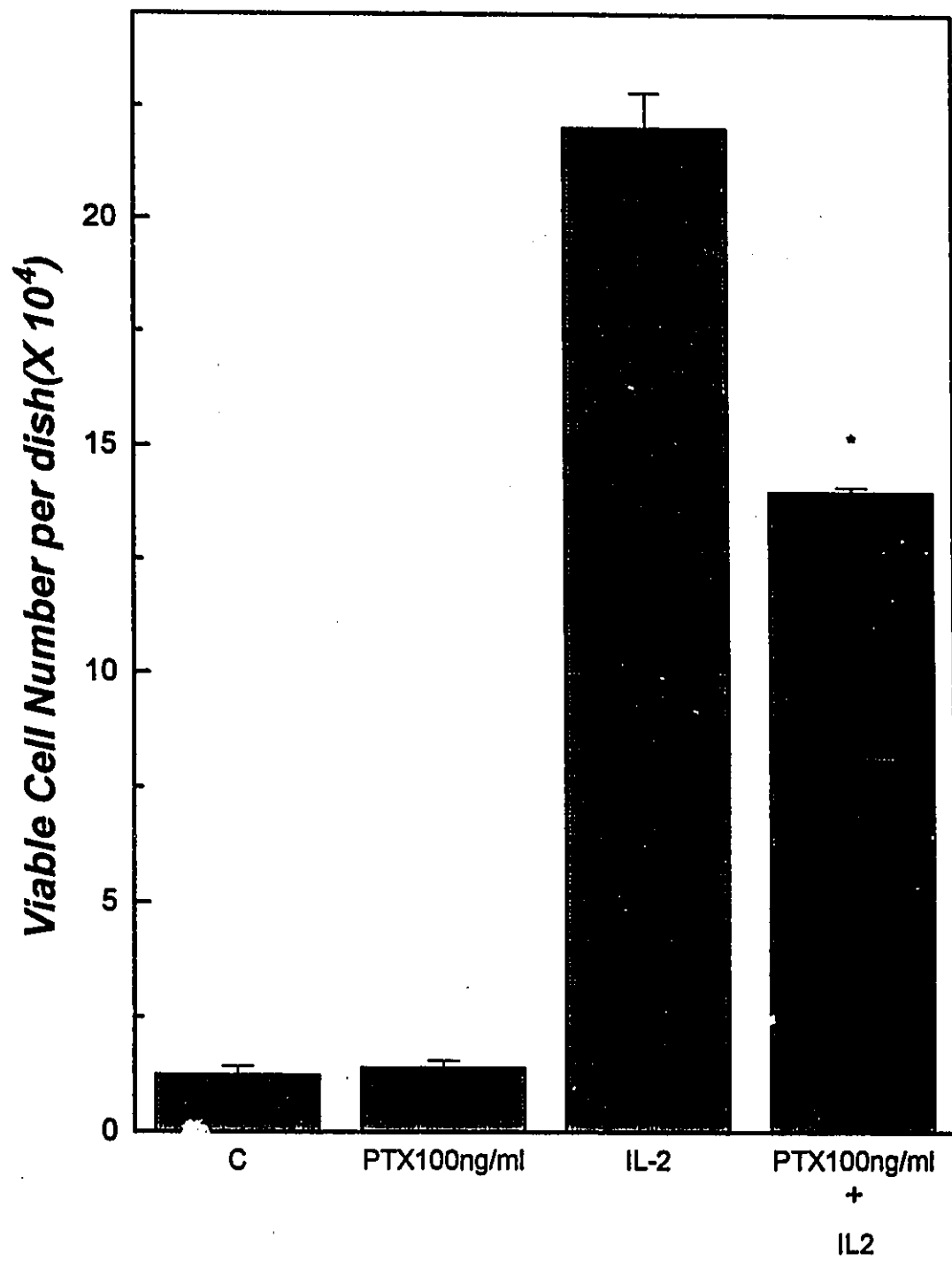


Figure 3.7 IL-2 stimulated DAG accumulation in CTLL-2 cells

Exponentially growing CTLL-2 cells were labeled with [^3H]-arachidonic acid (AA, 0.05 $\mu\text{Ci/ml}$, 100 Ci/mmol) for 24 hour and then placed in RPMI medium without serum and IL-2 for 4 hour. 100 U/ml of IL-2 were added to the cells for the indicated times at 37°C. The stimulation was then stopped by adding ice-cold PBS and total cellular lipids were extracted as described in *Method*. Neutral lipids were separated by TLC, stained with iodine vapor, and radioactivity co-migrating with DAG standards determined by liquid scintillation counting as described in *Methods*. Values are the percentage of [^3H] in DAG relative to total radioactivity on TLCs of 12×10^6 cells, and are representative of three separate experiments.

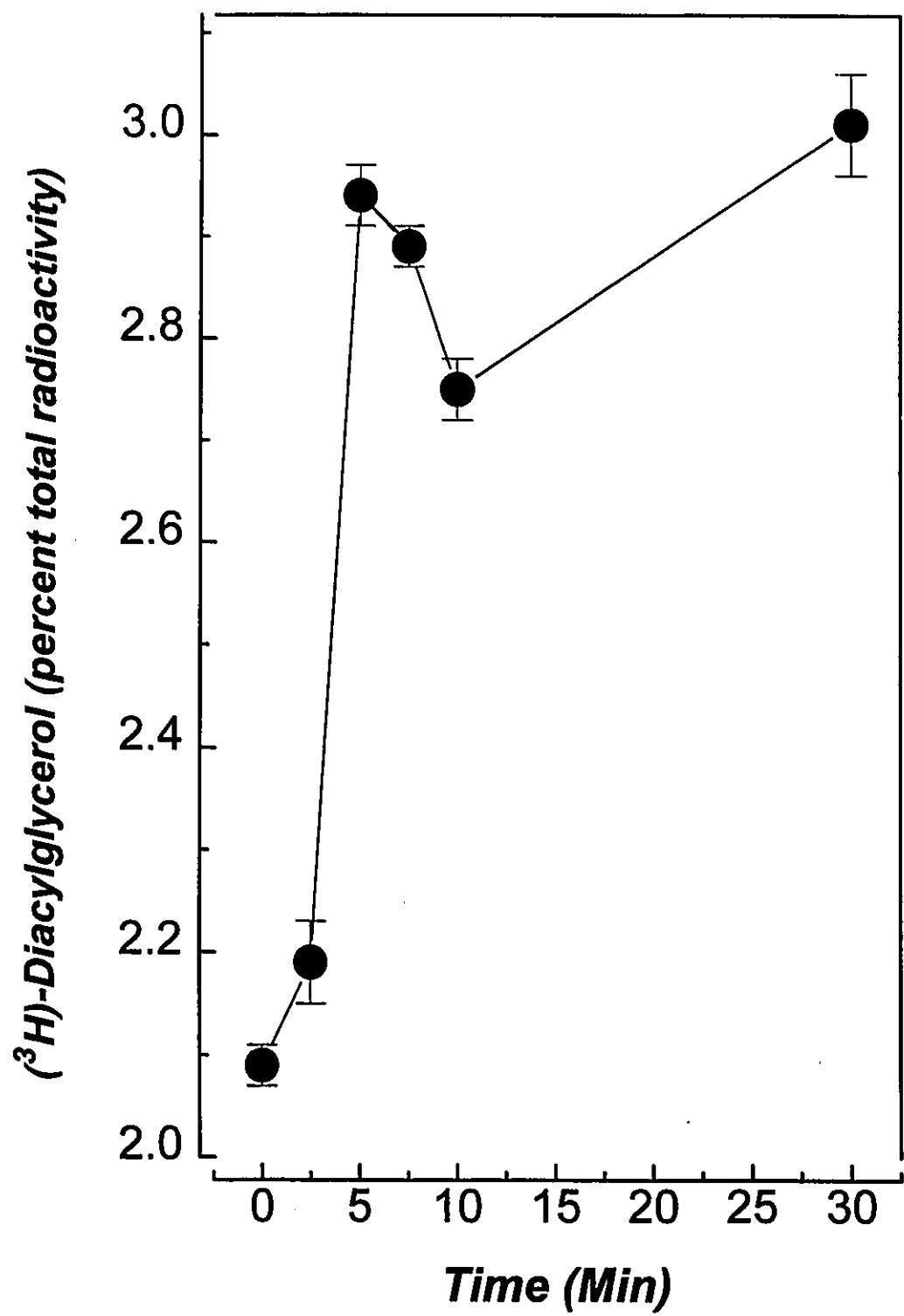


Figure 3.8 The effect of exogenous DAG on intact CTLL-2 cells PKC activity

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium and incubated for 4 hour at 37°C. Cells were then stimulated with the indicated concentrations of exogenous DAG (1-oleoyl, 2-acetyl glycerol) for 10 min at 37°C. The reaction were stopped by adding ice cold PBS and the cells were then lysed hypotonically. Membrane and cytosolic fractions were isolated as described in *Methods* and membrane PKC activity was measured by the direct PKC assay as described in *Methods*. The values are the means $\pm$ SEM of triplicate determinations and are representative of two separate experiments.

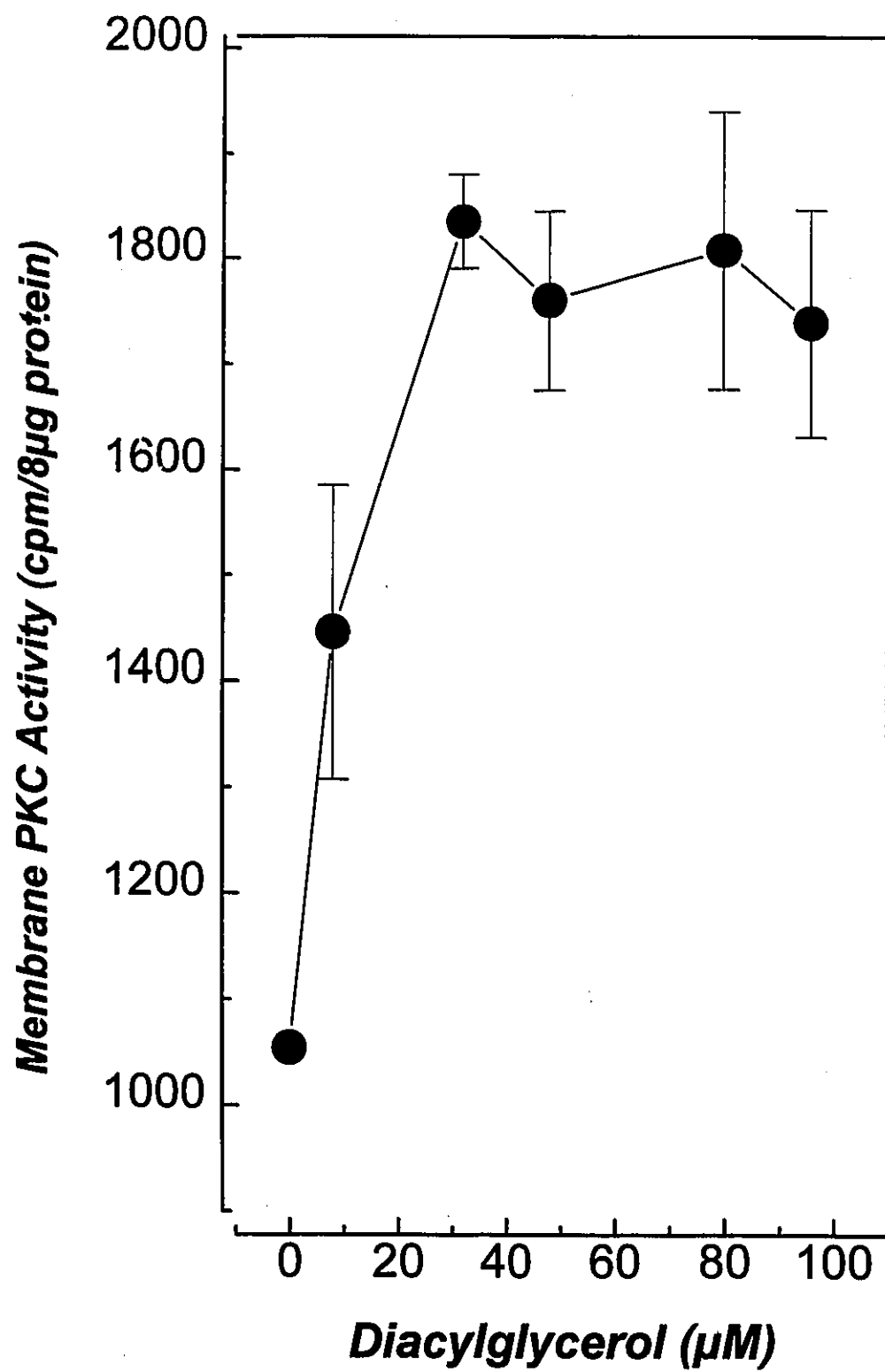


Figure 3.9 The effects of exogenous DAG on inactive membrane associated PKC activity

Exponentially growing CTLL-2 cells (40×10^6 cells) were washed twice, resuspended in unsupplemented RPMI medium and incubated for 4 hour at 37°C. The cells were then lysed hypotonically and membrane and cytosolic fractions isolated as described in *Methods*. The indicated concentrations of exogenous DAG (1-oleoyl, 2-acetyl glycerol) were added to membrane and membrane PKC activity was determined by the direct PKC assay as described in *Methods*. The values are the means $\pm$ SEM of triplicate determinations and are representative of two experiments.

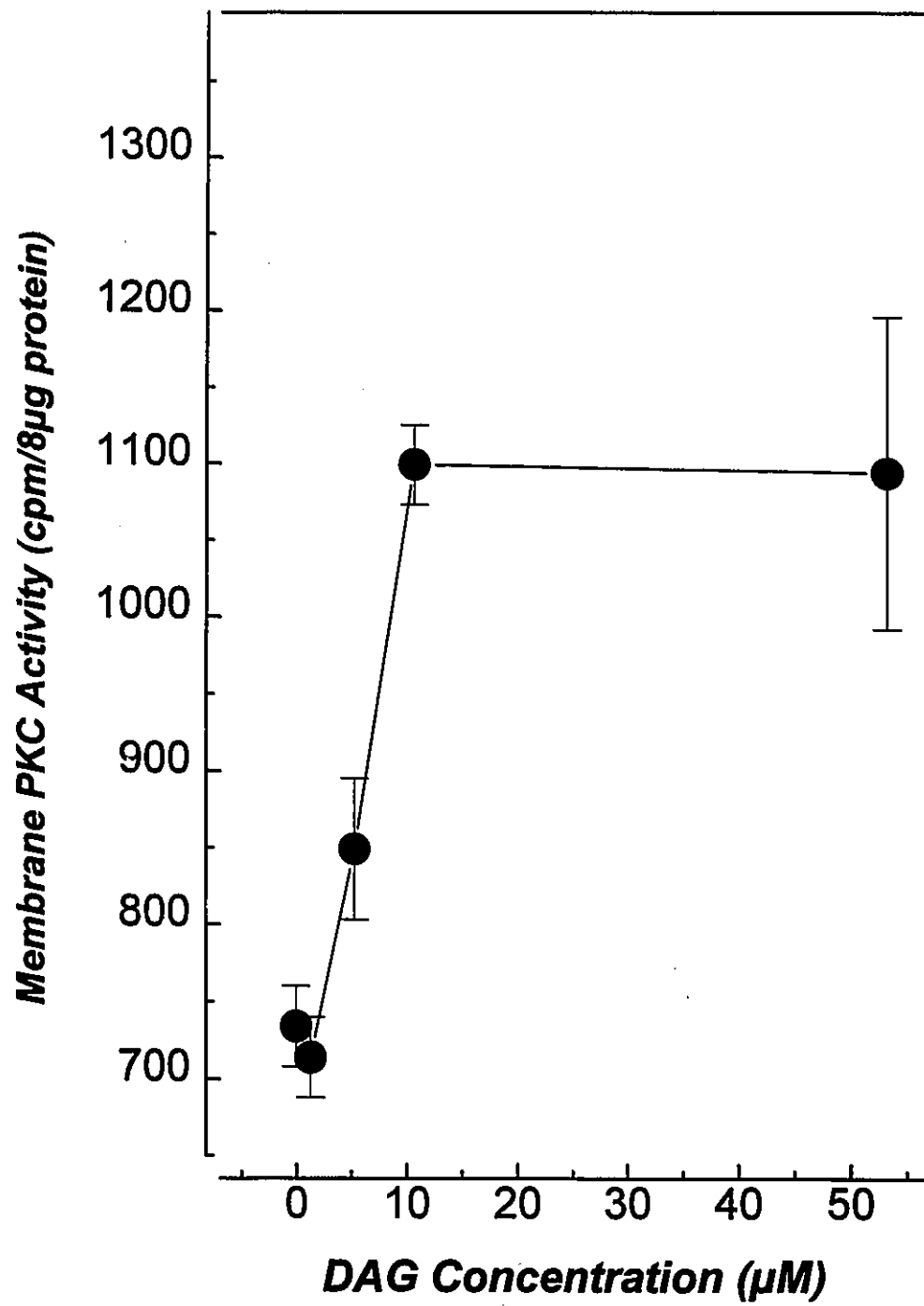


Figure 3.10 The effect of IL-2 on inositol phosphatide turnover

(A) Exponentially growing CTLL-2 cells were resuspended in RPMI medium with 5% serum and 25 U/ml IL-2 and labeled with [^3H]-myo-inositol (6 $\mu\text{Ci/ml}$, 20.5 Ci/mmol) for 24 hour and then placed in RPMI medium without serum and IL-2 for 4 hour in the presence of [^3H]-myo-inositol (6 $\mu\text{Ci/ml}$, 20.5 Ci/mmol). The cells were washed three times, resuspended in RPMI medium with LiCl (20 mM) without serum and IL-2 for 10 min at 37°C. The indicated concentrations of IL-2 were added to the cultures and incubated for 10 min at 37°C. The cells were then rapidly pelleted by centrifugation, and 10 % trichloroacetic acid (TCA) was added to each sample. Cellular lipids were extracted and inositol phosphates separated by DOWEX-AG 1X8-formate anion exchange chromatography as described in *Methods*. (B) As a positive control for these experiments, confluent rat type I astrocytes grown in 60 mm Petri dishes were prelabeled with [^3H]-myo-inositol (2.5 $\mu\text{Ci/ml}$) for 16-18 h in serum-and inositol-free DMEM. Unbound [^3H]-myo-inositol was washed thoroughly with DMEM. LiCl (20mM) was added to the cells for 10 min at 37°C. Endothelin-1 (ET, 20nM) was added to astrocytes in the presence of 20 mM LiCl for 10 min. The reaction was stopped by replacing the medium with cold 0.3 M TCA, and cells were scraped, briefly sonicated, and sedimented by centrifugation. Cellular lipids were extracted, inositol phosphates were separated, and measured as described in *Methods*. In all cases (^3H)-IP's measured were expressed relative to the levels of protein present in the TCA precipitated formed. The values are the average of two experiments performed in duplicate.

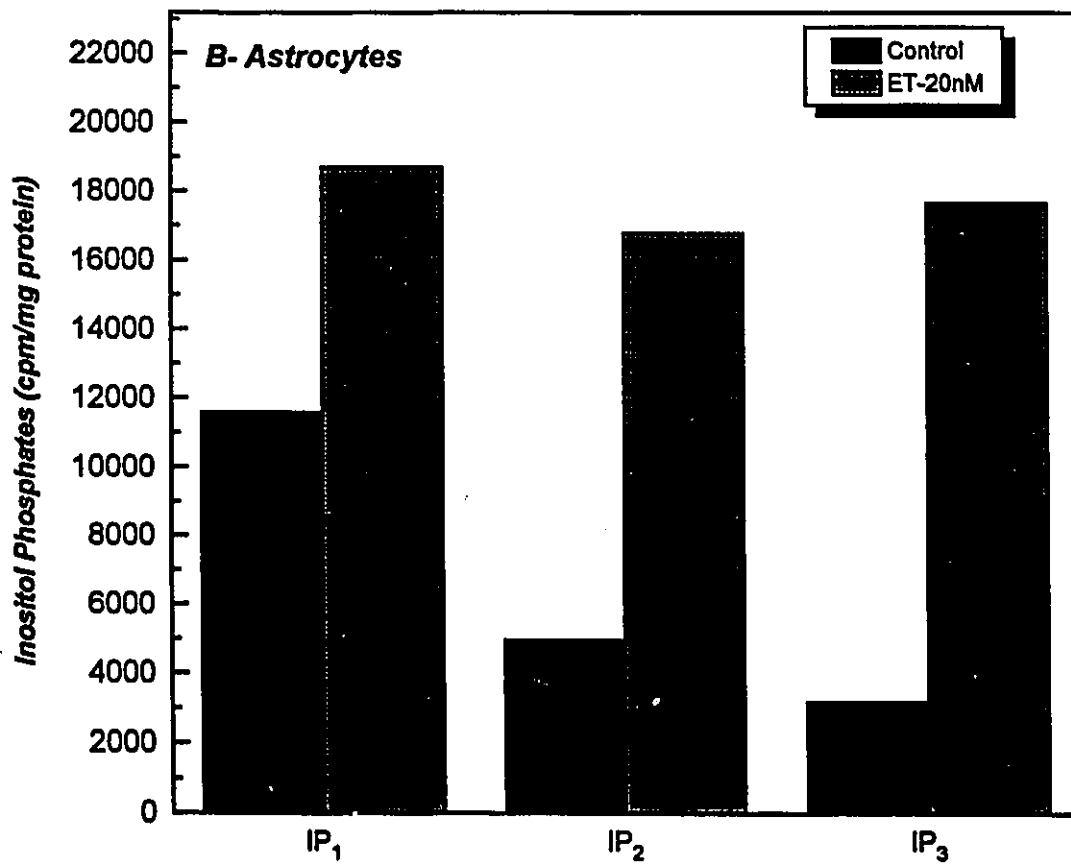
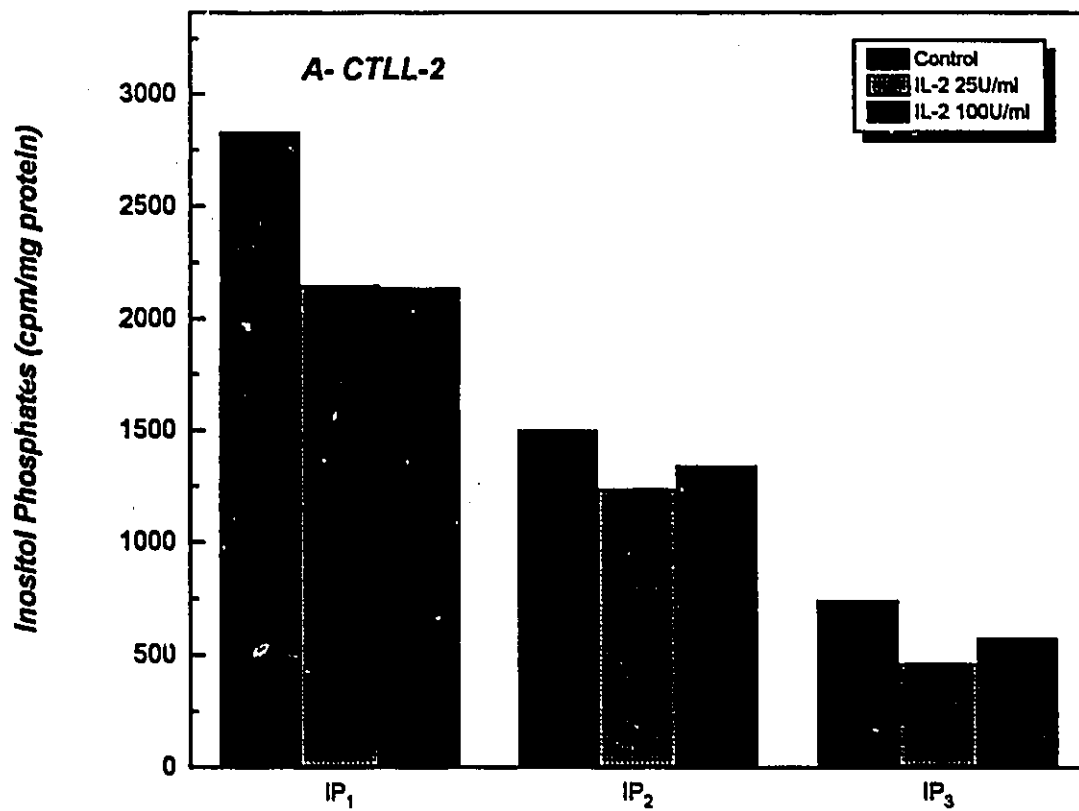


Figure 3.11 The effects of IL-2 on intracellular Ca^{2+} levels

Exponentially growing CTLL-2 cells were washed twice and incubated for 2 hour in unsupplemented RPMI medium. Cells were then loaded with fura-2 by incubation for 30 min at 37°C in a normal buffer solution (NBS) containing 5 μM fura-2-acetoxymethyl ester, washed three time in fura-free NBS, and incubated for 30 min in NBS at room temperature to ensure full hydrolysis of the fura-2 ester. Experiments were conducted at room temperature on 5×10^6 suspended cells in a cuvette placed in a CM3 cation measurement spectrofluorimeter. Measurements were performed using 350-nm and 380-nm excitation wavelengths alternating at a frequency of 1 Hz as described in *Methods*. In figure A-G, the arrows indicate the time of addition of the specified agents directly to the cuvette. Ionomycin and EGTA were added at 5 μM and 2 mM, respectively. The results shown are tracings of representative experiments, similar results were observed in cells obtained from different cell preparations.

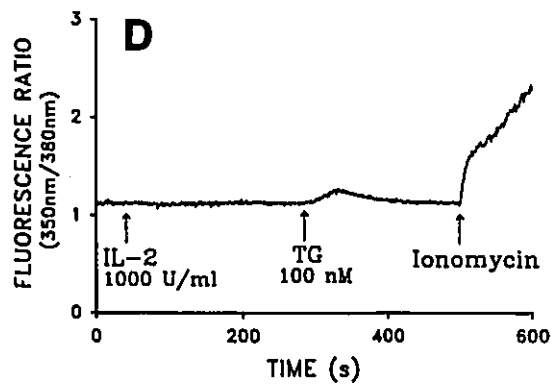
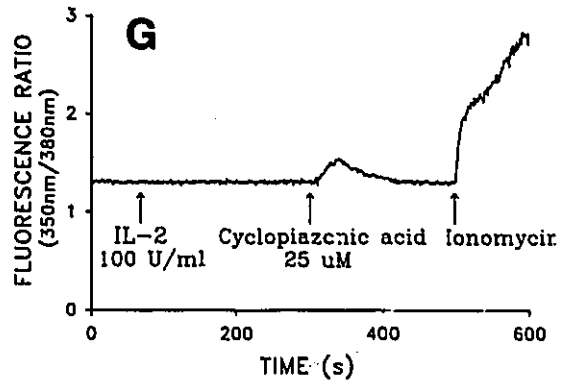
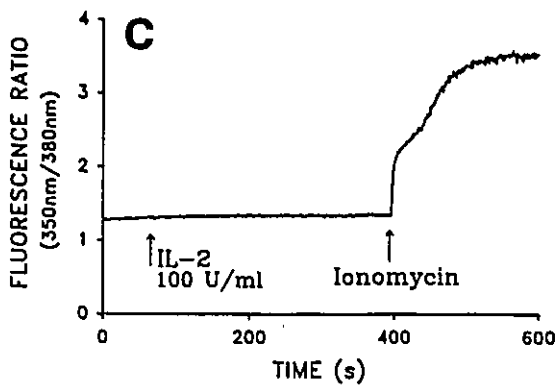
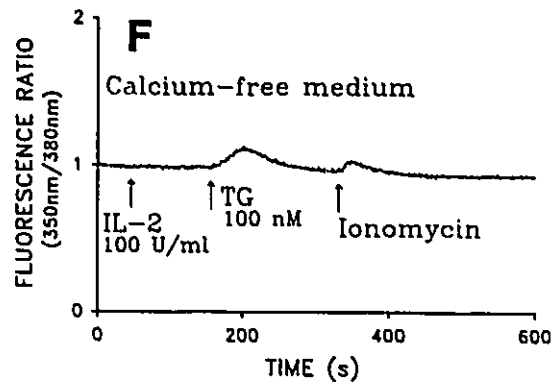
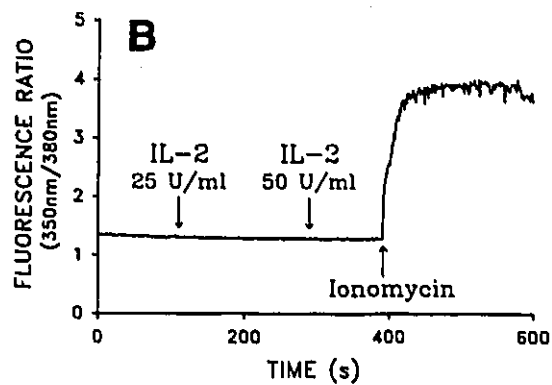
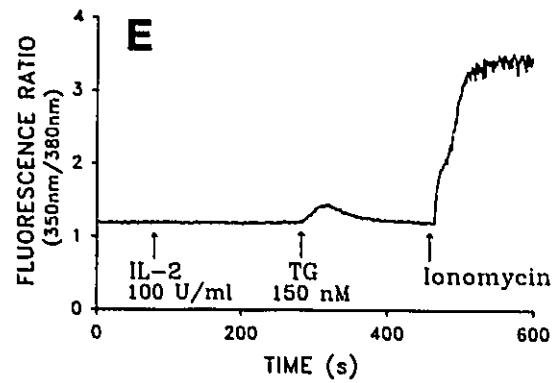
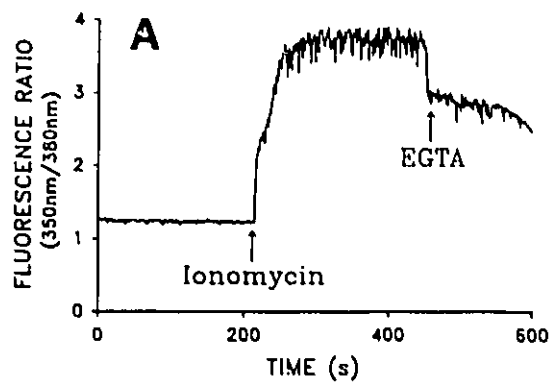


Figure 3.12 IL-2-stimulated AA accumulation in CTLL-2 cells

Exponentially growing CTLL-2 cells were labeled with [^3H]-arachidonic acid (0.05 $\mu\text{Ci/ml}$, 100 Ci/mmol) for 24 hour at 37°C at a density of 2.5×10^5 cells/ml and then incubated in unsupplemented RPMI medium for 4 hour. 100 U/ml of IL-2 (●) or unsupplemented medium (▲) were added to the cells for the indicated times at 37°C after which the reaction was stopped by adding ice-cold PBS, and total cellular lipids were extracted as described in the *Methods*. Neutral lipids were separated by TLC, stained with iodine vapor, and regions corresponding to standard AA preparation (added to each sample) were excised and counted for [^3H] in a liquid scintillation counter as described in *Methods*. Values are the percentage of [^3H] AA relative to total radioactivity on TLCs and are representative of three separate experiments.

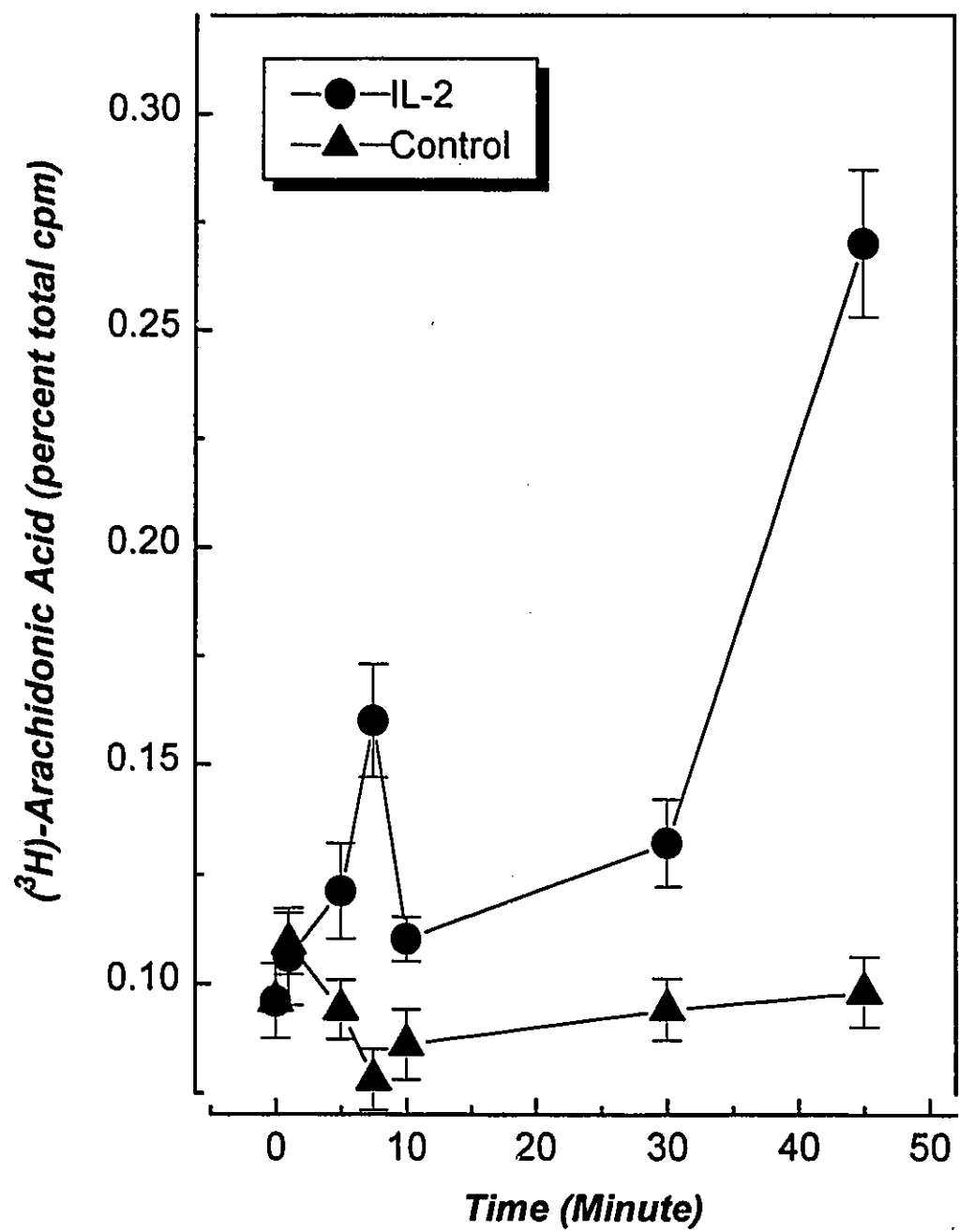


Figure 3.13 The effect of exogenous AA on CTLL-2 cell PKC activity

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium and incubated for 4 hour at 37°C. The cells were then stimulated with the indicated concentrations of exogenous AA for 10 min at 37°C. The reaction was stopped by adding ice cold PBS and the cells were then lysed hypotonically and membrane and cytosolic fractions isolated as described in *Methods*. Membrane PKC activity was measured by the direct PKC assay as described in *Methods*. The values are the means $\pm$ SEM of triplicate determinations and are representative of two separate experiments.

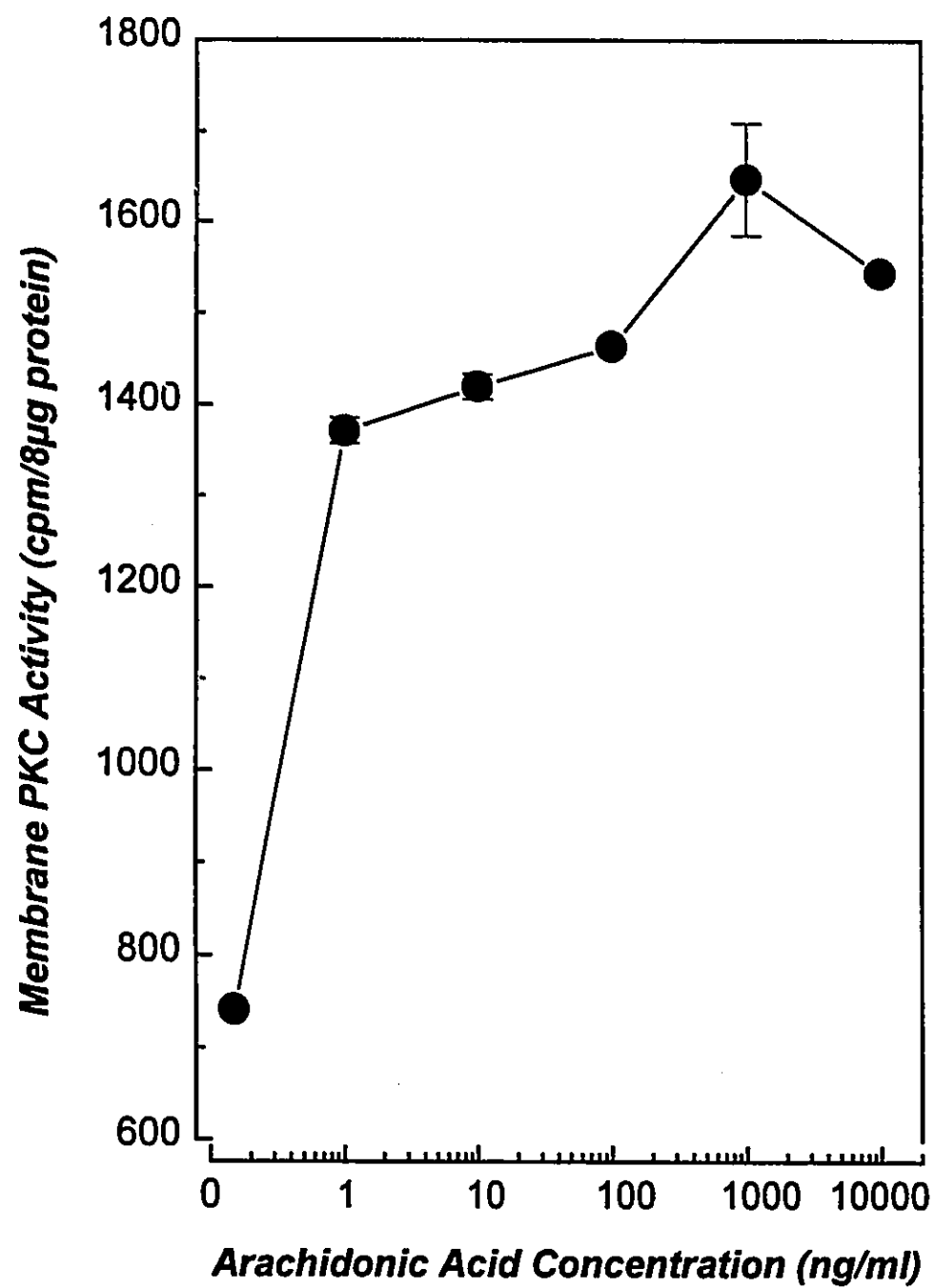


Figure 3.14 The effect of exogenous AA on inactive membrane associated PKC activity in isolated CTLL-2 membranes

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium and incubated for 4 hour at 37°C. The cells were then lysed hypotonically and membrane and cytosolic fractions isolated as described in *Methods*. The indicated concentrations of exogenous AA were added to membrane and membrane PKC activity was determined by the direct PKC assay. The values are the means $\pm$ SEM of triplicate determination and are representative of two separate experiments.

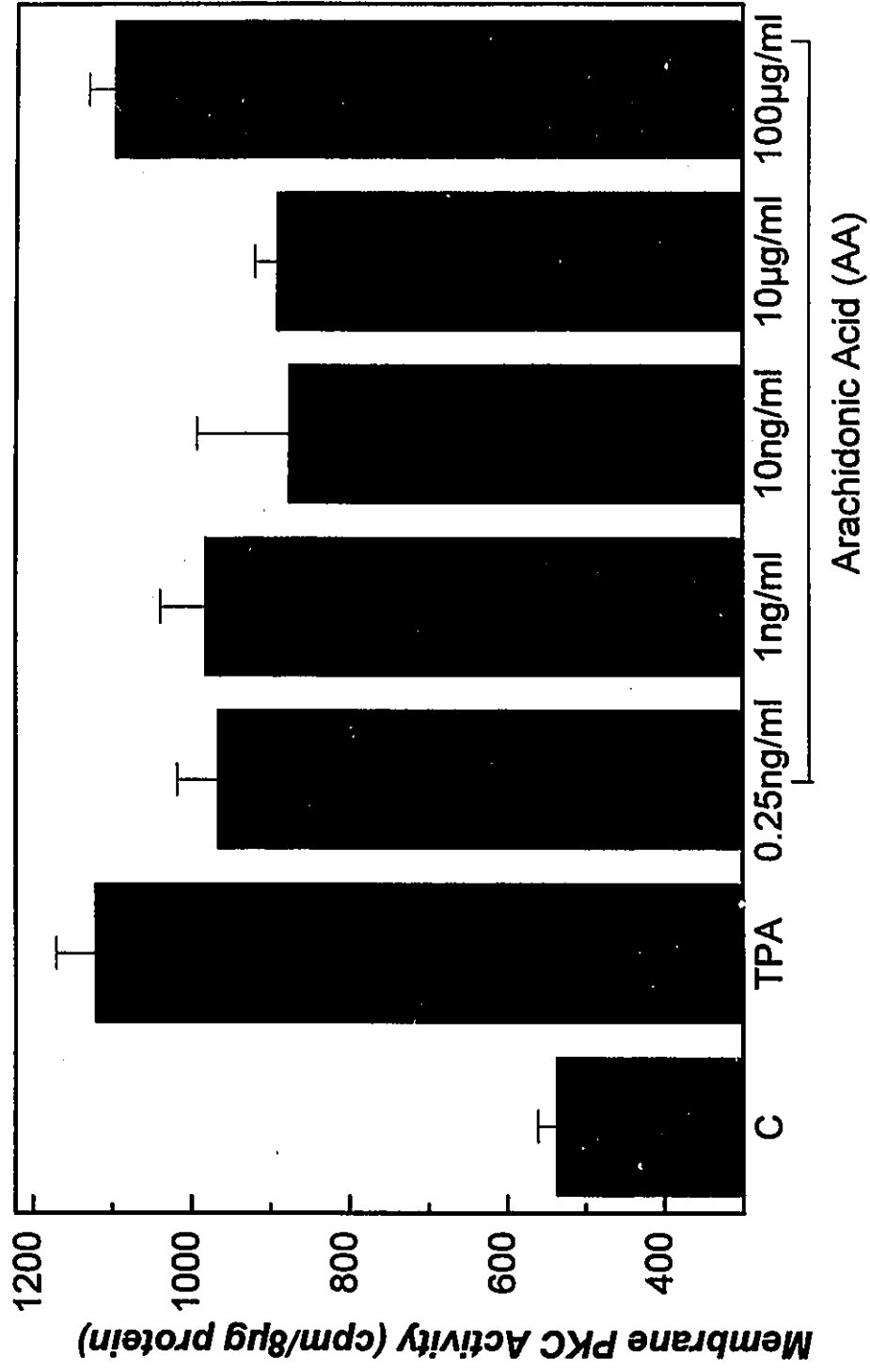


Figure 3.15 The effects of the PLA₂ inhibitor oleoyloxyethyl phosphocholine (OOPC) on IL-2 stimulated PKC activity

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium and incubated for 4 hour at 37°C. The cells were then incubated with the indicated concentrations of PLA₂ inhibitor, OOPC, for 30 min at 37°C before adding 50 U/ml IL-2 for 10 min. The reaction was stopped by adding ice cold PBS and the cells were then lysed hypotonically and membrane and cytosolic fractions isolated as described in *Methods*. Membrane PKC activity was measured by the direct PKC assay. The values are the means $\pm$ SEM of triplicate determination and are representative of two separate experiments.

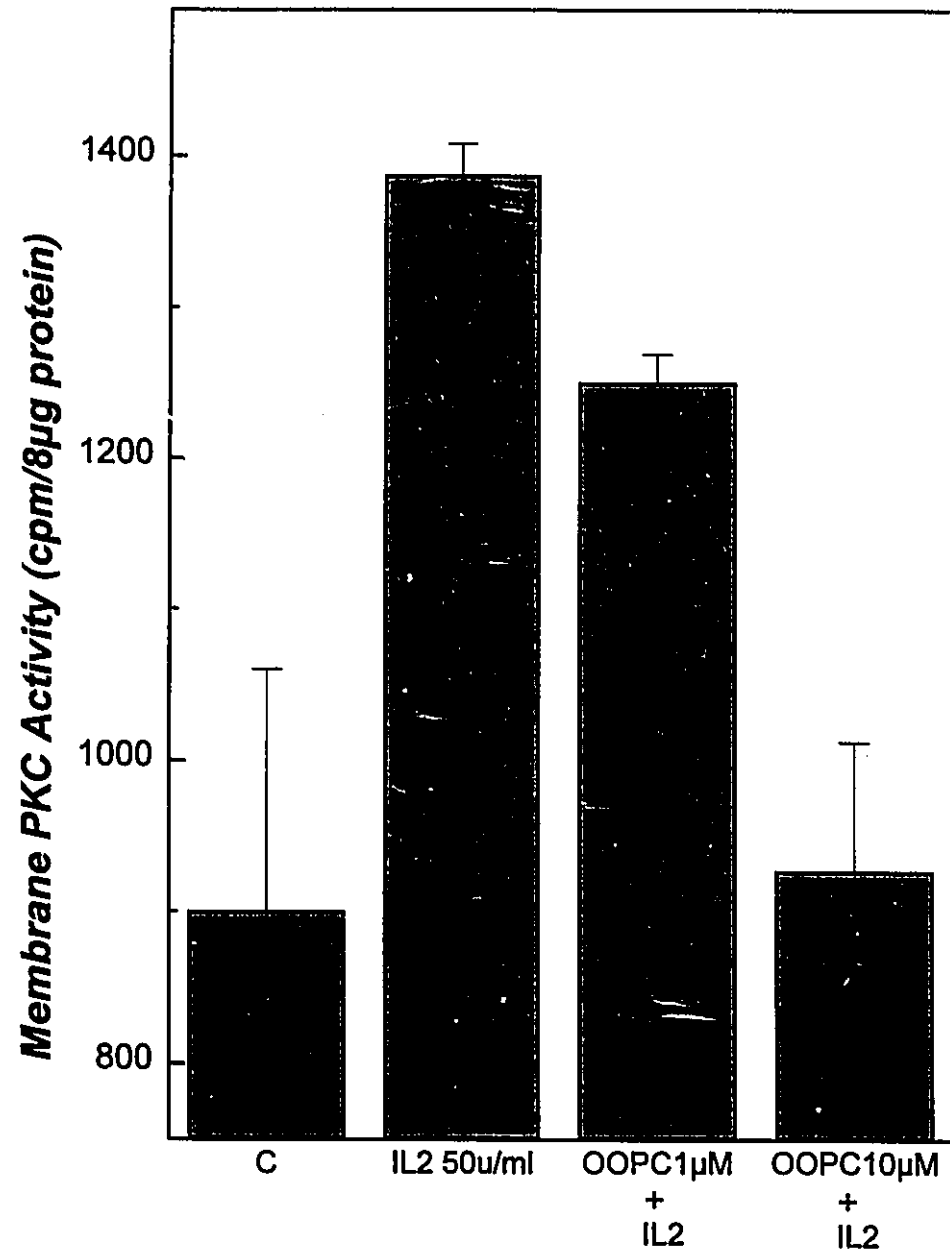


Figure 3.16 The effects of the PLA₂ inhibitor, AACF, on IL-2 stimulated PKC activity

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium and incubated for 4 hour. The cells were then incubated with indicated concentrations of PLA₂ inhibitor, AACF, for 30 min at 37°C before adding 50 U/ml IL-2 for 10 min. The reaction was stopped by adding ice cold PBS and the cells were then lysed hypotonically and membrane and cytosolic fractions isolated as described in *Methods*. Membrane PKC activity were measured by the direct PKC assay. The values are the means $\pm$ SEM of triplicate determination.

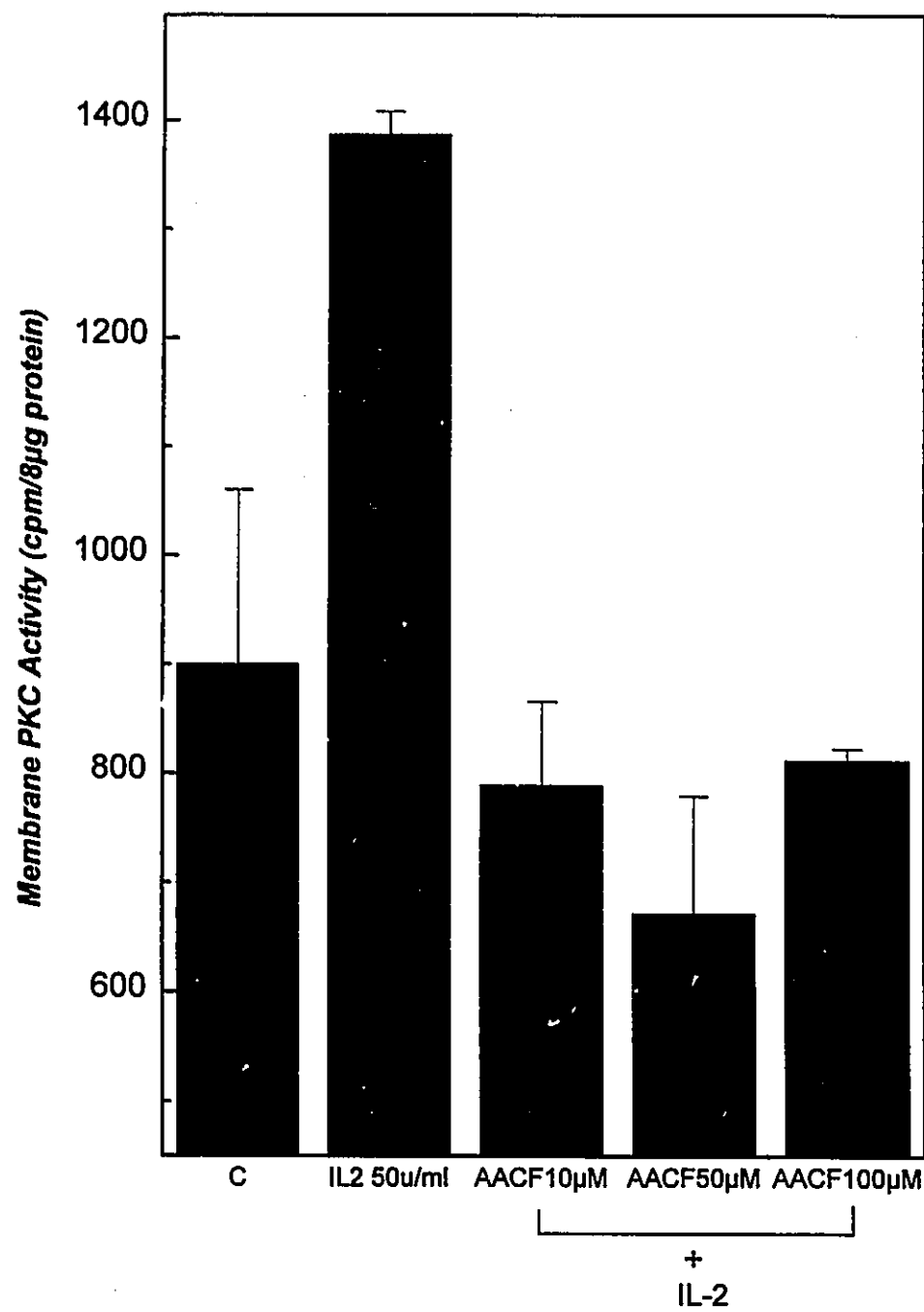


Figure 3.17 PLA₂ inhibitor, U73122, on IL-2 stimulated PKC activity

Exponentially growing CTLL-2 cells were washed twice, resuspended in unsupplemented RPMI medium and incubated for 4 hour. The cells were then incubated with indicated concentrations of PLA₂ inhibitor, U73122, for 30 min at 37°C before adding 100 U/ml IL-2 for 10 min. The reaction was stopped by adding ice cold PBS and the cells were then lysed hypotonically and membrane and cytosolic fractions isolated as described in *Methods*. Membrane PKC activity were measured by the direct PKC assay. The values are the means $\pm$ SEM of triplicate determination and are representative of two separate experiments.

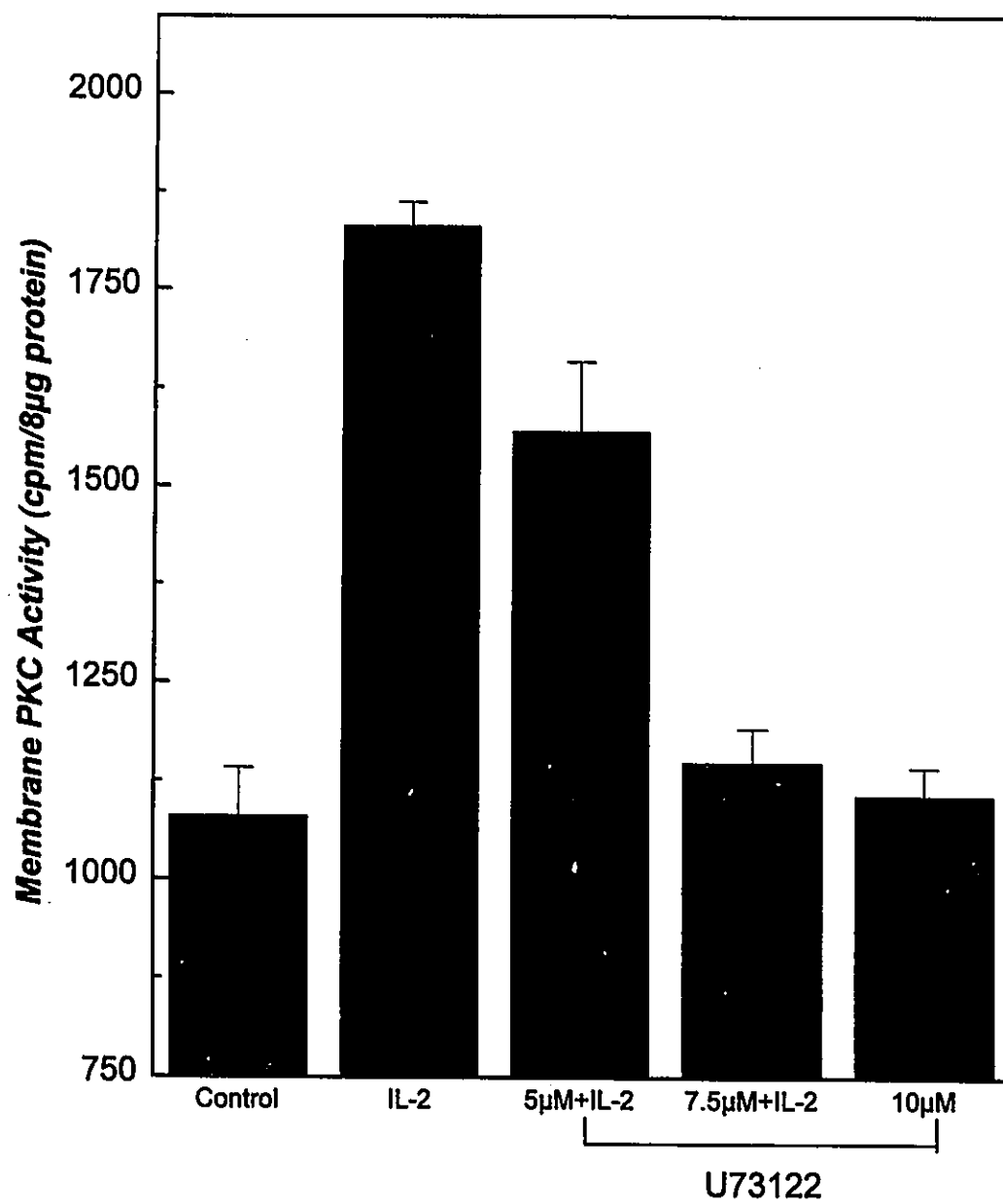


Figure 3.18 IL-2 treated cytosol, but not control cytosol, activates membrane PKC

Exponentially growing CTLL-2 cells (25×10^6 cell) were washed twice and resuspended in unsupplemented RPMI medium for 4 hour at 37°C . The cells were then stimulated with 50Units/ml IL-2 for 10 min at 37°C or left unstimulated. The cells were lysed immediately thereafter and the cytosolic fractions isolated as described in *Methods*. Membranes (20 μg protein), isolated from unstimulated CTLL-2 cells as described in *Methods*, were incubated with 200 μl of either the cytosol from unstimulated CTLL-2 cells (i.e. control cytosol) or IL-2 treated cells (i.e. IL-2 cytosol) for 10 min at 27°C . Other membrane preparation were incubated in 200 μl lysis buffer (i.e. for control and TPA stimulated preparation). The membranes were isolated by centrifugation and subjected to a direct PKC assay in the presence or absence of 1 μM TPA as described in *Methods*. The values are the mean $\pm$ SEM of triplicate determinations and are representative of four separate experiments.

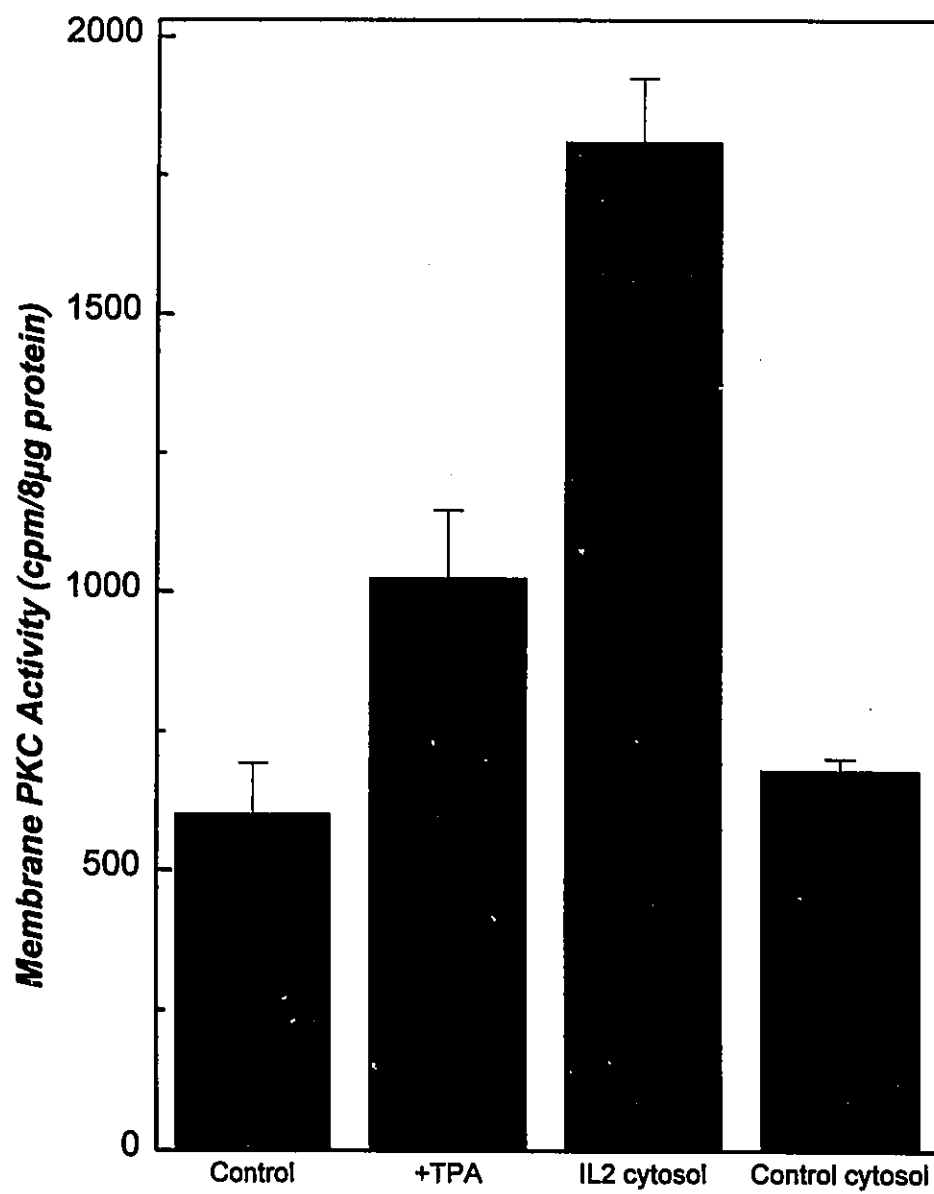


Figure 3.19 IL-2 treated cytosol increases membrane PKC activity in a dose dependent manner

Exponentially growing CTLL-2 cells (30×10^6 cell) were washed twice, resuspended in unsupplemented RPMI medium and incubated for 4 hour at 37°C. The cells were then lysed hypotonically and the membrane and cytosolic fractions isolated as described in *Methods*. Membranes were incubated with the indicated amount of IL-2 stimulated CTLL-2 cell cytosol at 27°C for 10 min (the final volume of each reaction was the same). IL-2 stimulated cytosol was generated as described in *Methods*. Membrane and cytosol fractions were separated, and membrane PKC activity was determined by the direct PKC assay as described in *Methods*. The values are the mean $\pm$ SEM of triplicate determinations.

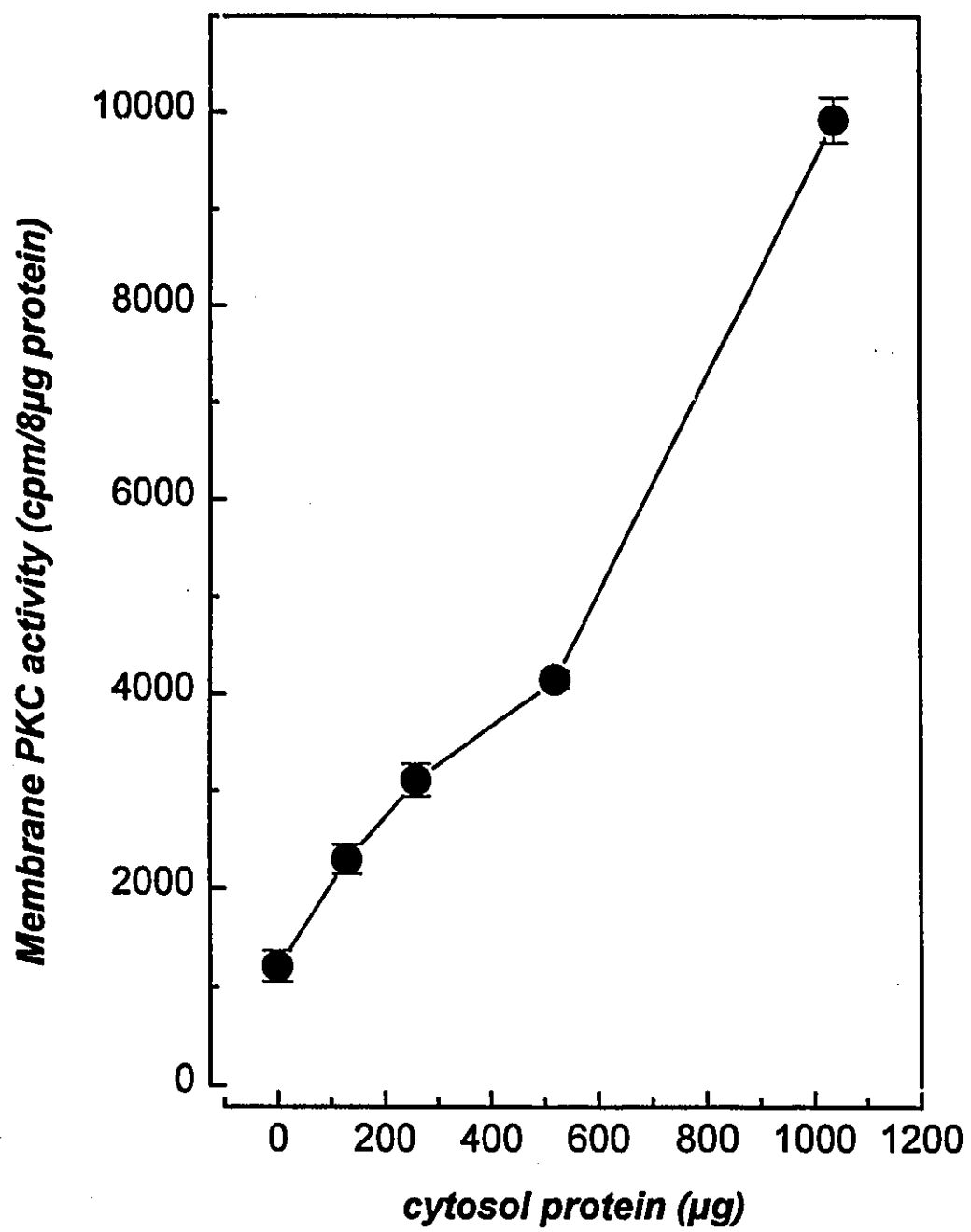


Figure 3.20 The effect of trypsin on the ability of IL-2 cytosol to stimulate membrane PKC activity

IL-2 cytosol and control cytosol were generated as described in *Methods*. 400 μ l of control and IL-2 cytosol were either left untreated, or were treated with trypsin (Sigma) at 0.05 μ g per μ g cytosol protein for 10 min at 27°C. Soybean trypsin inhibitor (Sigma: 0.2 μ g/ μ g cytosol protein) was then added to all tubes to stop any further trypsin activity. Control CTLL-2 membrane (20 μ g protein), isolated as described in the *Methods* section, was incubated with 200 μ l of either IL-2 cytosol (IL2cyto), trypsin treated IL-2 cytosol (TrypIL2cyto), control cytosol (cont.cyto) or trypsin-treated control cytosol (tryp.c.cyto) at 27°C for 10 min. Membranes and cytosol were separated and membrane PKC activity measured by the direct PKC assay as described in *Methods*. The values are the means $\pm$ SEM of triplicate determination and are representative of two separate experiments.

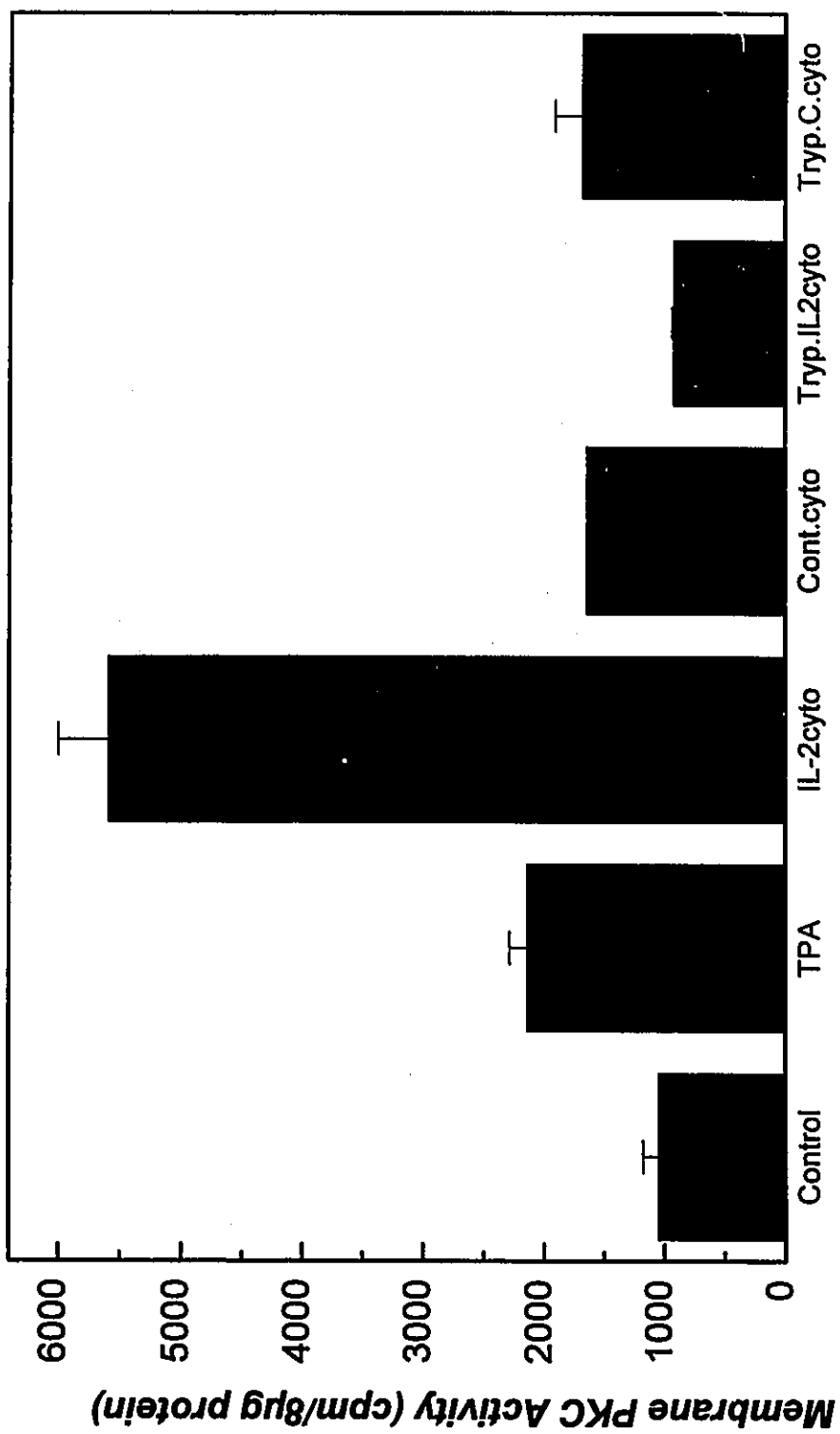


Figure 3.21 The effect of IL-2 cytosol with molecular mass less than 10 kDa on membrane PKC activity

IL-2 cytosol and control cytosol was isolated as described in *Methods*. One milliliter of these preparation were fractionated using ultrafree-MC filter units (10,000NMWL; Millipore Corp.) by centrifugation at 2000 g for 3-4 hours at 4°C. The eluant (i.e. < 10K material) was collected. Isolated membranes (20 µg protein) from IL-2/serum-deprived CTLL-2 cells, prepared as described in *Methods*; were incubated for 10 min at 27°C with 200µl of either IL-2 treated cytosol (IL2cyto), control cytosol (cont.cyto), fractioned IL2 cytosol (IL2cyto<10K) or fractioned control cytosol (c.cyto<10K). Membranes were isolated by centrifugation and subjected to a direct assay in the presence or absence of 1µM TPA as described in *Methods*. The values are the mean ± SEM of triplicate determinations and representative of two experiments.

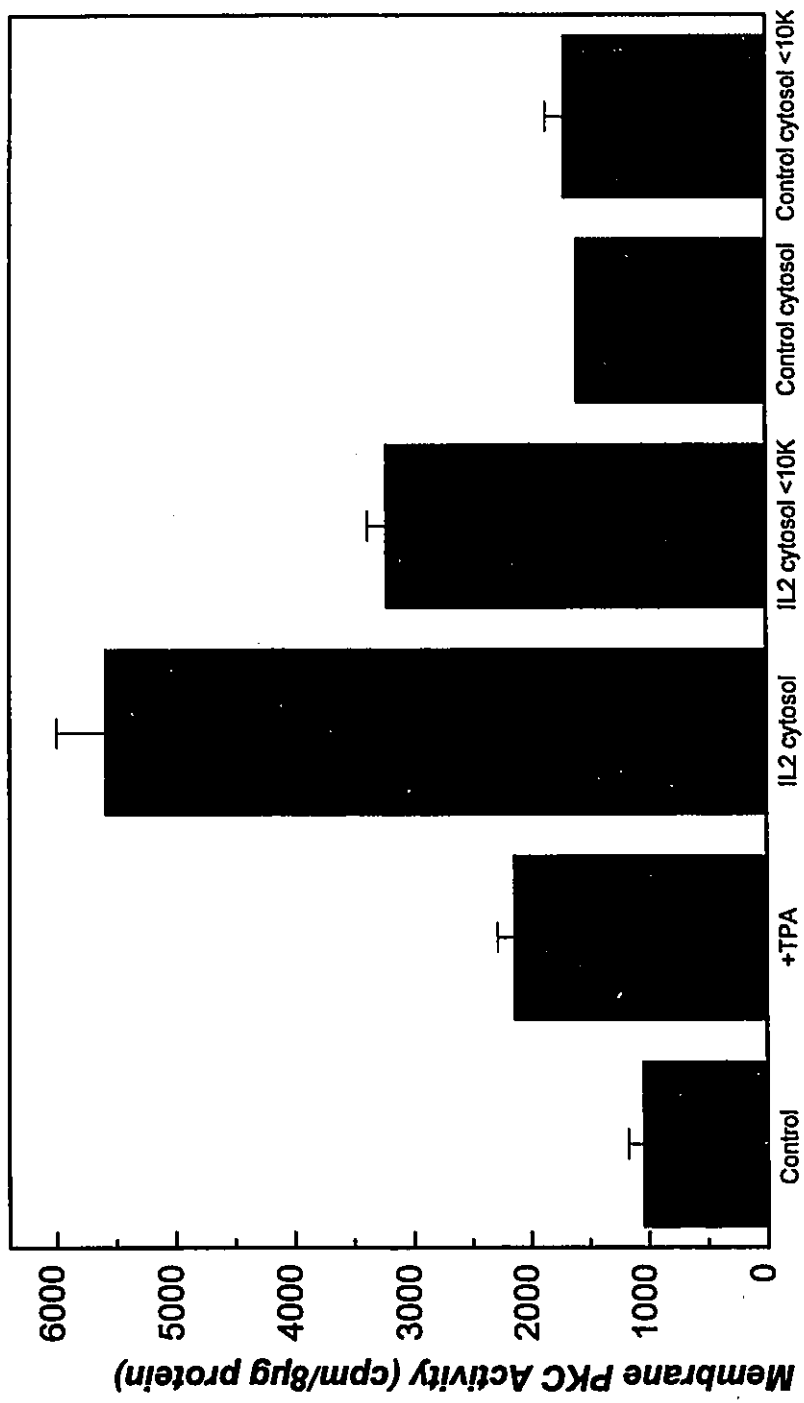


Figure 3.22 The effect of okadaic acid on the ability of IL-2 cytosol to stimulate membrane PKC activity

IL-2 cytosol was prepared as described in *Methods*. In some cases IL-2 cytosol was fractionated through a spin column (10,000NMWL) as described in the legend to Fig. 3.21. Control membrane (20 μ g), isolated from serum/IL2 deprived CTLL-2 cells as described in *Methods*, were incubated at 27°C for 10 min with 200 μ l of either lysis buffer (C), 1 μ M TPA in lysis buffer (TPA), IL-2 cytosol (IL2cyto), fractionated IL-2 cytosol (IL2cyto<10K), 1 μ M okadaic acid in lysis buffer (OKA), or IL-2 cytosol or fractionated IL-2 cytosol plus 1 μ M okadaic acid (IL2cyto+OKA, and IL2cyto<10K+OKA, respectively). After the reaction period the membrane were isolated and subjected to a direct PKC assay as described in the *Methods*. The values are the mean $\pm$ SEM of triplicate determinations and are representative of two separate experiments.

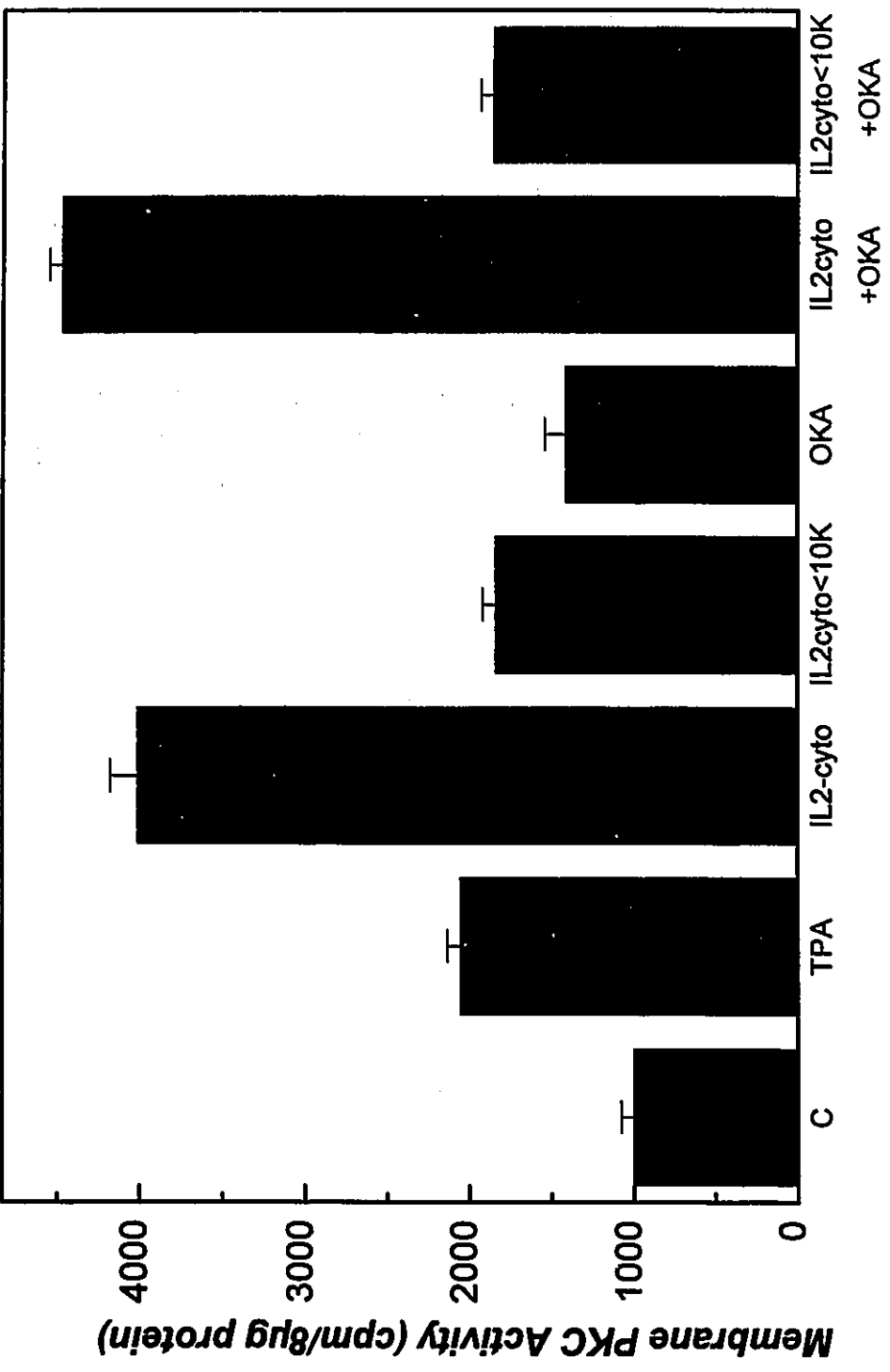


Figure 3.23 The effect of the G-protein inhibitor, pertussis toxin, on the ability of compound X to activate membrane PKC

IL-2 cytosol was prepared as described in the *Methods* section. Exponentially growing CTLL-2 cells were labeled with $^3\text{H-AA}$ ($0.05\ \mu\text{Ci/ml}$, $100\ \text{Ci/ml}$) for 24 hour at 37°C . The labeled cells were washed 3 times in unsupplemented RPMI medium, and incubated for 4 hour in the presence or absence of pertussis toxin ($75\ \text{ng/ml}$). Cells were then lysed hypotonically and the membrane and cytosolic fractions isolated as described in *Methods*. Both PTX-treated and untreated unstimulated membranes were incubated with $200\ \mu\text{l}$ IL-2 cytosol or lysis buffer (i.e. control) at 27°C for 10 min. Membrane and cytosol were separated and membrane PKC activity measured by the direct PKC assay as described in *Methods*. The values are the means $\pm$ SEM of triplicate determinations and are representative of two separate experiments.

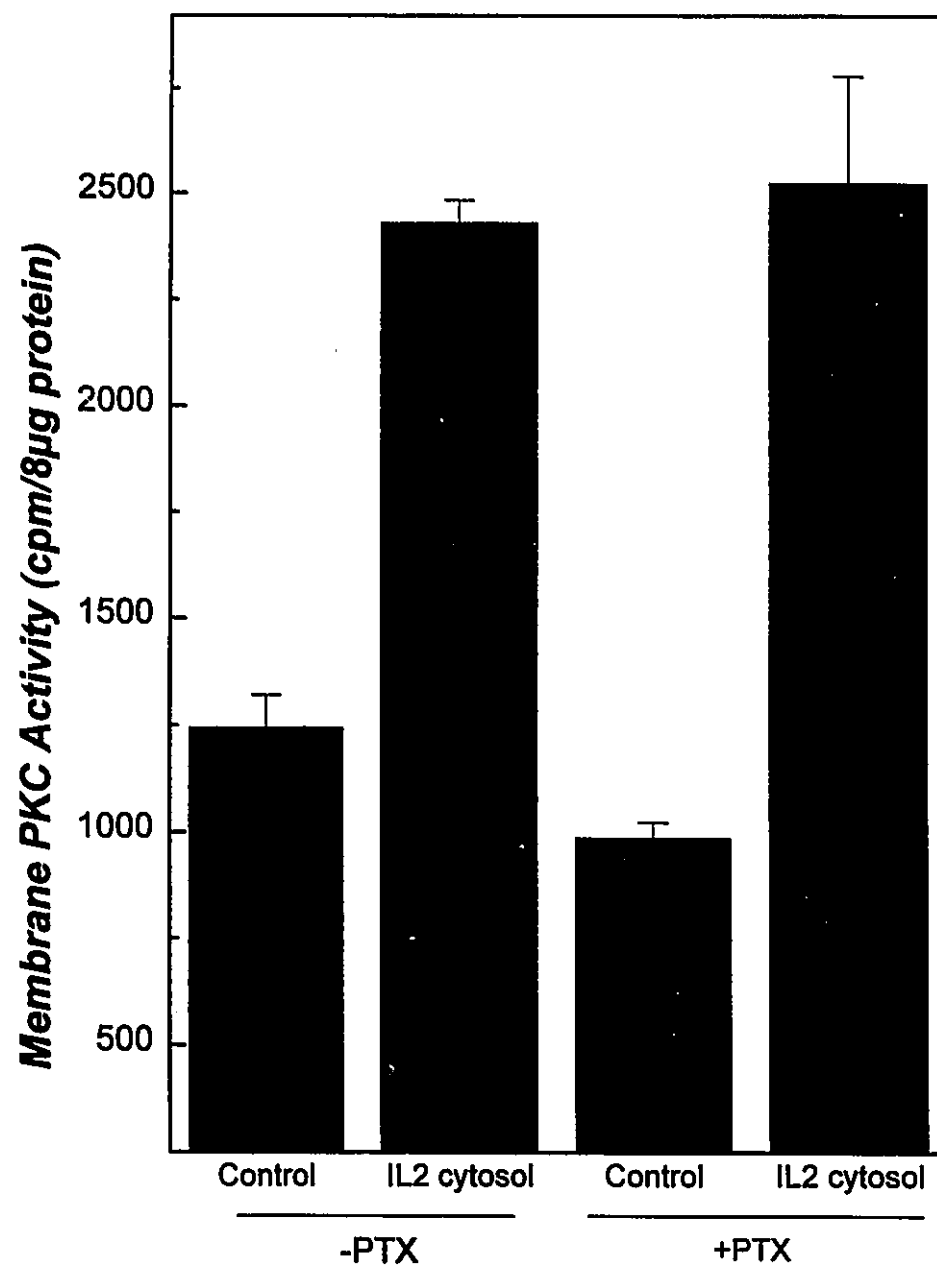
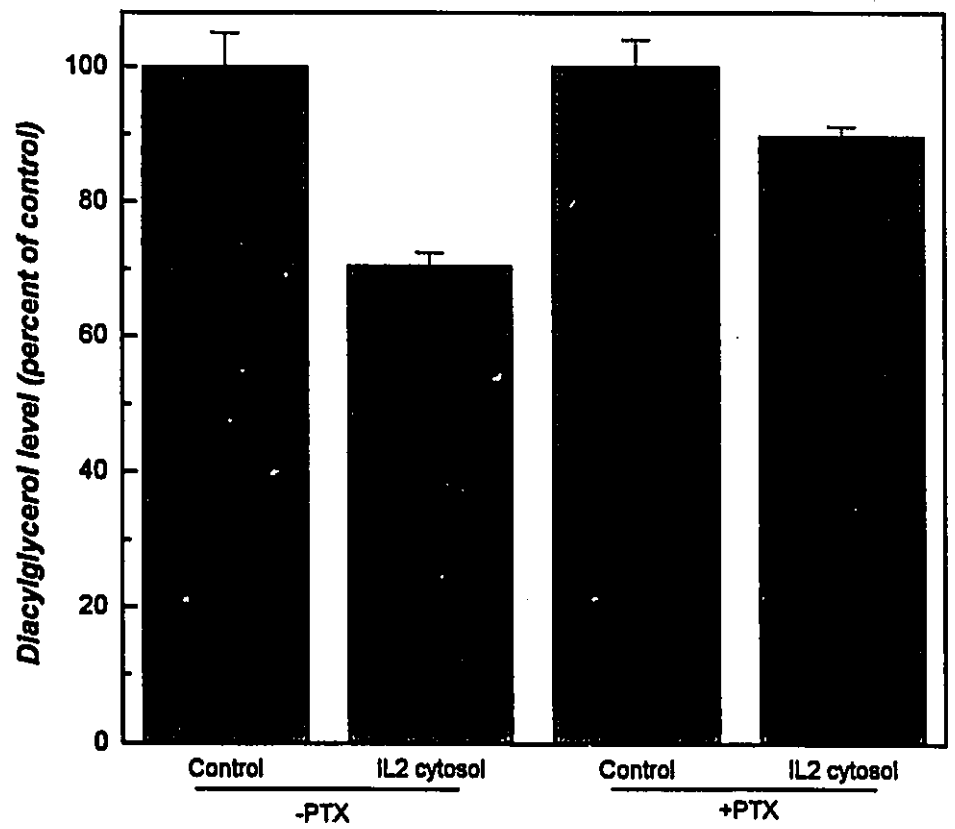
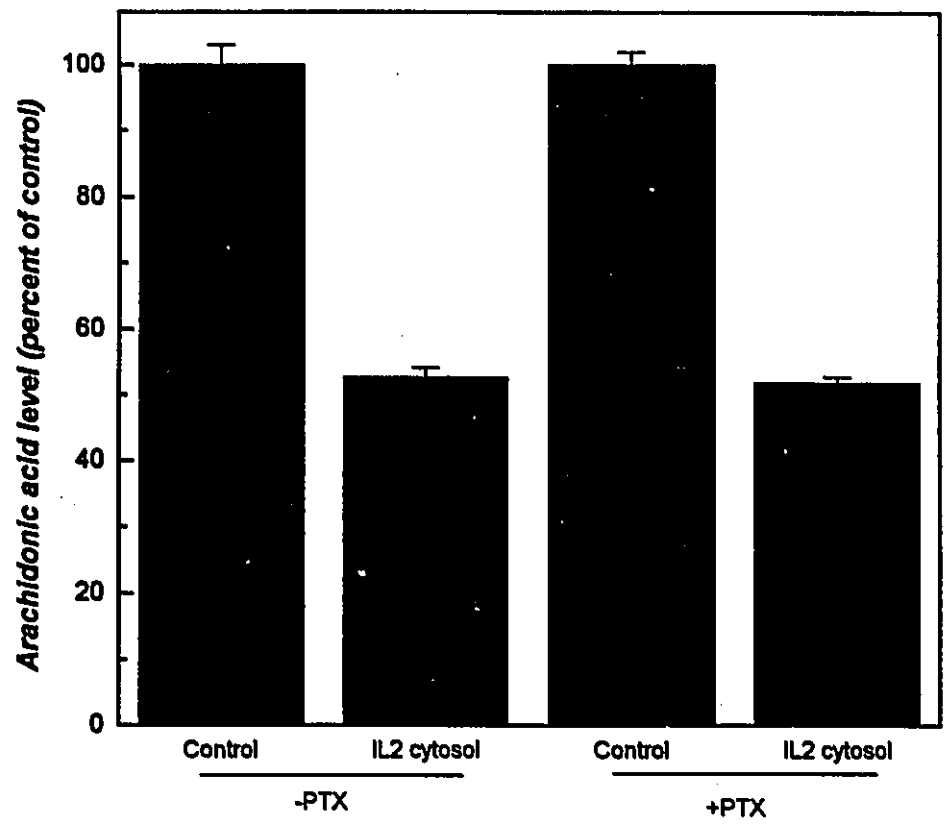


Figure 3.24 The effect of compound X on AA and DAG level

Exponentially growing CTLL-2 cells were labeled with ^3H -AA (0.05 $\mu\text{Ci/ml}$, 100 Ci/mmol) for 24 hours at 37°C. The labeled cells were washed 3 times in unsupplemented RPMI medium, and incubated for 4 hour at 37°C either in the presence or absence of pertussis toxin (75 ng/ml). Cells were lysed hypotonically and the membrane and cytosolic fractions isolated as described in *Methods*. Both PTX treated and untreated unstimulated membranes were incubated with 200 μl IL-2 treated cytosol or lysis buffer (i.e. control) at 27°C for 10 min. Membrane and cytosol were separated, total lipid was extracted from the membrane and neutral lipids was separated by TLC as described in *Methods*. The radioactivity of [^3H] DAG, or AA was counted in liquid scintillation counter. The values are the percentage of [^3H] AA or DAG relative to total radioactivity on TLCs and are representative of two separate experiments.



3.5 DISCUSSION

3.5.1 *The role of protein tyrosine kinases in PKC activation*

Although it is well established that PIP_2 breakdown generates IP_3 and DAG which induce the release of intracellular Ca^{++} and PKC activation respectively, relatively less is known about the complex signals triggering PLC activation in cells. Several studies indicate that PLC activation can occur subsequent to the activation of cellular tyrosine kinases (reviewed by Rhee and Choi 1992). With respect to IL-2, it has been demonstrated that the IL-2R β is physically associated with the nonreceptor tyrosine kinase, p56^{lck}, and that the activity of this enzyme is increased after IL-2 stimulation (Hatakeyama et al., 1991, Horak et al., 1991). Thus, a link between tyrosine kinase activity and IL-2 stimulated PKC activation is a real possibility- one that needed to be examined. As shown in Fig. 3.1, IL-2 stimulated a significant increase in tyrosine kinase activity in serum/IL-2 deprived CTLL-2 cells. However, neither herbimycin A nor genistein, two potent and specific tyrosine kinase inhibitors, were able to block IL-2 stimulated PKC activation (Fig.3.2), suggesting that tyrosine kinase activity was not likely linked to IL-2 stimulated PKC activation in these cells. At the concentrations used for PKC experiments, both inhibitors were shown to functionally obstruct tyrosine kinase activity in that cell proliferation and DNA synthesis (IL-2 stimulated processes known to be tyrosine kinase dependent [Asao et al., 1990, Mills et al., 1990]) were effectively blocked by these agents.

The data suggested a role for tyrosine kinase activity in CTLL-2 cell proliferation. The results presented in Figs.3.3 and 3.4 show that tyrosine kinases are needed for the IL-2-mediated cell proliferation. These observations were consistent with previous reports suggesting a role for tyrosine kinases in IL-2 -mediated cell proliferation (Rayhel et al., 1988, Mills et al., 1991). Nevertheless, the current studies clearly demonstrate that a *direct* link between PKC and tyrosine

kinases is unlikely in that tyrosine kinase inhibitors failed to block IL-2 stimulated PKC activation. Thus, tyrosine kinase and PKC activation likely work independently to stimulate cell proliferation and maintain cell survival respectively. Collectively, the results suggest that IL-2 stimulated tyrosine kinase activity is not linked to PKC activation, and given the fact that both herbimycin A and genistein are relatively specific, potent and broad spectrum tyrosine kinase inhibitors (Uehara et al., 1986, 1989a, 1989b, Akiyama et al., 1987), the possibility that an inhibitor-resistant tyrosine kinase activates PKC in IL-2 treated CTLL-2 cells is quite low.

3.5.2 *The role of G-proteins in IL-2 stimulated PKC activity*

It is well established that PI-PLC- $\beta 1$ is activated by members of the G_q class of pertussis toxin-insensitive G proteins (Rhee 1992). There are currently four distinct members ($G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$, $G_{\alpha 16}$) of the G_q subfamily, none of which contain a site for pertussis toxin (PTX) modification.

Taylor et al. (1991) have shown that a purified G-protein α -subunit, GPA-42, which is immunologically related to G_q is able to activate PLC $\beta 1$ but not $\gamma 1$ or $\delta 1$. PLC activation was observed only in the presence of ALF $_4$, and not with the nonhydrolyzable GTP analog GTP γ S. Exton and co-workers (1993) have also reported that $G_{\alpha q}$, $G_{\alpha 11}$ activate PLC- $\beta 1$, but not PLC- $\gamma 1$. It has been proposed (Rhee 1992) that the interaction of the heterotrimeric GDP-bound G_q with appropriate ligand-occupied receptors cause its dissociation into a $\beta\gamma$ subunit and the active GTP-bound $G_{\alpha q}$. PLC- $\beta 1$ is subsequently activated as a result of binding the GTP-bound $G_{\alpha q}$, probably via the carboxyl-terminal region of both proteins.

It has been reported that IL-2 stimulates G-protein activity in IL-2 sensitive cells (Evans et al., 1987c). However, until the current study it was not known whether this G-protein

activation led to downstream signaling events, especially those responsible for PKC activation. It was demonstrated that PTX prevented PKC activation by IL-2 while having no effect on basal PKC activity (Fig.3.5). Nevertheless, PTX treatment only partially blocked IL-2's ability to maintain cell viability (Fig. 3.6). It is tempting to speculate that both basal PKC activity and that stimulated by IL-2 are part of the mechanism by which cell viability is maintained by IL-2, and that the profound loss in cell viability in the presence of the potent PKC inhibitors BIS and AMG was due to the fact that both these PKC pools were abated.

Because of its PTX sensitivity, the G-protein(s) linked to IL-2 stimulated PKC activation in CTLL-2 cells is not likely a member of the G_q family, but most probably a G_i or G_o -like protein which, by definition, is sensitive to PTX. However, it has been reported that PTX itself can behave as a T-cell mitogen (Tamura et al., 1983). It has been speculated that this mitogenic action of PTX arises from its ability to induce the cross-linking and clustering of membrane-binding proteins which triggers intracellular signaling cascades leading to cell proliferation (Ui 1990). However, the concentrations required for this mitogenic effect (>1 mg/ml) were considerably higher than that used in this study to effect ADP ribosylation (< 100 ng/ml).

It has been reported that PTX had no effect on the stimulation of PI-PLC by thrombin and other agonists in Swiss 3T3 fibroblasts, but that PLA_2 activation in these cells was totally blocked by prior exposure to this agent (Murayama and Ui 1985). The possibility that G-proteins regulate PC hydrolysis by interacting with PC-specific PLCs and PLDs has also been reported (see details in Introduction). Therefore, it is possible that a pertussis toxin sensitive G protein may be linked to PKC activation via either PC-PLC and/or PC-PLD, or PLA_2 connected pathways.

3.5.3 Mechanisms of DAG generation by IL-2 in CTLL-2 cells

It was established that IL-2 did not stimulate PIP₂ breakdown in CTLL-2 cells since neither an increase in IP3 nor intracellular Ca²⁺ levels was detected after IL-2 stimulation (Figs. 3.10, 3.11). However, IL-2 did stimulate increases in DAG (Fig 3.7), increases that were likely responsible for PKC activation (Fig 3.8, 3.9). These results suggest that the generation of DAG by IL-2 likely arose from sources other than phosphatidylinositol turnover. This situation has been found with several other hematopoietic active agents. For example, it has been convincingly shown that TNF α activates PC-PLC in the absence of PI-PLC activation, Ca²⁺ mobilization and PC-PLD activation (Schutz et al., 1991). Similarly, interferon- α , IL-1, IL-3 and colony-stimulating factor 1 (CSF-1) have all been reported to stimulate DAG production from a PC-PLC linked pathway in the absence of PI hydrolysis (Rosoff et al., 1988, Durino et al., 1989, Imamura et al., 1990, Pfeffer et al., 1990). Since IL-2 receptors are also members of the cytokine receptor superfamily, it was not entirely surprising to discern that IL-2 likely stimulates DAG production via a PC-PLC or PC-PLD linked signalling pathway.

3.5.4 The role of arachidonic acid in IL-2 stimulated PKC activation

Over the years it has become increasingly clear that PKC activation in cells is mechanistically much more complex than initially imagined. One clear example is the realization that AA and other unsaturated long-chain fatty acids activate PKC, and that this activation is independent of phospholipids and Ca²⁺ (McPhail et al., 1984, Lester 1990, Lester et al., 1991). In the current study it was demonstrated that IL-2 not only stimulated DAG increases in CTLL-2 cells, but also increases in cellular AA (Fig. 3.12). The increase in AA probably participated in IL-2 mediated PKC activation since exogenous AA was a potent stimulator of membrane PKC activity when applied to intact cells or isolated CTLL-2 cell membranes (Fig 3.13, 3.14). The

fact that two different PLA₂ inhibitors blocked IL-2 stimulated PKC activation (Fig.3.15, 3.16) suggested that PLA₂ activity was likely responsible for the generation of AA, and consequently for the activation of PKC. The results indicate that IL-2 stimulated both DAG and AA production in CTLL-2 cells, and that both events could account for the activation of PKC effected by the cytokine. It is possible that the mechanisms by which AA and DAG stimulate membrane PKC may be quite different, and that AA may be another potent second messenger in CTLL-2 cells, one that may act in concert with DAG and IP₃ to regulate PKC activity under both physiologically and pharmacologically relevant conditions. This apparent redundancy probably reflects the importance of PKC activity in a wide range of cellular functions- cell survival being only one of many PKC-dependent processes likely occurring in CTLL-2 cells.

3.5.5 *IL-2 generates a soluble component capable of activating PKC*

The cytosolic component, factor X, generated within 10 min of IL-2 stimulation of CTLL-2 cells was able to activate inactive membrane associated PKC as well as cause PKC translocation from the cytosol to membranes (Fig. 3.18, 3.21, Table 3.1). PKC activation was observed only with IL-2 treated cytosol and not with cytosol isolated from unstimulated cells, suggesting that some factor was rapidly generated or activated in the cytosol during the process of IL-2 receptor stimulation. IL-2 cytosol was clearly able to induce the translocation of PKC from the cytosol to membranes. However, the data suggested that IL-2 cytosol was also able to activate membrane resident enzyme since increases in membrane PKC were observed using a <10kDa fraction of IL-2 cytosol that was devoid of measurable PKC activity (Fig. 3.21). These results are consistent with the growing realization that cells contain multiple pools of membrane PKC, some of which are not activated by phorbol esters, such as aPKCs (reviewed by Nishizuka 1992, 1995) and enzyme actively suppressed by endogenous membrane-associated PKC

inhibitors (Chakravarthy et al., 1995). The PKC-stimulatory activity of IL-2 cytosol was totally lost (i.e., both translocation and the activation of membrane resident enzyme [see Fig. 3.20]) when exposed to trypsin, suggesting that factor X may be a protein with molecular weight under 10,000. Since factor X was active at dilutions of cellular cytosol estimated to be greater than 100,000 (based on cytosolic volume calculations), it is most difficult to imagine that the activation of PKC arose from IL-2 induced changes in the concentrations of Ca^{2+} , ATP, GTP, DAG or any other PKC-cofactor present in the cytosolic preparation. Further studies will be needed to confirm that factor X is indeed a protein and not a PKC stimulatory lipid, or other known PKC activator of small molecular weight.

With respect to studies designed to explore possible mechanisms by which factor X stimulated PKC activity, it appeared unlikely that the cytosolic component was responsible for the generation of DAG and AA. The data clearly showed that IL-2 cytosol activated membrane PKC activity without increasing the cellular levels of either AA and DAG (Fig.3.23, 3.24). In fact, IL-2 cytosol reduced the amounts of both compounds in CTLL-2 membranes. It is possible that factor X directed the conversion of AA and DAG to eicosanoids and phosphatidic acid or other compounds capable of stimulating inactive membrane PKC. With respect to this later point, it is interesting to note that the AA derivative, lipoxin A, has been reported to activate PKC (Piomelli 1993). This study has also shown that pertussis toxin-sensitive G-proteins are likely involved in IL-2 stimulated PKC activation (Fig.3.5), yet pertussis toxin did not block factor X's ability to activate PKC *in vitro* (Fig.3. 23). Thus, it is unlikely that factor X activated PKC via steps upstream of the pertussis toxin-sensitive G-protein in the signalling pathway. Thus, the mechanism responsible for factor X stimulated PKC activation is not clear at present. It is possible that factor X directly stimulates PKC on membranes, but this possibility is most

difficult to determine experimentally. It is also possible that the factor stimulated PKC by pathways independent of a PTX-sensitive G protein as well as DAG and AA production in CTLL-2 cells. These and other possibilities will need to be examined in future attempts to determine what physiological role, if any, factor X plays in IL-2 signalling and IL-2 mediated PKC activation in CTLL-2 cells.

3.6 CONCLUSION

The studies embodied in this chapter have been able to cast significant light on the mechanism(s) responsible for the activation of membrane PKC in CTLL-2 cells by IL-2. In general, it can be stated that there appears to be a set of complex and interactive mechanisms responsible for this process in CTLL-2 cells, ranging from well established membrane-linked signalling steps to a surprising and likely novel cytoplasmic component. The specific conclusions reached were:

- 3.6.1 While protein tyrosine kinases are activated after IL-2 stimulation, they are not likely linked to PKC activation.
- 3.6.2 A pertussis toxin sensitive G-protein(s) is likely involved in IL-2 stimulated PKC activation.
- 3.6.3 DAG production is likely at least partially responsible for PKC activation, but it is probably produced from sources other than PIP_2 hydrolysis.
- 3.6.4. AA production from PLA_2 is also likely at least partially responsible for IL-2 stimulated PKC activity.
- 3.6.5 A cytosolic component, designated factor X, is able to activate inactive-membrane associated-PKC as well as to cause PKC translocation from cytosol to membranes. Factor X is likely a protein with a molecular mass less than 10kDa, and is not a tyrosine kinase or phosphatase. The mechanism(s) of PKC activation by factor X is unlikely through the generation of AA, DAG, or the activation of pertussis toxin sensitive G-protein. It might act directly on PKC or through other unknown signaling pathways.

GENERAL CONCLUSIONS

IL-2, one of the most important lymphokines secreted by activated helper T-cells, plays a vital role in the generation and regulation of the immune response (Taniguchi et al., 1986, Smith 1988a). IL-2 has pleiotropic effects on T, B, and natural killer (NK) lymphocytes, as well as nonlymphoid cells. Numerous biological activities have been ascribed to IL-2, including the stimulation of T-cell proliferation and survival (Morgan 1976, Walker et al., 1993), the generation of lymphokine-activated killer cells (Lotze et al., 1981, Grimm et al., 1983), the augmentation of natural killer cell activity (Henney et al., 1981), the induction of cytotoxic T-cell reactivity (Gillis et al., 1979), and the stimulation of B cell growth and differentiation (Kishi et al., 1985, Mond et al., 1985). However, in contrast to the wealth of information regarding the biological actions of IL-2, the molecular mechanisms involved in the IL-2-stimulated signal transduction remains elusive, in particular, the physiological role of protein kinase C and the mechanisms of PKC activation. In this thesis I have studied the role of PKC in IL-2 signal transduction and the mechanisms responsible for its activation in the cytotoxic T-cell line, CTLL-2.

In Chapter 1 it was established that CTLL-2 cells constitute a relevant model to study the role of PKC in IL-2 signaling. It was initially demonstrated that IL-2 is critical for CTLL-2 cell survival and for the cytolytic activity associated with this cytotoxic T-cell line. It was also shown that changes in cellular function and behavior occurring in response to the withdrawal and/or addition of IL-2 took place within experimentally acceptable timeframes. It was then shown that CTLL-2 cells possess measurable levels of PKC activity, and having done this, the distribution of the various PKC isozymes was examined. A pivotal finding was the determination

that PKC activity, measured by the direct assay, was indeed responsive to IL-2 withdrawal and addition. This step permitted an in depth analysis of the mechanism of IL-2 initiated PKC activation in these cells which formed the basis of the studies presented in subsequent chapters.

In Chapter 2, It was determined that PKC is not translocated from the cytosol to membrane after IL-2 stimulation. This finding was in agreement with the previous study of Mukaida and co-workers who also were unable to detect PKC translocation after IL-2 stimulation (Mukaida et al., 1988). However, there have been an increasing number of reports over the past few years indicating that PKC activity can be stimulated in cells in the absence of detectable translocation. In most cases this activation has been shown to arise from the activation of inactive membrane PKC already resident on membranes (Chakravarthy et al., 1991, 1992, 1994, 1995, Durkin et al., 1992, Jouishomme et al., 1992) or due to hyper-stimulation of the enzyme of some kind chemical modification (Pelech et al., 1990b). In the case of CTLL-2 cells, I determined despite the absence of detectable translocation, PKC was nevertheless activated in these cells after IL-2 stimulation. This finding was made possible by the use of the "direct" PKC assay (Chakravarthy et al., 1991), which enables one to distinguish between active and inactive PKC associated with cell membranes. As discussed in the General Introduction (Section I-4.6), this direct PKC assay measures enzyme activity directly in its native membrane-associated state without prior detergent solubilization and reconstitution of the enzyme with the exogenous PKC activating co-factors, PS, TPA, and Ca^{2+} . As demonstrated in Chapter 2, a significant proportion of membrane PKC in CTLL-2 cells exists in an inactive, but primed state which can be readily activated by IL-2. It was shown that the cellular levels of this pool of inactive membrane PKC increased after withdrawing IL-2 from the media of exponentially proliferating CTLL-2 cells, and that it underwent rapid reactivation when IL-2 were restored. This

reversible activation of a membrane-resident pool of inactive PKC was similar to that reported previously in other cell types stimulated by a variety of mitogens and other stimulatory agents (Chakravarthy et al., 1992, 1994; Durkin et al., 1993,1995).

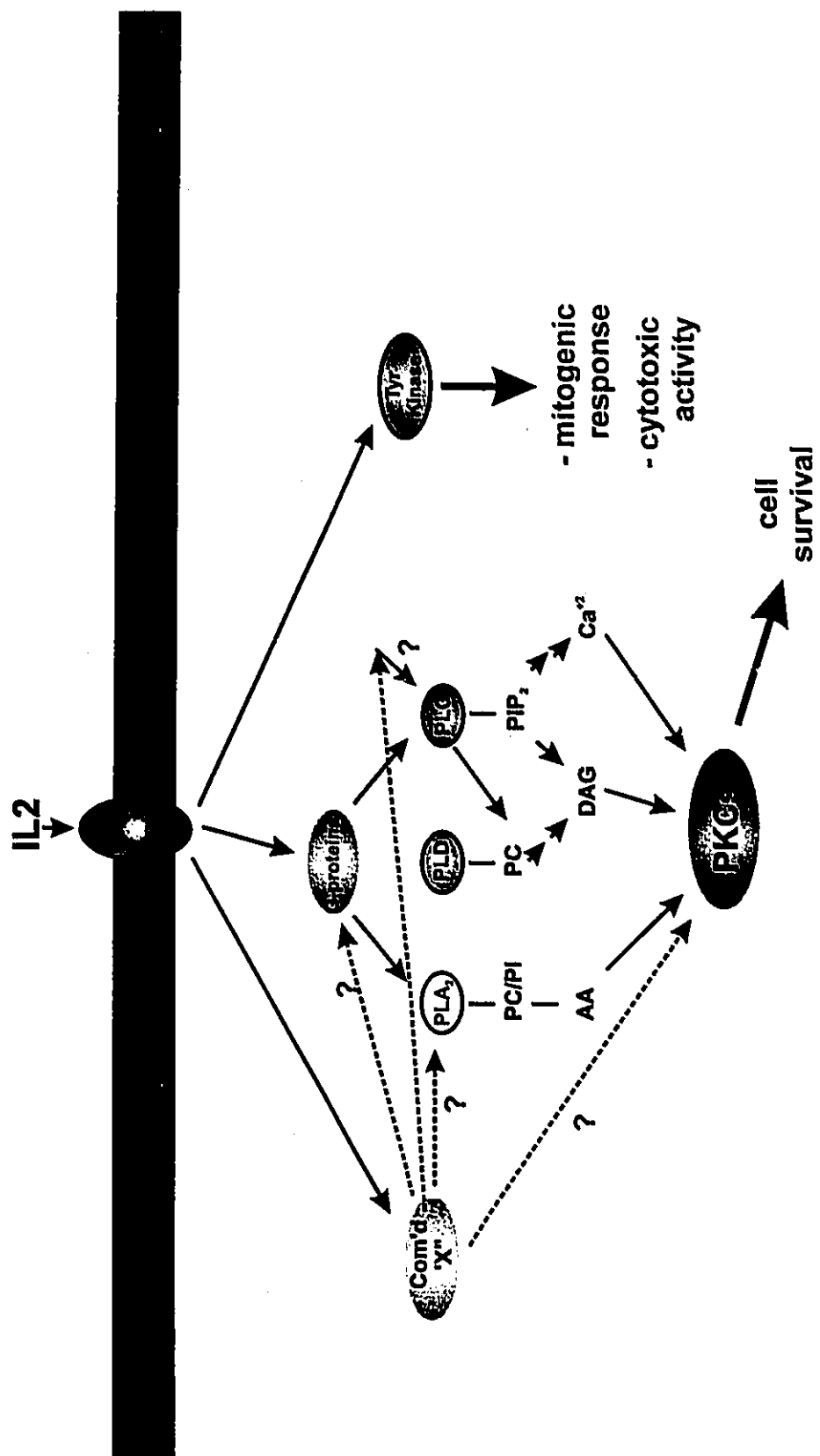
Substantial data was presented in Chapter 2 indicating that IL-2's ability to maintain cell viability was dependent on PKC activity. Two specific PKC inhibitors, BIS and AMG were able to block IL-2's ability to suppress CTLL-2 cell death as measured by viable cell numbers. Moreover, DNA laddering experiments demonstrated that PKC activity was necessary for the IL-2 induced suppression of apoptosis, since blocking PKC activity at the time of restoring IL-2 to IL-2/serum deprived cultures effectively reversed the cytokine-mediated suppression of DNA fragmentation. While the data clearly suggested a role of PKC in the mechanism by which IL-2 prevented apoptosis, it was more difficult to definitively conclude that it was the transient increase in inactive membrane PKC within the first few minutes of IL-2 addition that was the critical PKC activity involved in the suppression of cell death. However, one important piece of data was wholly consistent with the view that PKC activation was crucial to this process. The results of Fig.2.8 indicated that inhibiting PKC before IL-2 stimulation, but not 2 hours after the addition of IL-2, blocked the ability of the cytokine to rescue CTLL-2. From this experiment it was apparent that an early and transient requirement of PKC activity was indeed needed for the IL-2 induced suppression of apoptosis, a finding which supports the contention that the transient activation of PKC by IL-2 was an important step in this process. Collectively, the results of Chapter 2 suggested that the activation of inactive membrane-associated PKC is linked to important cellular functions in CTLL-2 cells, notably the ability of IL-2 to maintain cell survival. On the other hand, PKC is unlikely involved in other IL-2-mediated processes, such as cytotoxic activity.

In Chapter 3, the mechanism(s) of IL-2 induced PKC activation in CTLL-2 cells was investigated. It was shown that while tyrosine kinases were activated after IL-2 stimulation, it was unlikely that they were linked to the activation of PKC. The data did indicate that a pertussis toxin sensitive-G-protein was likely involved in PKC activation since PTX blocked IL-2 stimulated PKC activation. This result was consistent with, and extended, the previous finding of Evans (1987c) that IL-2 stimulates G-protein activity in IL-2 dependent cells. The observation that DAG, but not IP₃ and intracellular calcium, increased after IL-2 stimulation, indicated that DAG was unlikely being generated from the breakdown of PI. It was probable that the DAG produced by IL-2 in CTLL-2 cells arose via the PC-PLC or PC-PLD pathways. This conclusion is consistent with a growing list of reports indicating that cytokines can generate physiologically relevant DAG by routes independent of the PI-PLC pathway (Rosoff et al., 1988, Durino et al., 1989, Imamura et al., 1990, Pfeffer et al., Schutz et al., 1991). It was also shown that the increase in DAG by IL-2 was likely responsible for the ensuing activation of inactive membrane PKC since exogenously applied DAG stimulated similar increases in PKC in both intact cells and in isolated membranes. IL-2 also stimulated the levels of AA in CTLL-2 cells. This increase likely resulted from increased PLA₂ activity since PLA₂ inhibitors effectively blocked the IL-2 stimulated activation of PKC. As was the case with DAG, the addition of exogenous AA to intact cells and to isolated membranes caused a rapid increase in membrane PKC activity, suggesting that the endogenous production of AA by IL-2R stimulation was likely linked to PKC activation in CTLL-2 cells. These data are consistent with previous reports that AA is able to activate PKC in vitro and in vivo by a Ca²⁺ and phospholipid independent mechanism (McPhail et al., 1984), and that AA can act in collaboration with DAG to stimulate PKC activity (Lester 1990, Lester et al., 1991). These results suggest that the PLA₂ pathway is likely part of the

mechanism responsible for IL-2 stimulated PKC activation, with AA itself behaving as a second messenger with properties similar to but distinguishable from DAG. Future studies will be needed to determine whether the PLA₂ pathway stimulated by IL-2 is coupled to G-protein activation and to determine the exact role of G-proteins play in the PLA₂ and /or PC-PLC/PLD pathways in IL-2 stimulated cells.

The possibility of a cytoplasmic factor rapidly generated or activated by the stimulation of the IL-2 receptor(s) in CTLL-2 cells added an interesting and novel aspect to the mechanistic study of PKC activation in these cells. This compound, designated factor X, is likely a protein(s) with a molecular weight of less than 10 kDa. Much more work will be necessary before a proper characterization of the factor can be made. Its role in the pathway leading to PKC activation after IL-2R stimulation is unclear, although it appears not to act upstream or to be linked to the G-protein requirement or the generation of AA and DAG. Understanding the exact nature of this factor and the role it plays in PKC activation by IL-2 and perhaps other cytokines would be most illuminating, and would constitute an interesting follow-up project.

In the following illustration I propose a scheme summarizing the findings made in Chapter 3 with respect to IL-2 stimulated activation of PKC in CTLL-2 cells. It is postulated that the signals transduced from the IL-2R to either PLA₂, PC-PLC, PC-PLD is mediated by a pertussis toxin sensitive G-protein. PC-PLC and /or PC-PLD activation is seen as being responsible for DAG generation while PLA₂ activation causes an increase in AA production. Both AA and DAG are responsible for the overall increase in PKC activation possibly through different mechanisms, since AA activates PKC in the absence of Ca²⁺ and phospholipid. It is reasonable to propose that DAG and AA activate different sub-pools of PKC or that they stimulate PKC in an isoform-specific manner. The placement of factor X in the scheme is not



clear at present. It is possible that factor X activates PKC directly or through some mechanism unrelated to the above.

In summary, during the course of thesis study I have observed that IL-2 stimulates PKC in CTLL-2 cells not through the translocation of cytosolic enzyme to membrane, but via activation of inactive membrane associated PKC. This PKC activation is involved in IL-2's ability to suppress apoptosis in these cells. The mechanism(s) of PKC activation by IL-2 is substantially more complicated than we initially thought, and it likely involving multiple second messengers, including G-proteins, DAG, AA and factor X.

REFERENCES

- Abou-Samra, A.-B., Jueppner, H., Westerberg, D., Potts, J.T., Jr., Segre, G.V. (1989) Parathyroid hormone causes translocation of protein kinase C from cytosol to membrane in rat osteosarcoma cells. *Endocrinology* 124, 1107-1113.
- Aderem, A. (1992) The MARCKS brothers: a family of protein kinase C substrates. *Cell*, 71, 713-16.
- Akiyama, T., Ishida, J., Nakagawa, S., Ogewara, H., Watanabe, S., Itoh, N., Shibuye, M., Fukami, Y. (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* 262, 5592-95.
- Anderson, W.B., Estival, A., Tapiovaara, H., Gopalakrishna, R (1985) Altered subcellular distribution of protein kinase C (a phorbol ester receptor). Possible role in tumor promotion and the regulation of cell growth: relationship to changes in adenylate cyclase activity. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* 19, 287.
- Anthes J.C., Eckel, S., Siegel, M.I., Egan, R.W., Billah, M.M. (1989) Phospholipase D in homogenates from HL-60 granulocytes: implications of calcium and G-protein control. *Biochem. Biophys. Res. Commun.* 163,657.
- Arima, N., Kamio, M., Imada, K., Hori, T., Hattori, T., Tsudo, M., Okuma, M., Uchiyama, T. (1992) Pseudo-high affinity interleukin 2 (IL-2) receptor lacks the third component that is essential for functional IL-2 binding and signaling. *J. Exp. Med.* 176, 1265-72.
- Asao, H., Takeshita, T., Nakamura, M., Nagata, K., and Sugamura, K. (1990) Interleukin 2 (IL-2)-induced tyrosine phosphorylation of IL-2 receptor p75. *J. Exp. Med.* 171, 637-44.
- Asao, H., Takeshita, T., Ishii, N., Kumaki, S., Nakamura, M., and Sugamura, K. (1993) Reconstitution of functional interleukin 2 receptor complexes on fibroblastoid cells: involvement of the cytoplasmic domain of the γ chain in two distinct signaling pathways. *Proc. Natl. Acad. Sci. USA.* 90, 4127-31.
- Asaoka, Y., Nakamura, S., Yoshida, K. and Nishizuka, Y. (1992a) Protein kinase C, calcium and phospholipid degradation. *TIBS* 17, 414.
- Asaoka, Y., Yoshida, K., Oka, M. Shinomura, T., Hishima, H., Matsushima, S. Nishizuka, Y. (1992b) The signal-induced phospholipid degradation cascade and protein kinase C activation. In: *Interactions among cell signaling systems*, Eds.: Wiley, Chichester (Ciba Foundation symposium 164)p50-65.
- Ashendel, C.L. (1985) The phorbol ester receptor: a phospholipid-regulated protein kinase. *Biochim. Biophys. Acta* 822, 219.

- Bartoli, F. Lin, H-K, Ghomashchi, F., Gelb, M.H., Jain, M.K., Apitz-Castro, R. (1994) Tight binding inhibitors of 85-kDa phospholipase A₂ but not 14-kDa phospholipase A₂ inhibit release of free arachidonate in thrombin-stimulated human platelets. *J. Biol. Chem.* **269**, 15625-30.
- Bazan, J.F. (1990a) Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. USA* **87**, 6934-38.
- Bazan, J.F. (1990b) Hematopoietic receptors and helical cytokines. *Immunol. Today* **11**, 350.
- Bazzi, M.D. and Nelsestuen, G.L. (1987) Role of substrate in determining the phospholipid specificity of protein kinase C activation. *Biochem.* **26**, 5002-8.
- Bazzi, M.D. and Nelsestuen, G.L. (1988) Constitutive activity of membrane-inserted protein kinase C. *Biochem. Biophys. Res. Comm.* **152**, 336-43.
- Beadling, C., Guschin, D., Witthuhn, B.A., Zieniecki, A., Ihle, J.N., Kerr, I.M., Cantrell, D.A. (1994) Activation of JAK kinases and STAT proteins by interleukine-2 and interferon alpha, but not the T cell antigen receptor in human T lymphocytes. *EMBO J.* **13**, 5605-15.
- Beckner, S.K., and Farrar, W.L. (1986) Interleukin 2 modulation of adenylyate cyclase. Potential role of protein kinase C. *J. Biol. Chem.* **261**, 3043.
- Beckner, S.K. and Farrar, W. L. (1987) Inhibition of adenylyate cyclase by IL-2 in human T lymphocytes is mediated by protein kinase C. *Biochem. Biophys. Res. Commun.* **145**, 176.
- Bell, R.L., Kennerley, D.A., Stanford, N., Jagerus, P.W. (1979) Diglyceride lipase: a pathway for arachidonate release from human platelets. *Proc. Natl. Acad. Sci. USA* **76**, 3238-41.
- Bell, R.M. (1986) Protein kinase C activation by diacylglycerol second messengers. *Cell* **45**, 631-32.
- Bell, R.M. and Burns, D.J. (1991) Lipid activation of protein kinase C. *J. Biol. Chem.* **266**, 4661.
- Ben-Avi, P., and Liscovitch, M. (1989) Phospholipase D activation by the mitogens platelet-derived growth factor and 12-o-tetradecanoyl phorbol 13-acetate in NIH 3T3 cells. *FEBS. Lett* **259**, 64-6.
- Benevento, E.N., Merrill, J.E. (1986) Stimulation of oligodendroglial proliferation and maturation by interleukin-2. *Nature* **321**, 610-13.

Benjamini E, and Leskowitz S. (1988) In: Immunology: a short course, (Alan R. Liss, Inc., New York) pp15-291.

Berridge, M., Downes, P., Hanley, M.R. (1982) Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem. J.* 206, 587-95.

Berridge, M., and Irvine, R.F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* 312, 315-21.

Berridge, M. (1987) Inositol lipids and cell proliferation. *Biochim. Biophys. Acta* 907, 33-45.

Berry, N., Ase, K., Kishimoto, A. and Nishizuka, Y. (1990a) Activation of resting human T cells requires prolonged stimulation of protein kinase C. *Proc. Natl. Acad. Sci. USA* 87, 2294-98.

Berry, N. and Nishizuka, Y. (1990b) Protein kinase C and T cell activation. *Eur. J. Biochem.* 189, 205-14.

Besterman, J.M., Pollenz, R.S., Booker E.L. Jr. (1986) Diacylglycerol-induced translocation of diacylglycerol kinase: use of affinity-purified enzyme in a reconstitution system. *Proc. Natl. Acad. Sci. USA* 83, 9378-82.

Bierman, A.J., Koenderman, L., Tool, A.J., De Laat, S.W. (1990) Epidermal growth factor and bombesin differ strikingly in the induction of early responses in Swiss 3T3 cells. *J. Cell. Physiol.* 142, 441-8.

Billah, M.M., Lapetina, E.G., Cuatrecasas, P.J. (1981) Phospholipase A₂ activity specific for phosphatidic acid: a possible mechanism for the production of arachidonic acid in platelets. *J. Biol. Chem.* 256, 5399-5403.

Billiah, M.M., Pai, J.-K., Mullmann, T.J., Egan, R.W., Siegel, M.I. (1989) Regulation of phospholipase D in HL-60 granulocytes: Activation by phorbol ester, diglyceride, and calcium ionophore via protein kinase C-independent mechanisms. *J. Biol. Chem.* 264, 9069-76.

Billah, M.M., Anthes, J.C. (1990) The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.* 269, 281-91.

Blackmen, M.A., Tigges, M.A., Minie, M.E., Koshland, M.E. (1986) A model system for peptide hormone action in differentiation: interleukin 2 induces a B lymphoma to transcribe the J chain gene. *Cell* 47, 609-17.

Blackshear P.J. (1993) The MARCKS family of cellular protein kinase C substrates. *J. Biol. Chem.* 268, 1501-4.

- Bleasdale, J.E., Bundy, G.L., Bunting, S., Fitzpatrick, F.A., Huff, R.M., Sun, F.F., Pike, J.E. (1989) Inhibition of phospholipase C dependent processes by U-73122. IN: *Advances in Prostaglandin, Thromboxane, and Leukotriene Research*, Eds.: Samuelsson, B., Wong, P.Y.-K., and Sun, F.F., (Raveb Press, Ltd., New York), pp590-593.
- Bocckino, S.P., Blackmore, P.F., Wilson, P.B., Exton, J.H. (1987) Phosphatidate accumulation in hormone-treated hepatocytes via a phospholipase D mechanism. *J. Biol. Chem.* **262**, 15309-15.
- Boni, L.T., and Rando, R.R. (1985) The nature of protein kinase C activation by physically defined phospholipid vesicles and diacylglycerols. *J. Biol. Chem.* **260**, 10819-24.
- Bonser, R.W., Thompson, N.T., Randall, R.W., Garland, L.G. (1989) Phospholipase D activation is functionally linked to superoxide generation in the human neutrophil. *Biochem. J.* **264**, 617-20.
- Brandhuber, B.J., Boone, T., Kenney, W.C., McKay, D.B. (1987) Three-dimensional structure of interleukin-2. *Science* **238**, 1707-9.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-53.
- Brooks, S. F., Herget, T., Erusalimsky, J.D., and Rozengurt, E. (1991) Protein kinase C activation potently down-regulates the expression of its major substrate, 80K, in Swiss 3T3 cells. *EMBO J.* **10**, 2497-05.
- Brooks, G. (1994) The role of 80K/MARCKS, a specific substrate of protein kinase C, in cell growth and tumor progression. *Pigment Cell Res.* **7**, 451-7.
- Burch, R.M. (1989) G protein regulation of phospholipase A₂. *Molec. Neur.* **3**, 155-71.
- Burch, R.M. (1990) Regulation of phospholipase A₂ by G proteins. In: *G proteins and calcium signaling*, Eds.: Nacache, P.H. (CRC press, Inc., Boca Raton), pp47-58.
- Cano, E., Munoz-Fernandez, M.A., Fresno, M. (1992) Regulation of interleukin-2 responses by phosphatidic acid. *Eur. J. Immunol.* **22**, 1883-90.
- Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., Soltoff, S. (1991) Oncogenes and signal transduction. *Cell* **64**, 281-90.
- Cantrell, D., Davies, A.A., Londei, M., Feldman, M. Crumpton, M.J. (1987) Association of phosphorylation of the T3 antigen with immune activation of T lymphocytes. *Nature* **325**, 540-43.

- Carroll, M.P., Clark-Lewis, I., Rapp, U.R., May, W.S. (1990) Interleukin-3 and granulocyte-macrophage colony-stimulating factor mediate rapid phosphorylation and activation of cytosolic c-raf. *J. Biol. Chem.* 265, 19812-16.
- Casey, P.J., Gilman, A.G. (1988) G protein involvement in receptor-effector coupling. *J. Biol. Chem.* 263, 2577-80.
- Cass, W.A., Larson, G., Fitzpatrick, F.A., Zahniser, N.R. (1991) Inhibitors of arachidonic acid metabolism: effects on rat striatal dopamine release and uptake. *J. Pharma. Exp. Ther.* 257, 990-6.
- Chaikin, E., Ziltener, H.J. and Razin, E. (1990) Protein kinase C plays an inhibitory role in interleukin 3- and interleukine 4-mediated mast cell proliferation. *J. Biol. Chem.* 265, 22109-22116.
- Chakravarthy, B.R. (1985) In Turnover of fatty acyl chains in the phospholipids of neuroblastoma cells and cellular membranes. Ph.D. thesis, (Dalhousie University) p1-150.
- Chakravarthy, B., Bussey, A., Whitfield, J.F., Sikorska, M., Williams, R.E. and Durkin, J.P. (1991) The direct measurement of protein kinase C in isolated membranes using selective peptide substrate. *Anal. Biochem.* 196, 144-50.
- Chakravarthy, B.R., Tremblay, R., Macdonald, P., Krsmanovic, V., Whitfield, J.F., and Durkin, J.P. (1992) The activation of inactive membrane-associated protein kinase C is associated with DMSO-induced erythroleukemia cell differentiation. *Biochimica. Biophysica Acta*, 1136, 83-90.
- Chakravarthy, B.R., Whitfield, J.F., Durkin, J.P. (1994) Inactive membrane protein kinase Cs: a possible target for receptor signaling. *Biochemical J.* 304, 809-16.
- Chakravarthy, B.R., Wong, J., Durkin, J.P. (1995) Evidence that the modulation of membrane-associated protein kinase C activity by an endogenous inhibitor plays a role in NIE-115 murine neuroblastoma cell differentiation. *J. Neurochem.* 65, 1569-79.
- Chalifour, R.J., and Kanfer, J.N. (1980) Microsomal phospholipase C of rat brain and lung tissue. *Biochem. Biophys. Res. Commun.* 96, 742-7.
- Chalifour, R.J., and Kanfer, J.N. (1983) The presence of phospholipase C in rat central nervous system axolemma. *J. Neurochem.* 40, 1189-91.
- Chapline, C., Ramsay, K., Klauck, T., and Jaken, S. (1993) Interaction cloning of protein kinase C substrates. *J. Biol. Chem.* 268, 6858-61.
- Chen, K.-H., Peng, Z.-G., Lavu, S., Kung, H.-F. (1989) Molecular cloning and sequence analysis of two distinct types of *Xenopus laevis* protein kinase C. *Second messengers and phosphoproteins* 12, 251-6.

- Clemens, M.J., Trayner, I., Menaya, J. (1992) The role of protein kinase C isoenzymes in the regulation of cell proliferation and differentiation. *J. Cell Sci.* 103, 881-8.
- Cleveland, J.L., Rapp, U.R., Farrer, W.L. (1987) Role of c-myc and other genes in interleukin 2 regulated CT6 T lymphocytes and their malignant variants. *J. Immunol.* 138, 3495-3501.
- Cohen, J.J., Duke, R.C., Chervenak, R., Sellins, K.S., Olson, L.K. (1985) DNA fragmentation in target of CTL: an example of programmed cell death in the immune system. *Adv. Exp. Med. Biol.* 184, 493-8.
- Cohen, F.E., Kosen, P.A., Kuntz, I.D., Epstein, L.B., Ciardelli, T.L., and Smith, K.A. (1986) Structure-activity studies of interleukin-2. *Science* 234, 349-52.
- Cohen, J.J. and Duke, R.C. (1992) Apoptosis and programmed cell death in immunity. *Annu. Rev. Immunol.* 10, 267-93.
- Colamonici, O.R., Neckers, L.M., Rosolen, A. (1990) Putative γ -subunit of the IL-2 receptor is detected in low, intermediate, and high affinity IL-2 receptor-bearing cells. *J. Immunol.* 145, 155-60.
- Conscience, J.F., Verrier, B., Martin, G. (1986) Interleukin-3-dependent expression of the c-myc and c-fos proto-oncogenes in hemopoietic cell lines. *EMBO J.* 5, 317-24.
- Cook, S.J., and Wakelam, M.J.O. (1992) Phospholipase C and D in mitogenic signal transduction. *Rev. Physiol. Biochem. Pharmacol.*, 119, 13-45.
- Cosman, D., Lyman, S.D., Idzerda, R.L., Beckmann, M.P., Park, L.S., Goodwin, R.G., March, C.J. (1990) A new cytokine receptor superfamily. *TIBS* 15, 265-70.
- Cotter, T.G., Lennon, S.V., Martin, S.J. (1990) Apoptosis: Programmed cell death. *J. Biomed Sci* 1, 72-80.
- Crebtree, G.R. (1989) Contingent genetic regulatory events in T lymphocyte activation. *Science* 243, 355-61.
- Darnell, J.E., JR., Kerr, I.M., Stark, G.R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264, 1415-21.
- Dean, N.M., Kanemitsu, M., Boynton, A.L. (1989) Effects of the tyrosine-kinase inhibitor genistein on DNA synthesis and phospholipid-derived second messenger generation in mouse 10T1/2 fibroblasts and rat liver T51B cells. *Biol. Biophys. Res. Commun.* 165, 795-801.
- Dekker, L.V., Parker, P.J. (1994) Protein kinase C - a question of specificity. *Trends Biochem. Sci.* 19, 73-7.

- Demaurex, N., Lew, D.P., Krause, K.-H. (1992) Cyclopiazonic acid depletes intracellular Ca^{2+} stores and activates an influx pathway for divalent cations in HL-60 cells. *J. Biol. Chem.* 267, 2318-24.
- Dukovich, M., Wano, Y., Thuy, L.B., Katz, P., Cullen, B.R., Kehrl, J.H., and Greene, W.C. (1987) A second human interleukin-2 binding protein that may be a component of high-affinity interleukin-2 receptors. *Nature* 327, 518-22.
- Doi, T., Hatakeyama, M., Minamoto, S., Kono, T., Mori, H., Taniguchi, T. (1989) Human IL-2 receptor β chain allows transduction of IL-2-induced proliferation signal(s) in a murine cell line. *Eur. J. Immunol.* 19, 2375-81.
- Dourmashkin, R., Deteix, P., Simone, C.B., Henkart, P. (1980) Electron microscope demonstration of lesions on target cell membranes associated with antibody dependent cellular cytotoxicity. *Clin. Exp. Immunol.* 43, 554-60.
- Downes, C.P., Michell, R.H. (1985) In: *Molecular aspects of cellular regulation. Vol.4, Molecular aspects of transmembrane signaling*, Eds.: Cohen, P. and Houslay, M.D. (Elsevier, Amsterdam) pp3-56.
- Duke, R.C., Cohen, J.J. (1986) IL-2 addiction: withdrawal of growth factor activates a suicide program in dependent-T cells. *Lymphokine Res.* 5, 289-94.
- Durkin, J.P., Chakravarthy, B.R., Tremblay, R., Macdonald, P., Jouishomme, H., Whitfield, J.F., Biquard, J.M., and Krsmanovic, V. (1992) Evidence that a novel human differentiation-inhibiting protein (DIP) blocks DMSO-induced differentiation of erythroleukemia cells by inhibiting the activation of membrane protein kinase C. *Cancer research*, 52, 6329-34.
- Durkin, J.P., Tremblay, R., Blosser, J., Chakravarthy, B., Mealing, G., Morley, P., and Song, D. (1995) An early loss in membrane protein kinase C activity precedes the delayed death of primary cortical neurons by excitatory amino acids. *J. Neurochem.* in press.
- Durino, V., Nip, L., Pelech, S.L. (1989) Interleukin 3 stimulates phosphatidylcholine turnover in a mast/megakaryocyte cell line. *Biochem. Biophys. Res. Commun.* 164, 804-10.
- Eardley, D.D., Koshland, M.E. (1991) Glycosylphosphatidylinositol: A candidate system for interleukin-2 signal transduction. *Science* 251, 78-81.
- Eisen, H.N. (1990) In: *General immunology*, Eds.: McAllister, L., Winters, R., Bellus, J., Littlewood, B. (J. B. Lippin Cott Company) pp11-215.
- Epand, R.M., Stafford, A.R., Bottega, R., Ball, E.H. (1989) Studies on the mechanism of action of a bilayer stabilizing inhibitor of protein kinase C: cholesteryl phosphoryldimethylethanolamine. *Biosci. Rep.* 9, 315-28.

- Erusalimsky, J.D., Brooks, S. F., Herget, T., Morris, C., and Rozengurt, E. (1991) Molecular cloning and characterization of the acidic 80-kDa protein kinase C substrate from rat brain: Identification as a glycoprotein. *J. Biol. Chem.* 266, 7073-80.
- Evans, S.W. and Farrar, W.L. (1987a) Interleukin 2 and diacylglycerol stimulate phosphorylation of 40 S ribosomal S6 protein. *J. Biol. Chem.* 262, 4624-30.
- Evans, S.W., Farrar W.L. (1987b) Identity of common phosphoprotein substrates stimulated by interleukin 2 and diacylglycerol suggests a role of protein kinase C for IL2 signal transduction. *J. Cell. Biochem.* 34, 47-51.
- Evans, S.W., Beckner, S.K., Farrar, W.L. (1987c) Stimulation of specific GTP binding and hydrolysis activities in lymphocytes membrane by interleukin-2. *Nature* 325, 166-8.
- Exton, J.H. (1990) Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* 265, 1-4.
- Exton, J.H. (1993) Role of G proteins in activation of phosphoinositide phospholipase C. *Adv. Sec. Messen. Phosphopro. Res.* 28, 65-72.
- Exton, J.H. (1994) Phosphatidylcholine breakdown and signal transduction. *Biochim. Biophys. Acta* 1212, 26-42.
- Farago, A., Nishzuka, Y. (1990) Protein kinase C in transmembrane signaling. *FEBS Lett.*, 268, 350-4.
- Farrar, W.L., Johnson, H.M., and Farrar, J.J. (1981) Regulation of the production of immune interferon and cytotoxic T lymphocytes by interleukin 2. *J. Immunol.* 126, 1120-26.
- Farrar, W.L., Anderson, W.B. (1985) Interleukin-2 stimulates association of protein kinase C with plasma membrane. *Nature* 315, 233-35.
- Farrar, W.L., Cleveland, J.L., Beckner, S.K., Bonvini, E., and Evans, S.T. (1986) Biochemical and molecular events associated with interleukin-2 regulation of lymphocyte proliferation. *Immunological Review* 92, 49-65.
- Farrar, W.L., Ferris, D.K. (1989) Two-dimensional analysis of interleukin 2-regulated tyrosine kinase activation mediated by the p70-75 β subunit of the interleukin 2 receptor. *J. Biol. Chem.* 264, 12562-6.
- Farrar, W.L., Linnekin, D., Brini, A.T., Kelvin, D.J., Michiel, D.F. (1990) The interleukin-2 receptor complex: structure, gene regulation, and signal transduction. In: ligands, receptors, and signal transduction in regulation of lymphocyte function, Eds.: Cambien, J.C., p267-90.

- Ferris, D.K., Willette-Brown, J., Ortaldo, J.R., Farrar, W.L. (1989) IL-2 regulation of tyrosine kinase activity is mediated through the p70-75 β -subunit of the IL-2 receptor. *J. Immunol.* 143, 870-8.
- Folch, J., Lees, M., Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497-503.
- Forbes, I.J., Zalewski, P.D., Giannakis, C., Cowled, P.A. (1992) Induction of apoptosis in chronic lymphocytic leukemia cells and its prevention by phorbol esters. *Exp. Cell. Res.* 198, 367-72.
- Fu, X.Y. (1992a) A transcription factor with SH2 and Sh3 domains is directly activated by an interferon alpha-induced cytoplasmic protein tyrosine kinase(s). *Cell* 70, 323-7.
- Fu, X., Schindler, C., Improtta, T., Aebersold, R., Darnell J.E.J. (1992b) The proteins of ISFG-3, the interferon α -induced transcriptional activator, define a gene family involved in signal transduction. *Proc. Natl. Acad. Sci. USA* 89, 7840-5.
- Gajewski, T.F., Schell, S.R., Nau, G. Fitch, R.W. (1989) Regulation of T-cell activation: Differences among T-cell subsets. *Immunol. Rev.* 111, 79-110.
- Garcia, I., Martinou, I., Tsujimoto, Y., Martinou, J-C. (1992) Prevention of programmed cell death of sympathetic neurons by the *bcl-2* proto-oncogene. *Science* 258, 302-4.
- Grassmann, M., Amrein, K.E. and Burn, P. (1993) CD4:P56^{lck} association studied *in vivo* using antibody-induced capping and double indirect immuno-fluorescence microscopy. *J. Receptor Res.* 13, 711-8.
- Gaulton, G.N., Eardley, D.D. (1986) Interleukin 2-dependent phosphorylation of interleukin 2 receptors and other T cell membrane proteins. *J. Immunol.* 136, 2470-6.
- Gelfand, E.W., Mills, G.B., Cheung, R.K., Lee, J.W.W., and Grinstein, S. (1987) Transmembrane ion fluxes during activation of human T lymphocytes: role of Ca⁺⁺, Na⁺/H⁺ exchange and phospholipid turnover. *Immunol. Rev.* 95, 59-75.
- Gillis, S., and Smith, K.A. (1977) Long term culture of tumour-specific cytotoxic T cells. *Nature* 268, 154-6.
- Gillis, S., Ferm, M.W., OU, W. and Smith, C.A. (1978) T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120, 2027-31.
- Gillis, S., Banker, P.E., Union, N.A. and Smith, K.A. (1979) The *in vitro* generation and sustained culture of nude cytotoxic T-lymphocytes. *J. Exp. Med.* 149, 1460-7.
- Gillis, S., Mochizuki, D.Y., Conlon, P.J., Hefeneider, S.H., Ramthun, C.A., Gillis, A.E., Frank, M.B., Henney, C.S., Watson, J.D. (1982) Molecular characterization of interleukin 2. *Immunological Rev.* 63, 167-209.

- Graff, J.M., Young, T.N., Johnson, J.D., and Blackshear, P.J. (1989a) Phosphorylation-regulated calmodulin binding to a prominent cellular substrate for protein kinase C. *J. Biol. Chem.* **264**, 21818-23.
- Graff, J.M., Stumpo, D.J., and Blackshear, P.J. (1989b) Molecular cloning, sequence, and expression of a cDNA encoding the chicken myristoylated alanine-rich C kinase substrate (MARCKS) *Mol. Endocrinol.* **3**, 1903-6.
- Graff, J.M., Rajan, R.R., Randall, R.R., Nairn, A.C., and Blackshear, P.J. (1991) Protein kinase C substrate and inhibitor characteristics of peptides derived from the myristoylated alanine-rich C kinase substrate (MARCKS) protein phosphorylation site domain. *J. Biol. Chem.* **266**, 14390-8.
- Grimm, E.A., Mazumder, A., Zhang, H.Z., Rosenberg, S.A. (1982) Lymphokine-activated killer cell phenomenon: lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J. Exp. Med.* **155**, 1823-30.
- Gross, J., Herblin, W., Do, U., Pounds, J., Buenaga, L., Stephens, L. (1990) Characterization of specific [³H]dimethylstaurosporine binding to protein kinase C. *Biochem. Pharmacol.* **40**, 343-7.
- Grynkiewicz, G., Poenie, M., Tsien, R. Y. (1985) A new generation of Calcium indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-8.
- Gutman, A., Wasylyk, B. (1991) Nuclear targets for transcription regulation by oncogenes. *Trends Genet.* **7**, 49-56.
- Halsey, D.L., Girard, P.R., Kuo, J.F., Blackshear, P.J. (1987) Protein kinase C in fibroblasts: characteristics of its intracellular location during growth and after exposure to phorbol esters and other mitogens. *J. Biol. Chem.* **262**, 2234-43.
- Hamblin, A.S. (1993) Cytokines and cytokines receptors, Eds.: Male, D.(IRL press at Oxford University Press), p5-197.
- Hannun, Y.A., Loomis, C.R. Bell, R.M. (1985) Activation of protein kinase C by Triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine. *J. Biol. Chem.* **260**, 10039-44.
- Hannun, Y.A., Loomis, C.R., Merrill, A.H.J., Bell, R.M. (1986) Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. *J. Biol. Chem.* **261**, 12604-10.

- Harlan, D.M., Graff, J.M., Stumpo, D.J., Eddy, R.L., Jr., Shows, T.B., Boyle, J.M., and Blackshear, P.J. (1991) The human myristoylated alanine-rich C kinase substrate (MARCKS) gene (MACS): analysis of its gene product, promoter, and chromosomal localization. *J. Biol. Chem.* 266, 14399-4-5.
- Hartwig, J.H., Thelen, M., Rosen, A., Janmey, P.A., Nairn, A.C., and Aderem, A. (1992) MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature* 356, 618-22.
- Hatakeyama, M., Mori, H., Doi, T., and Taniguchi. (1989) A restricted cytoplasmic region of IL-2 receptor β chain is essential for growth signal transduction but not for ligand binding and internalization. *Cell* 59,837-41.
- Hatakeyama, M., Kono, T., Kobayashi, N., Kawahara, A., Levin, S.D., Perlmutter, R.M., Taniguchi, T. (1991) Interaction of the IL-2 receptor with the src-family kinase p56^{lck}: identification of novel intermolecular association. *Science* 252, 1523-8.
- Hatakeyama, M., Kawahara, A., Mori, H., Shibuya, H., Taniguchi, T. (1992) c-fos gene induction by interleukin 2: Identification of the critical cytoplasmic regions within the interleukin 2 receptor β chain. *Proc. Natl. Acad. Sci. USA* 89, 2022-6.
- Heemskerk, F.M.J., Chen, H.C., Huang, F.L. (1993) Protein kinase C phosphorylates Ser¹⁵², Ser¹⁵⁶, and Ser¹⁶³, but not Ser¹⁶⁰ of MARCKS in rat brain. *Biochem. Biophys. Res. Commun.* 190, 236-41.
- Henkart, P.A. (1985) Mechanism of lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* 3, 31-45.
- Henkart, P.A., Williams, M.S., Nakajima, H. (1995) Degranulating cytotoxic lymphocytes inflict multiple damage pathways on target cells. *Cur. Top. Microbiol. Immunol.* 198, 75-93.
- Henney, C.S., Kuribayashi, K., Kern, D.E., and Gillis, S. (1981) Interleukin-2 augments natural killer activity. *Nature* 291, 335-8.
- Herbert, J.M., Seban, E., Maffrand, J.P. (1990) Characterization of specific binding sites for [³H]-staurosporine on various protein kinases. *Biochem. Biophys. Res. Commun.* 171, 189-96.
- Hidaka, H., Inagaki, M., Kawamoto, S., Sasaki, Y. (1984) Isoquinolinesulfonamides, novel second potent inhibitors of cyclic nucleotide-dependent protein kinase and protein kinase C. *Biochemistry* 23, 5036-42.
- Hockenberry, D., Nunez, G., Milliman, C., Schreiber, R.D., Korsmeyer, S.J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348, 334-336.

Holbrook, N.J., Smith, K.A., Foranace, A.J., Jr., Comeau, C.M., Wiskocil, R.L., and Crabtree, G.R. (1984) T-cell growth factor: complete nucleotide sequence and organization of the gene in normal and malignant cells. *Proc. Natl. Acad. Sci. USA* 81, 1634-38.

Horak, I.D., Gress, R.E., Lucas, P.J., Horak, E.M., Waldmann, T.A., Bolen, J.B. (1991) T-lymphocyte interleukin 2-dependent tyrosine protein kinase signal transduction involves the activation of p56^{lck}. *Proc. Natl. Acad. Sci. USA* 88, 1996-2000.

House, C., Kemp, B. (1987) Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. *Science* 238, 1726-31.

House, C., Kemp, B. (1990) Protein kinase C pseudosubstrate prototope: structure-function relationships. *Cell. Signaling* 2, 187-90.

Huang, F.L., Yoshida, Y., Nakabayashi, H., Knopf, J.L., Young, W.S. III Huang, K.-P. (1987a) Immunochemical identification of protein kinase C isozymes as products of discrete genes. *Biochem. Biophys. Res. Commun.* 149, 946-52.

Huang, F.L., Yoshida, Y., Nakabayashi, H. and Huang, K.-P. (1987b) Differential distribution of protein kinase C isozymes in the various regions of brain. *J. Biol. Chem.* 262, 15714-20.

Huckle, W.R., Hepler, J.R., Rhee, S.G., Harden, T.K., Earp, H.S. (1990) Protein kinase C inhibits epidermal growth factor-dependent tyrosine phosphorylation of phospholipase C gamma and activation of phosphoinositide hydrolysis. *Endocrinol.* 127, 1697-705.

Hug, H. Sarre, T.F. (1993) Protein kinase C isoenzymes: divergence in signal transduction? *Biochem. J.* 291, 329-43.

Hunter, T., Cooper, J.A. (1985) Protein-tyrosine kinases. *Annu. Rev. Biochem.* 54, 897-905.

Hunter, T. (1991) Cooperation between oncogenes. *Cell* 64, 249-57.

Ihle, J.N., Witthuhn, B.A., Aueller, F.W., Yamanoto, K., Thierfelder, W.E., Kreider, B., Silvenninen, O. (1994) Signaling by the cytokine receptor superfamily: JAKs and STATs. *TIBS*, 19, 222-7.

Imamura, K., Dianoux, A., Nakamura, T., Kufe, D. (1990) Colony-stimulating factor 1 activates protein kinase C in human monocytes. *EMBO J.* 9, 2423-9.

Inagaki, N., Ito, M., Nakano, T., Inagaki, M. (1994) Spatiotemporal distribution of protein kinase and phosphatase activities. *TIBS* 19, 448-52.

- Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. *J. Biol. Chem.* **252**, 7610-7.
- Irving, H.R., Exton, J.H. (1987) Phosphatidylcholine breakdown in rat liver plasma membranes: Role of guanine nucleotides and P2-purine agonists. *J. Biol. Chem.* **262**, 3440-3.
- Isfort, R.J., Stevens, D., Stratford May, W., Ihle, J.N. (1988) Interleukin-3 binds to a 140-kDa phosphotyrosine-containing cell surface protein. *Proc. Natl. Acad. Sci. USA* **85**, 7982-86.
- Ishii, T., Takeshita, T., Numata, N., and Sugamura, K. (1988) Protein phosphorylation mediated by IL-2/IL-2 receptor β -chain interaction. *J. Immunol.* **141**, 174-9.
- Jimenez, B., Pestana, A., Fernandez-Remart, M. (1989) A phospholipid-stimulated protein kinase from *dictyostelium discoideum*. *Biochem. J.* **260**, 557-61.
- Johnston, J.A., Kawamura, M., Kirken, R., Chen, Y., Blake, T.B., Shibuya, K., Ortaldo, J.R., McVicar, D.W., O'Shea, J.J. (1994) Phosphorylation and activation of the JAK3 Janus kinase in response to IL-2. *Nature*, **370**, 151-5.
- Jone, R.R., Antonetti, D.L., Reid, T.W. (1986) Aluminum ions stimulate mitosis in murine cells in tissue culture. *J. Cell. Biochem.* **30**, 31-7.
- Jouishomme, H., Whitfield, J.F., Chakravarthy, B., Durkin, J.P., Gagnon, L., Isaacs, R.J., MacLean, S., Neugebauer, W., Willick, G., Rixon, R.H. (1992) The protein kinase C-activation domain of the parathyroid hormone. *Endocrinol.* **130**, 53-8.
- Kalbag, S.S., Roginsky, M.S., Jelveh, Z., Sullmovici, S. (1991) Phorbol ester, prolactin, and relaxin cause translocation of protein kinase C from cytosol to membranes in human endometrial cells. *Biochim. Biophys. Acta* **1094**, 85-95.
- Kanfer, J.M. (1980) The base exchange enzymes and phospholipase D of mammalian tissue. *Can. J. Biochem.* **58**, 1370-80.
- Karnitz, L., Sutor, S.L., Torigoe, T., Reed, J.C., Bell, M.P., McKean, D.J., Leibson, P.J., Abraham, R.T. (1992) Effects of p56^{lck} deficiency on the growth and cytolytic effector function of an interleukin-2-dependent cytotoxic T-cell line. *Mol. Cell. Biol.* **12**, 4521-30.
- Kauser, K., Clark, J.E., Masters, B.S., Ortiz de Montellano, P.R., Ma, Y-H., Harder, D.R., Roman, R.J. (1991) Inhibitors of cytochrome P-450 attenuate the myogenic response of dog renal arcuate arteries. *Circulation Research* **68**, 1154-63.

- Kelly, K., Cochran, B.H., Stiles, C.C., Leder, P. (1983) Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. *Cell* **35**, 603-11.
- Kester, M., Simonson M.S., Mene, P., Sedor, J.R. (1989) Interleukin-1 generates transmembrane signals from phospholipids through novel pathways on cultured rat mesangial cells. *J. Clin. Invest.* **83**, 718-24.
- Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., Nihizuka, Y. (1982) Calcium-activated, phospholipid-dependent protein kinase from rat brain. *J. Biol. Chem.* **257**, 13341-7.
- Kikkawa, U., Ono, Y., Ogita, K., Fujii, T., Asaoka, Y., Sekiguchi, K., Kosaka, K., Igarashi, K., Nishizuka, Y. (1987) Identification of the structures of multiple subsequence of protein kinase C expressed in rat brain. *FEBS Lett.* **217**, 227-34.
- Kikkawa, U., Kishimoto, A., Nishizuka, Y. (1989) The protein kinase C family: heterogeneity and its implications. *Annu. Rev. Biochem.* **58**, 31-42.
- Kim, D.-K., Lancki, D.W., Hui, F.H. and Fitch, F.W. (1989) Protein kinase C-dependent and -independent mechanisms of cloned murine T cell proliferation: The role of protein kinase C translocation and protein kinase C activity. *J. Immunol.* **142**, 616-22.
- Kishi, H., Inui, S., Muraguchi, A., Hirano, T., Yamamura, Y. and Kishimoto, T. (1985) Induction of IgG secretion in a human B cell clone with recombinant IL-2. *J. Immunol.* **134**, 3104-10.
- Kiss, Z. Anderson, W.B. (1989) Phorbol ester stimulates the hydrolysis of phosphatidylethanolamine in leukemic HL-60, NIH 3T3, and baby hamster kidney cells. *J. Biol. Chem.* **264**, 1483-7.
- Kizaki, H., Shimada, H., Ishimura, Y. (1988) 12-o-tetradecanoylphorbol-13-acetate induces DNA cleavage at linker regions in mouse thymocytes. *J. Biochem.* **105**, 673-9.
- Kobayashi, M., Kanfer, J.N. (1987) Phosphatidylethanol formation via transphosphatidylolation by rat brain synaptosomal phospholipase D. *J. Neurochem.* **48**, 1597-1602.
- Kobayashi, E., Ando, K., Nakano, H., and Tamaoki, T. (1989a) UCN-1028A, a novel and specific inhibitor of protein kinase C, from *Cladosporium*. *J. Antibiot.* **42**, 1538.
- Kobayashi, E., Ando, K., Nakano, H., Iida, T., Ohno, H., Morimoto, M., and Tamaoki, T. (1989b) Calphostins (UCN-1028), novel and specific inhibitors of protein kinase C. I. Fermentation, isolation, physical-chemical properties and biological activities. *J. Antibiot.* **42**, 1479-83.

Kobayashi, E., Nakano, H., Morimoto, M., Tamaoki, T. (1989c) Calphostin C (UCN1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* 159, 548-56.

Kobayashi, N., Kono, T., Hatakeyama, M., Minami, Y., Miyazaki, T., Perlmutter, R.M., Taniguchi, T. (1993) Functional coupling of the *src*-family protein tyrosine kinases p59^{lck} and p53/56^{lyn} with the interleukin 2 receptor: Implications for redundancy and pleiotropism in cytokine signal transduction. *Proc. Natl. Acad. Sci. USA* 90, 4201-5.

Kolesnick, J., Paley, E. (1987) 1,2-diacylglycerols and phorbol esters stimulate phosphatidylcholine metabolism in GH3 pituitary cells. Evidence for separate mechanisms of action. *J. Biol. Chem.* 262, 9204-10.

Kondratyev, A.d., Popova, O.N., Severin, S.E., Choladze, M.A., Shmyrev, I.I., Tubasheva, I.A., Zotova, E.E., Posypanova, G.A, Severin, E.S. (1990) Nerve growth factor stimulates protein kinase C translocation in PC 12 cells. *FEBS Lett.* 264, 75-7.

Kono, T., Doi, T., Yamada, G., Hatakeyama, M., Minamoto, S., Tsudo, M., Miyasaka, M., Miyata, T., Taniguchi, T. (1990) Murine interleukin 2 receptor β chain: Dysregulated gene expression in lymphoma line EL-4 caused by a promoter insertion. *Proc. Natl. Acad. Sci. USA* 87, 1806-10.

Kornbluth, J., and Hoover R.G. (1988) Changes in gene expression associated with IFN- β and IL-2-induced augmentation of human natural killer cell function. *J. Immunol.* 141, 3234-41.

Koyasu, S. Tojo, A., Miyajima, A., Akiyama, T., Kasuga, M., Urabe, A., Schreurs, J., Arai, K., Takaku, F., Yahara, I. (1987) Interleukin 3-specific tyrosine phosphorylation of a membrane glycoprotein of Mr 150000 in multi-factor-dependent myeloid cell lines. *EMBO J.* 6, 3979-84.

Kraft, S.K., and Anderson, W.B. (1983) Phorbol esters increase the amount of Ca²⁺, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* 301, 621-3.

Kramer, I.M., Van der Bend, R.L., Tool, T.J., van Blitterswijk, W.J., Roos, D., and Verhoeven, A.J. (1989) 1-o-Hexadecyl-2-o-methylglycerol, a novel inhibitor of protein kinase C, inhibits the respiratory burst in human neutrophils. *J. Biol. Chem.* 264, 5876-84.

Kwast-Welfeld, J., de Belle, I., Walker, P.R., Isaacs, R.J., Whitfield, J.F., Sikorska, M. (1991) Changes in cyclic adenosine monophosphate-responsive element binding proteins in rat hepatomas. *Cancer Res.* 51, 528-35.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-8.

- Lane, D.P. (1993) A death in the life of p53. *Nature* 362, 786-7.
- Leonard, W.J., Depper, J.M., Robb, R.J., Waldmann, T.A. and Greene, W.C. (1983) Characterization of the human receptor for T-cell growth factor. *Proc. Natl. Acad. Sci. USA* 80, 6957-61.
- Leonard, W.J., Depper, J.M., Kanehisa, M., Kronke, M., Pfeffer, N.J., Svetlik, P.B., Sullivan, M., Greene, W.C. (1985) Structure of the human interleukin-2 receptor gene. *Science*, 220, 633-9.
- Lenardo, M.J. (1991) Interleukin-2 programs mouse $\alpha\beta$ T lymphocytes for apoptosis. *Nature* 353, 858-61.
- Lester, D.S. (1990) *In vitro* linoleic acid activation of protein kinase C. *Biochim. Biophys. Acta* 1054, 297-303.
- Lester, D.S., Collin, C., Etcheberrigaray, R., Alkon, D.L. (1991) Arachidonic acid diacylglycerol act synergistically to activate protein kinase *in vitro* and *in vivo*. *Biochem. Biophys. Res. Commun.* 179, 1522-8.
- LeVitzki, A. (1994) Signal-transduction therapy. A novel approach to disease management. *Eur. J. Biochem.* 2264, 1-34.
- Li, J., and Aderem, A. (1992) MacMARCKS, a novel member of the MARCKS family of protein kinase C substrates. *Cell* 70, 791-801.
- Liscovitch, M. (1992) Crosstalk among multiple signal-activated phospholipases. *TIBS* 17, 393-9.
- Liyanage, M., Frith, D., Livneh, E. and Stabel, S. (1992) Protein kinase C group B members PC-delta, -epsilon, -zeta and PKC-L (eta). Comparison of properties of recombinant proteins *in vitro* and *in vivo*. *Biochem J.* 283, 781-7.
- Lord, K.A., Abdollahi, A., Thomas, S.M., Demarco, M., Brugge, J.S., Hoffman-Liebermann, B., Liebermann, D.A. (1991) Leukemia inhibitory factor and interleukin-6 trigger the same immediate early response, including tyrosine phosphorylation, upon induction of myeloid leukemia differentiation. *Mol. Cell. Biol.* 11, 4371-9.
- Lotze, M.T., Grimm, E.A., Mazumder, A., Strausser, S.A., Rosenberg, S.A. (1981) Lysis of fresh and cultured autologous tumor by lymphocytes cultured in T-cell growth factor. *Cancer res.* 41, 4420-7.
- Lowry, O.H., Rosebrough, N.U., Farr, A.L., Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. chem.* 193, 265-75.

- MacKintosh, C., MacKintosh, R. (1994) Inhibitors of protein kinases and phosphatases. **TIBS**, **19**, 444-8.
- Magolda, R.L., Ripka, W.C., Galbraith, W., Johnson, P.R., Rudnick, M.S. (1985) Novel synthesis of potent site-specific phospholipase A₂ inhibitors, in: **Prostaglandins, Leukotrienes, and Lipoxins**, Eds.: Bailey J.M. (New York, Plenum Publishing Corp), p669-675.
- Mahadevan, L.C., Aitken, A., Heath, J., Foulkes, J.G. (1987) Embryonal carcinoma derived growth factor activates protein kinase C *in vivo* and *in vitro*. **EMBO J.** **6**, 921-6.
- Majno, G., Joris, I. (1995) Apoptosis, oncosis, and necrosis: an overview of cell death. **Am. J. Pathol.** **146**, 3-15.
- Makowske, M., Ballester, R., Cayre, Y., Rosen, O.M. (1988) Immunochemical evidence that three protein kinase C isozymes increase in abundance during HL-60 differentiation induced by dimethyl sulfoxide and retinoic acid. **J. Biol. Chem.** **263**, 3402-8.
- Margolis, B., Skolnik, E.Y. (1994) Activation of Ras by receptor tyrosine kinases. **J. Ame. Soc. Nephrol.** **5**, 1288-99.
- Martin, T.W. (1988) Formation of diacylglycerol by a phospholipase D-phosphatidate phosphatase pathway specific for phosphatidylcholine in endothelial cells. **Biochim. Biophys. Acta** **962**, 282-96.
- Martin, T.W., Michaelis, K.C. (1989) P₂-purinergic agonists stimulate phosphodiesteratic cleavage of phosphatidylcholine in endothelial cells. **J. Biol. Chem.** **264**, 8847-52.
- Martinek, R.G. (1972) A rapid ultraviolet spectrophotometric lactic dehydrogenase assay. **Clin. Chim. Acta** **40**, 91-7.
- May, W.S., Jr., Sahyoun, N., Wolf, M., Cuatrecasas, P. (1985) Role of intracellular calcium mobilization in the regulation of protein kinase C-mediated membrane processes. **Nature**, **317**, -51.
- Medema, R.H., Bos, J.L. (1993) The role of p21 ras in receptor tyrosine kinase signaling. **Crit. Rev. Oncog.** **4**, 615-61.
- Metz, S.A., Dunlop, M. (1991) Inhibition of the metabolism of phosphatidylethanol and phosphatidic acid, and stimulation of insulin release, by propranolol in intact pancreatic islets. **Biochem Pharmacol.** **41**, R1-R4.
- McConkey, D.J., Orrenius, S. (1991) Cellular signaling in thymocyte apoptosis, in: **Apoptosis: The molecular basis of cell death. Current communications in cell and molecular biology**, **3**, Eds.: Tomei, L.D. and Cope, F.O. (New York: Cold Spring Harbour Laboratory Press).

- McPhail, L., Clayton, C., Snyderman, R. (1984) A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+} -dependent protein kinase. *Science* **224**, 622-4.
- Merida, I., Gaulton, G.N. (1990a) Protein tyrosine phosphorylation associated with activation of the interleukin 2 receptor. *J. Biol. Chem.* **265**, 5690-6.
- Merida, I., Pratt, J.C., Gaulton, G.N. (1990b) Regulation of interleukin 2 dependent growth responses by glycosylphosphatidylinositol molecules. *Proc. Natl. Acad. Sci. USA* **87**, 9421-5.
- Merida, I., Diez, E., Gaulton, G.N. (1991) IL-2 binding activates a tyrosine phosphorylated phosphatidylinositol-3-kinase. *J. Immunol.* **147**, 2202-7.
- Mescher, M.F. (1995) Molecular interactions in the activation of effector and precursor cytotoxic T lymphocytes. *Immunol. Rev.* **146**, 177-210.
- McGlynn, E., Liebetanz, J., Reutener, S., Wood, J., Lydon, N.B., Hofstetter, H., Vanek, M., Meyer, T. and Fabbro, D. (1992) Expression and partial characterization of rat protein kinase C-delta and protein kinase C-zeta in insect cells using recombinant baculovirus. *J. Cell Biochem.* **49**, 239-50.
- Mier, J.W. and Gallo, R.C. (1980) Purification and some characteristics of human T-cell growth factor from phytohemagglutinin-stimulated lymphocyte-conditioned media. *Proc. Natl. Acad. Sci. USA* **77**, 6134-8.
- Mills, G.B., Cheung, R.K., Grinstein, S., and Gelfand, E.W. (1985a) Interleukin 2-induced lymphocyte proliferation is independent of increases in cytosolic-free calcium concentrations. *J. Immunol.* **134**, 2431-5.
- Mills, G.B., Stewart, D.J., Mellors, A., and Gelfand, E.W. (1986) Interleukin 2 does not induce phosphatidylinositol hydrolysis in activated T cells. *J. Immunol.* **136**, 3019-24.
- Mills, G.B., Girard, P., Grinstein, S., and Gelfand, E.W. (1988) Interleukin-2 induces proliferation of T lymphocyte mutants lacking protein kinase C. *Cell*, **55**, 91-100.
- Mills, G.B., May, C., McGill, M., Fung, M., Baker, M., Sutherland, R., Green, W.C. (1990) Interleukin 2-induced tyrosine phosphorylation. *J. Biol. Chem.* **265**, 3561-7.
- Mills, G.B., Zhang, N., Schmandt, R., Fung, M., Greene, W., Mellors, A., Hogg, D. (1991) Transmembrane signaling by interleukin 2. *Biochem. Soc. Transact.* **19**, 277-87.
- Minami, Y., Kono, T., Miyazaki, T., Taniguchi, T. (1993a) The IL-2 receptor complex: Its structure, function, and target genes. *Annu. Rev. Immunol.* **11**, 245-67.

Miura, O., D'Andrea, A., Kabat, D., Ihle, J.N. (1991) Induction of tyrosine phosphorylation by the erythropoietin receptor correlates with mitogenesis. *Mol. Cell. Biol.* **11**, 4895-4904.

Miyajima, A., Kitamura, T., Harada, N., Yokota, T., Arai, K. (1992) Cytokine receptors and signal transduction. *Annu. Rev. Immunol.* **10**, 295-306.

Miyazaki, T., Maruyama, M., Yamada, G., Hatakeyama, M., Taniguchi, T. (1991) The integrity of the conserved 'WS motif' common to IL-2 and other cytokine receptors is essential for ligand binding and signal transduction. *EMBO J.* **10**, 3191-9.

Miyazaki, T., Kawahara, A., Minami, Y., Liu, Z., silvennoinen, O., Witthuhn, B.A., Ihle, J.N., Taniguchi, T. (1994) Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. *Science* **266**, 1045-8.

Mond, J.J., Thompson, C.T., Finkelman, F.D., Farrar, J., Schaefer, M. and Robb, R.J. (1985) Affinity-purified interleukin-2 induces proliferation of large but not small B cells. *Proc. Natl. Acad. Sci. USA* **82**, 1518-21.

Morgan, D.A., Ruscetti, F.W. and Gallo, R. (1976) Selective *in vitro* growth of T lymphocytes from normal human bone marrows. *Science* **193**, 1007-8.

Mori, H., Barsoumian, E.L., Hatadeyama, M. and Taniguchi, T. (1991) Signal transduction by interleukin 2 receptor β chain: importance of the structural integrity as revealed by site-directed mutagenesis and generation of chimeric receptors. *Int. Immunol.* **3**, 149-54.

Morla, A.O., Schreurs, J., Miyajima, A., Wang, J.Y.J. (1988) Hematopoietic growth factors activate the tyrosine phosphorylation of distinct sets of proteins in interleukin 3-dependent murine cell lines. *Mol. Cell. Biol.* **8**, 2214-20.

Morley, P., Tsang, B.K., Whitfield, J.F., Schwartz, J-L. (1992) Thapsigargin increases cytoplasmic free Ca^{2+} without influencing steroidogenesis in chicken granulosa cells. *Cell Calcium* **13**, 263-71.

Morice, W.G., Brunn, G.J., Wiederrecht, G., Siekierka, J.J., Abraham, R.T. (1993) Rapamycin-induced inhibition of p34^{cdc2} kinase activation is associated with G_1/S -phase growth arrest in T lymphocytes. *J. Biol. Chem.* **268**, 3734-8.

Mueller, H.W., O'Flaherty, J.T., Green, D.G., Samuel, M.P., Wykle, R.L. (1984) 1-o-alkyl-linked glycerophospholipids of human neutrophils: distribution of arachidonate and other acyl residues in the ether-linked and diacyl species. *J. Lipid Res.* **25**, 383-8.

Mukaida, N., Yagisawa, H., Kawai, T., Kasahara, T. (1988) The role of protein kinase C activation in signal transmission by interleukin 2. *Biochem. Biophys. Res. Commun.* **154**, 187-93.

Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T., Kishimoto, T. (1991) Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. *Proc. Natl. Acad. Sci. USA* 88, 11349-53.

Murayama, T., and Ui, M. (1985) Receptor-mediated inhibition of adenylate cyclase and stimulation of arachidonic acid release in 3T3 fibroblasts. Selective susceptibility to islet-activating protein, pertussis toxin. *J. Biol. Chem.* 260, 7226-33.

Nakajima, K., Wall, R. (1991) Interleukin-6 signals activating junB and TIS 11 gene transcription in a B-cell hybridoma. *Mol. Cell. Biol.* 11, 1409-13.

Nakamura, Y., Russell, S.M., Mess, S.A., Friedmann, M., Erdos, M., Francois, C., Jacques, Y., Adelstein, S., Leonard, W.J. (1994) Heterodimerization of the IL-2 receptor β - and γ -chain cytoplasmic domains is required for signaling. *Nature* 369, 330-6.

Nakanishi, H. and Exton, J.H. (1992) Purification and characterization of the zeta isoform of protein kinase C from bovine kidney. *J. Biol. Chem.* 267, 16347-54.

Neer, E.J. (1995) Heterotrimeric G proteins: organizers of transmembrane signals. *Cell*, 80, 249-57.

Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R., Lefkowitz, J.B. (1986) Arachidonic acid metabolism. *Annu. Rev. Biochem.* 55, 69-102.

Nelson, B.H., Lord, J.D., Greenberg, P.D. (1994) Cytoplasmic domains of the interleukin-2 receptor beta and gamma chains mediate the signal for T-cell proliferation. *Nature*, 369, 333-6.

Nelsestuen, G.L., Bazzi, M. (1991) Activation and regulation of protein kinase C enzymes. *J. Bioenergetics and Biomembrane* 22, 43-61.

Newton, A.C. (1993) Interaction of proteins with lipid head groups: lessons from protein kinase C. *Annu. Rev. Biophys. Biomol. Struct.* 22, 1-14.

Nieto, M.A., Lopez-Rivas, A. (1989) IL-2 protects T lymphocytes from glucocorticoid-induced DNA fragmentation and cell death. *J. Immunol.* 143, 4166-72.

Nishibe, S., Wahl, M.I., Hernandezsotomayor S.M.T., Tonks, N.K., Rhee, S.G., Carpenter, G. (1990) Increase in the catalytic activity of phospholipase C- γ_1 by tyrosine phosphorylation. *Science* 250, 1253-8.

Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and turnover promotion. *Nature* 308, 693-8.

Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. *Science* 233, 305-312.

- Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* 334, 661-8.
- Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258, 607-14.
- Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* 9, 484-96.
- Ojima-Uchiyama, A., Masuzawa, Y., Suqiura, T., Waku, K., Saito, H., Yui, Y., Tomioka, H. (1988) Phospholipid analysis of human eosinophils: high levels of alkylacylglycerolphosphocholine (PAF precursor). *Lipids* 23, 815-20.
- O'Neal, K.D., Yu-Lee, L. (1993) The proline-rich motif (PRM): a novel feature of the cytokine/hematopoietic receptor superfamily. *Lymphokine cytokine Res.* 12, 309-14.
- Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., Nishizuka, Y. (1988) The structure, expression, and properties of additional members of the protein kinase C family. *J. Biol. Chem.* 263, 6927-32.
- Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., Nishizuka, Y. (1989a) Protein kinase C zeta subspecies from rat brain: its structure, expression, and properties. *Proc. Natl. Acad. Sci. USA* 86, 3099-103.
- Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U. and Nishizuka, Y. (1989b) Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. *Proc. Natl. Acad. Sci. USA* 86, 4868-71.
- Orr, J.W., Keranen, L.M., Newton, A.C. (1992) Reversible exposure of the pseudosubstrate domain of protein kinase C by phosphatidylserine and diacylglycerol. *J. Biol. Chem.* 267, 15263-8.
- Ortaldo, J.R., Yound, H.A., Varesio, L. (1989) Modulation of CD³⁺ large granular lymphocyte functions by agonist and antagonists of protein kinase C: effects on NK and lymphokine-activated killer activity and production of IFN- γ . *J. Immunol.* 143, 366-71.
- Paetkau, V., Mills, G.B. (1989) Cytokines and mechanisms of lymphocyte activation. *Immunol. Allergy Clins N. America* 9, 21-8.
- Park, D.J., Rho, H.W., Rhee, S.G. (1991) CD₃ stimulation causes phosphorylation of phospholipase C-gamma1 on serine and tyrosine residues in a human T-cell line. *Proc. Natl. Acad. Sci. USA* 88, 5453-6.
- Pauza, C.D. (1987) Regulation of human T-lymphocyte gene expression by interleukin 2: Immediate-response genes include the proto-oncogene c-myb. *Mol. Cell. Biol.* 7, 342-50.

Pelech, S.L., Vance, D.E. (1984) Regulation of phosphatidylcholine biosynthesis. *Biochem. Biophys. Acta* 779, 217-51.

Pelech, S.L., Meier, K.E., Crebs, E.G. (1986) Rapid microassay for protein kinase C translocation in Swiss 3T3 cells. *Biochemistry* 25, 8348-53.

Pelech, S.L., Paddon, H.B., Charest, D.L., Federspiel, B.S. (1990a) IL-3-induced activation of protein kinases in the mast cell/megakaryocyte R6-XE.4 line. *J. Immunol.* 144, 1759-66.

Pelech, S.L., Charest, D.L., Howard, S.L., Paddon, H.B., Salari, H. (1990b) Protein kinase C activation by platelet-activating factor is independent of enzyme translocation. *Biochim. Biophys. Acta* 1051, 100-7.

Pelosin, J.M., Vilgrain, I. and Chambaz, E.M. (1987) A single form of protein kinase C is expressed in bovine adrenocortical tissue, as compared to four chromatographically resolved isozymes in rat brain. *Biochem. Biophys. Res. Commun.* 147, 382-91.

Pfeffer, L.M., B. Strulovici, Saltiel, A.R. (1990) Interferon-alpha selectively activates the β isoform of protein kinase C through phosphatidylcholine hydrolysis. *Proc. Natl. Acad. Sci. USA.* 87, 6537-41.

Pike, L.J., Gallis, B., Casnellie, J.E., Bomstein, P. Krebs, E.G. (1982) Epidermal growth factor stimulates the phosphorylation of synthetic tyrosine-containing peptides by A431 cell membrane. *Proc. Natl. Acad. Sci. USA* 79, 1443-7.

Piomelli, D. (1993). Arachidonic acid in cell signalling. *Curr. Opin. Cell Biol.* 5, 274-80.

Plevin, R., Cook, S.J., Palmer, S., Wakelam, M.J.O. (1991) Multiple sources of sn-1,2-diacylglycerol in PDGF-stimulated Swiss 3T3 fibroblasts: evidence for activation of phosphoinositidase C and phosphatidylcholine-specific phospholipase C. *Biochem. J.* 279, 559-65.

Podack, E.R., Dennert, G. (1983) Assembly of two types of tubules with putative cytolytic function by cloned natural killer cells. *Nature* 302, 442-6.

Quelle, F.W., Wojchowski, D. M. (1991) Proliferative action of erythropoietin is associated with rapid protein tyrosine phosphorylation in responsive B6SUt.EP cells. *J. Biol. Chem.* 266,609-74.

Rayhel, E.J., Fields, T.J., Albright, J.W., Diamantstein, T. and Hughes, J.P. (1988) Interleukin 2 and lactogen regulate proliferation and protein phosphorylation in Jb2 cells. *Biochem. J.* 249, 333-8.

Redondo, J.M., Lopez-Rivas, A., Vila, V., Cragoe, E.J., Fresno, M. (1988) The role of protein kinase C in T lymphocyte proliferation. *J. Biol. Chem.* 263, 17467-70.

Reed, J.C., Sabath, D.E., Hoover, R.G., Prystowsky, M.B. (1985a) Recombinant interleukin 2 regulates levels of c-myc mRNA in a cloned murine T lymphocyte. *Mol. Cell. Biol.* 5, 3361-9.

Reed, J.C., Nowell, P.C., Hoover, R.G. (1985b) Regulation of c-myc mRNA levels in normal human lymphocytes by modulators of cell proliferation. *Proc. Natl. Acad. Sci. USA* 82, 4221-7.

Rhee, S.G., Suh, P.G., Ryu, S.H., Lee, S.Y. (1989) Studies of inositol phospholipid-specific phospholipase C. *Science* 244, 546-50.

Rhee, S.G., and Choi, K.D. (1992) Regulation of inositol phospholipid-specific phospholipase C isozymes. *J. Biol. Chem.* 267, 12393-6.

Rhee, S.G., Park, D.J. and Park, D. (1992) In: *Cellular and molecular mechanisms of inflammation: signal transduction*, Eds.: Cochrane, C.G. and Gimbrone, M.A. (Academic press, New York), pp25-55.

Robb, R.J., Smith, K.A. (1981) Heterogeneity of human T-cell growth factor due to glycosylation. *Mol. Immunol.* 18, 1087-92.

Robb, R.J., Greene, W.C. (1983) Direct demonstration of the identity of T-cell growth factor binding protein and the Tac antigen. *J. Exp. Med.* 158, 1332-41.

Robb, R.J., Rusk, C.M., Nepper, M.P. (1988) Structure-function relationships for the interleukin 2 receptor: Location of ligand and antibody binding sites on the Tac receptor chain by mutational analysis. *Proc. Natl. Acad. Sci. USA* 85, 5654-58.

Rodriguez-Tarduchy, G., Lopez-Rivas, A. (1989) Phorbol ester inhibit apoptosis in IL-2-dependent T lymphocytes. *Biochem. Biophys. Res. Commun.* 164, 1069-75.

Rosoff, P.M., Savage, N., Dinarello, C.A. (1988) Interleukin-1 stimulates diacylglycerol production in T lymphocytes by a novel mechanism. *Cell*, 54, 73-9.

Roy, C., Brown, D.L., Little, J., Valentine, B.K., Walker, P.R., Sikorska, M., LeBlanc, J. Chaly, N. (1992) The topoisomerase II inhibitor teniposide (VM-26) induces apoptosis in unstimulated mature murine lymphocytes. *Exp. Cell. Res.* 200, 416-21.

Rozengurt, E., Rodriguez-Pena, M., and Smith, K.A. (1983) Phorbol esters, phospholipase C, and growth factors rapidly stimulate the phosphorylation of a Mr. 80,000 protein in intact quiescent 3T3 cells. *Proc. Natl. Acad. Sci. USA* 80, 7244-8.

Ruscetti, F.W. and Gallo, R.C. (1981) Human T-lymphocyte growth factor: regulation of growth and function of T lymphocytes. *Blood* 57, 379-94.

Russell, S.M., Johnston, J.A., Noguchi, M., Kawamura, M., Bacon, C.M., Friedmann, M., Berg, M., McVicar, D.W., Witthuhn, B.A., Silvennoinen, O., Goldman, A.S., Schmalstieg, F.C., Ihle, J.N., O'Shea, J.J., Leonard, W.J. (1994) Interaction of IL-2 receptor β and γ chains with JAK1 and JAK3: Implications for XSCID and XCID. *Science* 266, 1042-8.

Ryu, S.H., Kim, U-H., Wahl, M.I., Brown, A.B., Carpenter, G., Huang, K-P., Rhee, S.G. (1990) Feedback regulation of phospholipase C- β by protein kinase C. *J. Biol. Chem.* 265, 17941-45.

Saito, N., Kikkawa, U., Nishizuka, Y. and Tanaka, C. (1988) Distribution of protein kinase C-like immunoreactive neurons in rat brain. *J. Neurosci.* 8, 369-82.

Saito, Y., Tadce, H., Sabe, H., Honjo, T. (1991) Biochemical evidence for a third chain of the interleukin-2 receptor. *J. Biol. Chem.* 266, 22186-93.

Sakai, K., Hirai, M., Kudoh, J., Minoshima, S., and Shimizu, N. (1992) Molecular cloning and chromosomal mapping of a cDNA encoding human 80K-L protein: major substrate for protein kinase C. *Genomics* 14, 175-8.

Saltzman, E.M., Thom, R.R., Casnellie, J.E. (1988) Activation of a tyrosine protein kinase is an early event in the stimulation of T lymphocytes by interleukin-2. *J. Biol. Chem.* 263, 6956-59.

Saltzman, E.M., White, K., Casnellie, J.E. (1990) Stimulation of the antigen and interleukin-2 receptors on T lymphocytes activates distinct tyrosine protein kinases. *J. Biol. Chem.* 265, 10138.

Sando, J.J., Maurer, M.C., Bolen, E.J., Grisham, C.M. (1992) Role of cofactors in protein kinase C activation. *Cell. signaling* 4, 595-602.

Saragovi, H., Malek, T.R. (1990) Evidence for additional subunits associated to the mouse interleukin 2 receptor p55/p75 complex. *Proc. Natl. Acad. Sci. USA* 87, 11-15.

Satoh, T., Nakafuku, M., Miyajima, A., Kaziro, Y. (1991) Involvement of ras in signal transduction pathways from interleukin-2, interleukin-3, and granulocyte-macrophage colony stimulation factor, but not from interleukin-4. *Proc. Natl. Acad. Sci. USA* 88, 3314-18.

Satoh, T., Minami, Y., Kono, T., Yamada, K., Kawahara, A., Taniguchi, T., Kaziro, Y. (1992) Interleukin 2-induced activation of ras requires two domains of interleukin 2 receptor β subunit, the essential region for growth stimulation and Lck-binding domain. *J. Biol. Chem.* 267, 25423-27.

Schaap, D., Parker, P. J., Bristol, A., Kriz, R., Knopf, R.J. (1989) Unique substrate specificity and regulatory properties of PKC-epsilon: a rationale for diversity. *FEBS Letters* 243, 351-7.

- Schaeffer, E., Smith, D., Mardon, G., Quinn, W., Zuker, C. (1989) Isolation and characterization of two new drosophila protein kinase C genes, including one specifically expressed in photoreceptor cells. *Cell* 57, 403-12.
- Schindler, C., Shuai, K., Prezioso V.R., Darnell J.E.J. (1992a) Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science* 257, 809-15.
- Schindler, C., Fu, X., Improta, T., Aebersold, R. Darnell, J.E.J. (1992b) Proteins of transcription factor ISGF-3: one gene encodes the 91- and 84-kDa ISGF-3 proteins that are activated by interferon α . *Proc. Natl. Acad. Sci. USA* 89, 7836-41.
- Schlessinger, J. (1986) Allosteric regulation of the epidermal growth factor receptor kinase. *J. Cell. Biol.* 103, 2067-72.
- Schorle, H., Holschke, T., Hunig, T., Schimpl, A., Horak, I. (1991) Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature*, 352, 621-24.
- Schutze, S., Berkovic, D., Tomsing, O., Unger, C., Kronke, M. (1991) Tumor necrosis factor induces rapid production of 1'2'diacylglycerol by a phosphatidylcholine-specific phospholipase C. *J. Exp. Med.* 174, 975-88.
- Seidler, N.W., Jona, I., Vegh, M., Martonosi, A. (1989) Cyclopiazonic acid is a specific inhibitor of the Ca^{2+} -ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* 264, 17816-23.
- Sharon, M., Klausner, R.D., Cullen, B.R., Chizzonite, R., Leonard, W.J. (1986) Novel interleukin-2 receptor subunit detected by cross-linking under high-affinity conditions. *Science* 234, 859-63.
- Sharon, M., Gnarr, J.R., Leonard, W.J. (1990) A 100-kilodalton protein is associated with the murine interleukin2 receptor: Biochemical evidence . *Proc. Natl. Acad. Sci. USA* 87, 4869-73.
- Shaw, P., Boverly, R., Tardy, S., Sahli, R., Sordat, B. (1992) Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. *Proc. Natl. Acad. Sci. USA* 89, 4495-9.
- Shearman, M.S., Sekiguchi, K. and Nishizuka, Y. (1989) Modulation of ion channel activity: a key function of the protein kinase C enzyme family. *Pharmacol. Rev.* 41, 211-32.
- Shen, S.S., Ricke, L.A. (1989) Protein kinase C from sea urchin eggs. *Comp. Biochem. Physiol.* 92B, 251-4.
- Shibuya, H., Yoneyama, M., Ninomiya-Tsuji, J., Matsumoto, K., Taniguchi, T. (1992) IL-2 and EGF receptors stimulate the hematopoietic cell cycle via different signaling pathways: Demonstration of a novel role for c-myc. *Cell* 70, 57-67.

- Shows, T., Eddy, R., Haley, L., Byers, M., Henry, M., Fujita, T., Matsui, H. and Taniguchi, T. (1984) Interleukin 2 (IL-2) is assigned to human chromosome 4. *Somatic Cell Mol. Genet.* 10, 315-18.
- Shuai, K., Schindler, C., Prezioso, V.R., Darnell, J.E.J. (1992) Activation of transcription by IFN- γ : tyrosine phosphorylation of a 91-kDa DNA binding protein. *Science* 258, 1808-14.
- Shuai, K., Stark, G.R., Kerr, I.M., Darnell, J.E.J. (1993) A signal phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. *Science* 261, 1744-8.
- Sigel, E. and Bauer, R. (1988) Activation of protein kinase C differentially modulates neuronal Na⁺, Ca²⁺, and gamma-aminobutyrate type A channels. *Proc. Natl. Acad. Sci. USA* 85, 6192-6.
- Simon, A.J., Milner, Y., Saville, S.P., Dvir, A., Mochly-Rosen, D., Orr, E. (1991) The identification and purification of a mammalian-like protein kinase C in the yeast *Saccharomyces cerevisiae*. *Proc. Roy. Soc. London, Ser. B: Biol. Sciences.* 243, 165-71.
- Slater, S.J., Cox, K.J.A., Lombardi, J.V., Kelly, M.B., Ho, C., Rubin, E., Stubbs, C.D. (1993) Inhibition of protein kinase C by alcohols and anaesthetics. *Nature* 364, 82-84.
- Slater, S.J., Kelly, M.B., Taddeo, F.J., Ho, C., Rubin, E., Stubbs, C.D. (1994) The modulation of protein kinase C activity by membrane lipid bilayer structure. *J. Biol. Chem.* 269, 4866-71.
- Slivka, S.R., and Insel, P.A. (1987) Alpha-adrenergic receptor-mediated phosphoinositide hydrolysis and prostaglandin E₂ formation in Madin-Darby canine kidney cells. *J. Biol. Chem.* 262, 4200-7.
- Smith, K.A. (1988a) Interleukin-2: inception, impact and implications. *Science* 240, 1169-76.
- Smith, K.A. (1988b) The interleukin 2 receptor. *Adv. Immunol.* 42, 165-76.
- Smith, M.K., Colbran, R.J., Soderling, T.R. (1990a) Specificities of autoinhibitory domain peptides for four protein kinases: Implications for intact cell studies of protein kinase function. *J. Biol. Chem.* 265, 1837-40.
- Smith, R.J., Sam, L.M., Justen, J.M., Bundy, G.L., Bala, G.A., Bleasdale, J.E. (1990b) Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase -dependent processes on cell responsiveness. *J. Pharmacol. Exp. Therap.* 253, 688-97.

- Souyri, M., Vigon, I., Penciolelli, J-F., Heard, J-M., Tambourin, P., Wendling, F. (1990) A putative truncated cytokine receptor gene transduced by the myeloproliferative leukemia virus immortalizes hematopoietic progenitors. *Cell* 63, 1137-42.
- Stabel, S. and Parker, P.J., Protein kinase C. (1991) *Pharmac. Ther* 51, 71-82.
- Stanimirovic, D.B., Ball, R., Mealing, G., Morley, P., Durkin, J.P. (1995) The role of intracellular calcium and protein kinase C in endothelin-stimulated proliferation of rat type I astrocytes. *Glia*, in press.
- Stern, J.B., Smith, K.A. (1986) Interleukin-2 induction of T-cell G1 progression and c-myc expression. *Science* 233, 203-7.
- Strom, T.B., Kelley, V.R., Murphy, J.R., Nichols, J., Woodworth, T.G. (1993) Interleukin-2 receptor-directed therapies: antibody- or cytokine-based targeting molecules. *Annu. Rev. Med.* 44, 343-53.
- Street, I.P., Lin, H-K., Laliberte, F., Ghomashchi, F., Wang, Z., Perrier, H., Tremblay, N.M., Huang, Z., Weech, P.K., Gelb, M.H. (1993) Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A₂. *Biochemistry*, 32, 5935-40.
- Stumpo, D.J., Graff, J.M., Albert, K.A., Greengard, P., and Blackshear, P.J. (1989) Molecular cloning, characterization, and expression of a cDNA encoding the "80- to 87-kDa" myristoylated alanine-rich C kinase substrate: a major cellular substrate for protein kinase C. *Proc. Natl. Acad. Sci. USA* 86, 4012-6.
- Suzuki, K., Tadakuma, T., Kizaki, H. (1991) Modulation of thymocyte apoptosis by isoproterenol and prostaglandin E₂. *Cell. Immunol.* 134, 235-40.
- Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum. *J. Biol. Chem.* 252, 7603-11.
- Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., Nishizuka, Y. (1979) Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.* 254, 3692-5.
- Takeshita, T., Asao, H., Ohtani, K., Ishii, N., Kumaki, S., Tanaka, N., Nakamura, M. and Sugamura, K. (1992) Cloning of the γ chain of the human IL-2 receptor. *Science*, 257, 379-82.
- Taki, T. and Kanfer, J.N. (1979) Partial purification and properties of a rat brain phospholipase. *J. Biol. Chem.* 254, 9761-5.

- Takuwa, N., Iwamoto, A., Kumada, M., Yamashita, K., Takuwa, Y. (1991) Role of Ca^{2+} influx in bombesin-induced mitogenesis in Swiss 3T3 fibroblasts. *J. Biol. Chem.* 266, 1403-9.
- Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., Tomita, F. (1986) Staurosporine, a potent inhibitor of phospholipid/ Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.* 135, 397-402.
- Tamura, M., Nogimori, K., Yajima, M., Ase, K., Ui, M. (1983) A role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of the biological effects on intact cells. *J. Biol. Chem.* 258, 6756-61.
- Tanaka, N., Asao, H., Ohbo, K., Ishii, N., Takeshita, T., Nakamura, M., Sasaki, H., Sugamura, K. (1994) Physical association of Jak1 and Jak2 tyrosine kinases with the interleukin 2 receptor β and γ chains. *Proc. Natl. Acad. Sci. USA* 91, 7271-8.
- Tang, E.K. and Houslay, M.D. (1992) Glucagon, vasopressin and angiotensin all elicit a rapid, transient increase in hepatocyte protein kinase C activity. *Biochem. J.* 283, 341-6.
- Taniguchi, T., Matsui, H., Fujita, T., Takaoka, C., Kashima, N., Yoshimoto, R. and Hamuro, J. (1983) Structure and expression of a cloned cDNA for human interleukin-2. *Nature* 302, 305-10.
- Taniguchi, T., Matsui, H., Fujita, T., Hatakeyama, M., Kashima, N., Fuse, A., Humuro, J., Nishi-Takaoka, C. and Yamada, G. (1986) Molecular analysis of the interleukin-2 system. *Immunol. Rev.* 92, 120-33.
- Taniguchi, T., Minami, Y. (1993) The IL-2/IL-2 receptor system: A current overview. *Cell* 73, 5-10.
- Taniguchi, T. (1995) Cytokine signaling through nonreceptor protein tyrosine kinases. *Science* 268, 251-255.
- Taylor, S.J., Chae, H.Z., Rhee, S.G., Exton, J.H. (1991) Activation of the β_1 isozyme of phospholipase C by α subunits of the G_q class of G proteins. *Nature* 350, 516-8.
- Tenniswood, M.P., Guenette, R.S., Lakins, J., Mooibroek, M., Wong, P., Welsh, J.E. (1992) Active cell death in hormone-dependent tissues. *Cancer Metastasis Rev* 11, 197-220.
- Teshigawara, K., Wang, H.-M., Kato, K., Smith, K.A. (1987) Interleukin 2 high-affinity receptor expression requires two distinct binding proteins. *J. Exp. Med.* 165, 223-38.
- Terai, C., Kornbluth, R.S., Pauza, C.D., Richman, D.D., Carson, D.A. (1991) Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1. *J. Clin. Invest* 87, 1710-22.

- Ting, A.T., Schoon, R.A., Abraham, R.T., Leibson, P.J. (1992) Interaction between protein kinase C-dependent and G protein-dependent pathways in the regulation of natural killer cell granule exocytosis. *J. Biol. Chem.* 267, 23957-62.
- Tomei, L.D., Kanter, P., Wenner, C.E. (1988) Inhibition of radiation-induced apoptosis in vitro by tumor promoters. *Biochem. Biophys. Res. Commun.* 155, 324-31.
- Torigoe, T., Saragovi, H.U., Reed, J.C. (1992) Interleukin 2 regulates the activity of the lyn protein-tyrosine kinase in a B-cell line. *Proc. Natl. Acad. Sci. USA* 89, 2674-8.
- Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baude, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D. and Kirilovskiy, J. (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase c. *J. Biol. Chem.* 266, 15771-81.
- Tracey, D.E., Wolfe, S.A., Durdik, J.M., and Henney, C.S. (1977) BCG-induced murine effector cells. I. Cytolytic activity in peritoneal exudates: an early response to BCG. *J. Immunol.* 119, 1145-54.
- Tritton, T.R. Cell death in cancer chemotherapy: the case of adriamycin, In: **Apoptosis: The molecular basis of cell death.** Current communications in cell and molecular biology, 3, Eds.: Tomei, L.D. and Cope, F.O. (New York: Cold Spring Harbour Laboratory Press), p66.
- Trouche, D., Robin, P., Robillard, O., Sassone-Corsi, P., Harel-Bellan, A. (1991) c-fos transcriptional activation by IL-2 in mouse CTLL-2 cells is mediated through two distinct signal transduction pathways converging on the same enhancer element. *J. Immunol.* 147, 2398-03.
- Tsuda, M., Kozak, R.W., Goldman, C.K., Waldmann, T.A. (1986) Demonstration of a non-Tac peptide that binds interleukin 2: a potential participant in a multi-chain interleukin 2 receptor complex. *Proc. Natl. Acad. Sci. USA* 83, 9694-8.
- Tsuda, M., Kitamura, F. and Miyasaka, M. (1989) Characterization of the interleukin 2 receptor β chain using three distinct monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* 86, 1982-8.
- Tuner, B., Rapp, U., App, H., Greene, M., Dobashi, K., Reed, J. (1991) Interleukin 2 induces tyrosine phosphorylation and activation of p72-74 Raf-1 kinase in T-cell line. *Proc. Natl. Acad. Sci. USA* 88, 1227-31.
- Uckun, F.M., Tuel-Ahlgren, L., Obuz, V., Smith, R., Dibirdik, I., Hanson, M., Langlie, M-C., Bedbetter, J.A. (1991) Interleukin 7 receptor engagement stimulates tyrosine phosphorylation, inositol phospholipid turnover, proliferation, and selective differentiation to the CD4 lineage by human feta thymocytes. *Proc. Natl. Acad. Sci. USA* 88, 6323-7.

- Uehara, Y., Hori, M., Takeuchi, T., Umezawa, H. (1986) Phenotypic change from transformed to normal induced by benzoquinonoid ansamycins accompanies inactivation of p60src in rat kidney cells infected with Rous sarcoma virus. *Mol. Cell. Biol.* **6**, 2198-206.
- Uehara, Y., Murakami, Y., Sugimoto, Y., Mizuno, S. (1989a) Mechanism of reversion of Rous sarcoma virus transformation by herbimycin A: reduction of total phosphotyrosin levels due to reduced kinase activity and increased turnover of p60v-src1. *Cancer Res.* **49**, 780-5.
- Uehara, Y., Fukazawa, H., Murakami, Y., Mizuno, S. (1989b) Irreversible inhibition of V-SRC tyrosine kinase activity by herbimycin A and its abrogation by sulfhydryl compounds. *Biochem. Biophys. Res. Commun.* **163**, 803-9.
- Ui, M. (1990) Pertussis toxin as a valuable probe for G-protein involvement in signal transduction. In: *ADP-ribosylation toxins and G proteins: insight into signal transduction*, Eds.: Moss, J. and Vaughan, M, p45-77.
- Ullrich, A. Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **51**, 203-212.
- Umekage, T., and Kato, K. (1991) A mouse brain cDNA encodes a novel protein with the protein kinase C phosphorylation site domain common to MARCKS. *FEBS Lett.* **286**, 147-51.
- Van Blitterswijk, W.J., van der Bend, R.L., Kramer, I.J.M., Verhoeven, A.J., Hilkmann, H., and de Widt, J. (1987a) A metabolite of an antineoplastic ether phospholipid may inhibit transmembrane signaling via protein kinase C. *Lipids* **22**, 842-6.
- Van Blitterswijk, W.J., Hilkmann, H., Storme, G.A. (1987b) Accumulation of an alkyl lysophospholipid in tumor cell membranes affects membrane fluidity and tumor cell invasion. *Lipids* **22**, 820.
- Valge, V.E., Wong, J.G.P., Datlof, B.M., Sinskey, A.J., Rao, A. (1988) Protein kinase C is required for responses to T cell receptor ligands but not to interleukin-2 in T cells. *Cell* **55**, 101-112.
- Vaux, D.L., Aguila, H.L., Weissman I.L. (1992) Bcl-2 prevents death of factor-deprived cells but fails to prevent apoptosis in targets of cell mediated killing. *Int. Immunol.* **4**, 821-4.
- Veals, S.A., Schindler, C., Leonard, D., Fu, X., Aebersold, R., Darnell, J.E.J., Levy, D.E. (1992) Subunit of an alpha-interferon-responsive transcription factor is related to interferon regulatory factor and myb families of DNA-binding proteins. *Mol. Cell. Biol.* **12**, 3315-21.
- Veillette, A., Bookman, M.A., Horak, E.M., Bolen, J.B. (1988) The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosin-protein kinase P56^{lck}. *Cell*, **55**, 301-9.

Vigon, I., Mornon, J-P., Cocault, L., Mitjavila, M-T., Tambourin, P., Gisselbrecht, S., Souyri, M (1992) Molecular cloning and characterization of MPL, the human homologue of the *v-mpl* oncogene: identification of a member of the hematopoietic growth factor receptor superfamily. *Proc. Natl. Acad. Sci, USA* 89, 5640-4.

Vujanovic, N.L., Herberman, R.B., Maghazachi, A. AL., Hiserodt, J.C. (1988) Lymphokine-activated killer cells in rats. III. A simple method for the purification of large granular lymphocytes and their rapid expansion and conversion into lymphokine-activated killer cells. *J. Exp. Med.* 167, 15-29.

Waldmann, T.A. (1991) The interleukin-2 receptor. *J. Biol. Chem.* 266, 2681-84.

Walker, P.R., Kwast-welfeld, J., Gourdeau, H., Leblanc, J., Neugebauer, W., Sikorska, M.(1993) Relationship between apoptosis and the cell cycle in lymphocytes: roles of protein kinase C, tyrosine phosphorylation and AP1. *Exp. Cell Res.* 207, 142-51.

Wang, A., Lu, S-D., Mark, D.F. (1984) Site-specific mutagenesis of the human interleukin-2 gene: structure-function analysis of the cysteine residues. *Science* 224, 1431.

Weaver, V.M., Lach, B., Walker, P.R., Sikorska, M. (1993) Role of proteolysis in apoptosis: involvement of serine proteases in internucleosomal DNA fragmentation in immature thymocytes. *Biochem. Cell. Biol.* 71, 488.

Weaver, V.M., Carson, C.E., Walker, P.R., Chaly, N., Lach, B., Raymond, Y., Brown, D.L., Sikorska, M. (1996) Degradation of nuclear matrix and DNA cleavage in apoptotic thymocytes. *J. Cell Science*, in press.

Williams, R.E., Chakravarthy, B.R., Sikorska, M., Whitfield, J.F., Durkin, J.P. (1992) In: *Peptides: Chemistry and biology*. Proceeding of the twelfth American Peptide symposium. Eds.: Smith, J.A. and Rivier, J.E. (Escom, Leiden, Germany), p923.

Wyllie, A.H., Kerr, J.F.R., Currie, A.R. (1980) Cell death: the significance of apoptosis. *Int. Cytol.* 68, 251-62.

White, D.A. (1973) in: *Form and Function of Phospholipids*, Eds.: Ansell, G.B., Hawthorne, J.N. and Dawson, R.M.C. pp444.

Whitman, M. and Cantley, L. (1988) Phosphoinositide metabolism and the control of cell proliferation. *Biochim. Biophys. Acta.* 948, 327-44.

Witthuhn, B.A., silvennoinen, O., Miura, O., Lai, K.S., Cwik, C, Liu, E.T., Ihle, J.N. (1994) Involvement of the JAK3 Janus kinase in IL-2 and IL-4 signaling in lymphoid and myeloid cells. *Nature* 370, 153-7.

Wolf, M., LeVine III, H., Stratford May Jr.W., Cuatrecasas, P. and Sahyoun, N. (1985) A model for intracellular translocation of protein kinase C involving synergism between Ca^{2+} and phorbol esters. *Nature* 317, 546-9.

Wroblewski and LaDue (1955) Serum glutamic oxalacetic transaminase activity as an index of liver cell injury: a preliminary report. *Annal. Internal Med.* 43, 345-8.

Yasuda, I., Kishimoto, A., Tanaka, S.-I., Tominaga, M., Sakurai, A., Nishizuka, Y. (1990) A synthetic peptide substrate for selective assay of protein kinase C. *Biochem. Biophys. Res. Comm.* 166, 1220-7.

Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., Oren, M. (1991) Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature* 352, 345-347.

Zmuidzinas, A., Mamon, H.J., Roberts, T.M., Smith, K.A. (1991) Interleukin-2-triggered Raf-1 expression, phosphorylation, and associated kinase activity increase through G1 and S in CD3-stimulated primary human T cells. *Mol. Cell. Biol.* 11, 2794-9.

APPENDIX : CHEMICAL SOLUTIONS**1. Complete CTLL-2 cell growing medium**

RPMI 1640 medium, 10% fetal bovine serum, 100 U/ml penicillin, 0.1mg/ml streptomycin, 0.2% sodium bicarbonate, 57 μ M 2-mercaptoethanol, and 100 U/ml IL-2.

2. Phosphate-buffered saline

140 mM NaCl, 4 mM KCl, 500 μ M Na₂HPO₄, and 150 μ M KH₂PO₄.

3. Solubilizing buffer for conventional PKC assay

50 mM Tris-HCl buffer (pH 7.5), 2 mM EGTA, 0.5 mM EDTA, and 0.1% Triton X-100.

4. Conventional assay buffer

50 mM Tris-HCl buffer (pH 7.5), 5 mM MgCl₂, 2 mM CaCl₂, 10 μ g phosphatidylserin, 1 μ M TPA, 100 μ M sodium vanadate, 100 μ M sodium pyrophosphate, 1 mM sodium fluoride, 100 μ M PMSF.

5. Direct PKC assay buffer

50mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 μ M CaCl₂, 100 μ M sodium vanadate, 100 μ M sodium pyrophosphate, 1 mM sodium fluoride, and 100 μ M PMSF.

6. Hypotonic Lysis buffer

1 mM NaHCO₃, 5 mM MgCl₂, 100 μ M phenylmethyl sulfonylfluoride.

7. SDS Solubilization buffer

10% glycerol, 5% β -mercaptoethanol, 3% SDS (w/v), 10mM Tris, pH 6.8, 0.01% (w/v) bromophenol blue.

8. Transfer buffer

192 mM glycine, 25 mM Tris-base (pH 8.3), 0.1% SDS in 20% (v/v) methanol.

9. Blocking Solution

1% BSA, 2.5 mM Tris-HCl, pH 8.0, 37.5 mM NaCl, 0.05% tween 20 (v/v).

10. TBST Solution

2.5 mM Tris-HCl, pH 8.0, 37.5 mM NaCl, 0.05% tween (v/v).

11. Color development solution

100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂, 0.01 % (w/v) nitro blue tetrazolium (NBT), 0.02 % (w/v) 5-bromo-4-chloro-3-indolylphosphate (BCIP).

12. Lysis buffer for detecting DNA fragmentation

5 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 0.5% Triton X-100.

13. Tris/EDTA buffer

10 mM Tris-HCl, pH 7.5, 1 mM EDTA.

14. Normal buffer solution (NBS)

140 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.1 mM MgCl₂, 2.6 mM dextrose, and 10 mM HEPES.

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(1) Full Papers:

D. Warren, V. Pasupuleti, Y. Lu, B. Platler and R. Horton. (1990) Tumor necrosis factor and interleukin-1 stimulate testosterone secretion in adult male rat Leydig cells in vitro. *J. Andrology* 11,353-360.

Y. Lu, A. McDonough, R. Farley and D. Warren. (1991) Regulation of testicular P450 cholesterol side chain cleavage and P450c17-20 lyase/c17 hydroxylase enzymes in the neonatal and adult rat. *Acta Endocrinologica (Copenh)* 123, 449-454.

Y. Lu, R. Tremblay, H. Jouishomme, B. Chakravarthy, and J.P. Durkin. (1994) Evidence that the activation of an inactive pool of membrane-associated protein kinase C is linked to the IL-2-dependent survival of T lymphocytes. *J. Immunol.* 153, 1495-1504.

(2) Abstracts:

1. V. Pasupuleti, R. Horton, Y. Lu, B. Platler, and D. Warren. "Tumor necrosis factor and interleukin-1 are potent secretagogues of Leydig cell secretion of testosterone". Presented at *the Western Society for Clinical Research*, Carmel, California, February 1989.

2. D. Warren, V. Pasupuleti, Y. Lu, B. Platler, and R. Horton. "Stimulatory effects of tumor necrosis factor and interleukin-2 on adult rat Leydig cells in culture". Presented at *the American Society of Andrology Annual Meeting*, New Orleans, Louisiana, April 1989.

3. D. Warren, V. Pasupuleti, B. Platler, Y. Lu, and R. Horton. "Tumor necrosis factor and interleukin-1 inhibit testosterone secretion in cultured fetal rat testis cells but stimulate testosterone production in adult rat testis cell". Presented at *the Endocrine Society Annual Meeting*, Washington, June 1989.

4. D. Warren, V. Pasupuleti, Y. Lu, B. Platler, and R. Horton. "Testosterone production by the adult and fetal rat testis in response to cytokines: Stimulation in the adult and inhibition in the fetus". Presented at *Sixth European Workshops on Molecular and Cellular Endocrinology of the Testis*, Mariehamn, Åland island, Finland, May 1990.

5. Y. Lu, Tremblay, B. Chakravarthy, and J.P. Durkin. "Interleukin-2 stimulates the rapid activation of membrane protein kinase C (PKC) activity without the translocation of cytosolic enzyme". Presented at *the CFBS annual meeting*, Windsor, Ontario, June 1993.

6. Y. Lu, R. Tremblay, B. Chakravarthy and J.P. Durkin. "The rapid activation of inactive membrane protein kinase C (PKC) activity by interleukin-2 is linked to the survival of IL-2-dependent Cells". Presented at *the CFBS annual meeting*, Montreal, Quebec, June 1994.

7. Y. Lu, J.P. Durkin. "The mechanisms of protein kinase C activation in T-lymphocytes: possible involvement of phospholipase C and phospholipase A₂". Presented in *Cytokines and Cytokine Receptors*, Lake George, New York, October 1995.