CONTROL OF PROLIFERATION IN THE RAT THYMUS

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SUMMARY

Quiescent rat thymocytes were stimulated to divide by a variety of agents. One such mitogen was the neurotransmitter acetylcholine which exhibited a biphasic action. Interaction with low affinity nicotinic receptors was linked with an obligatory requirement for magnesium ions whereas combination with high affinity muscarinic receptors induced mitosis only if calcium ions were present in the medium. Binding of acetylcholine to its muscarinic receptor enhanced calcium influx and increased intracellular calcium levels causing calmodulin activation, a necessary prelude to DNA synthesis and mitosis. Nicotinic receptor activation may be associated with a magnesium influx and stimulation of cells in a calmodulin-independent fashion.

Parathyroid hormone and its analogues exhibited only a monophasic mitogenic action. This response was linked to calcium influx, a rise in cytosolic calcium and calmodulin activation. Parathyroid hormone did not stimulate adenylate cyclase in thymocytes and decreased cellular cyclic AMP concentrations.

Picomolar amounts of interleukin-2 (IL-2) also stimulated division in thymocytes derived from 3-month old rats by binding to high affinity receptors. The response in thymocytes from newborn and foetal animals was greater reflecting the larger proportion of cells bearing receptors at this age. The mitogenic effect of IL-2 was abolished by a monoclonal antibody directed against the IL-2 receptor. Injections of IL-2 itself or the administration of IL-2 secreting activated syngeneic spleen cells also stimulated proliferation of both thymus and bone marrow cells in vivo. Likewise immunisation with pertussis toxin, which enhances endogenous IL2 production, also increased mitosis in these tissues. Calcium influx, increased cytosolic Ca^{2+} levels and calmodulin activation are associated features of the mitogenic action of IL-2.

Interleukin-1 was also found to be mitogenic in thymic lymphocyte cultures. The responses to this mitogen and to parathyroid hormone and acetylcholine were not inhibited by the anti-IL2 receptor antibody suggesting that the thymic lymphocyte bears discrete receptors for these agents. Subtle interactions of hormones, neurotransmitters and interleukins may thus contribute to the turnover and control of lymphoid cells in the thymus and perhaps bone-marrow.

Key words: Thymocytes
proliferation
Interleukins
hormones
cations

To

my parents

and

family

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CHAPTER ONE

STIMULUS-MITOSIS COUPLING

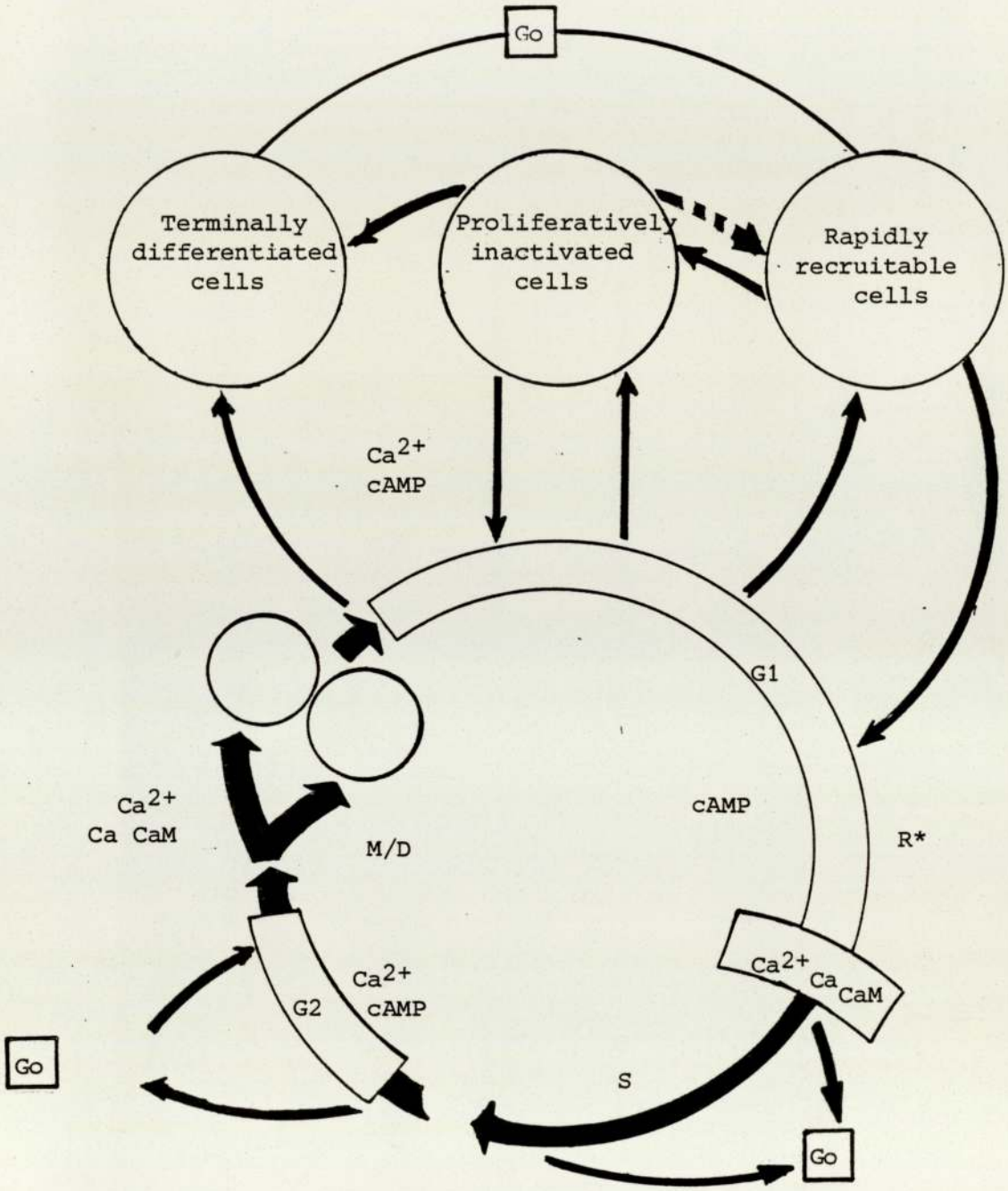
For cell growth and division to occur, a large variety of metabolic processes must be carefully co-ordinated. Through evolutionary pressures, specific hormones and growth factors have acquired the ability to trigger such a complex 'pleiotropic growth response' in target cells. This response is mediated by specific cellular receptors and intracellular messengers.

1.1 THE CELL CYCLE

The cell cycle is defined as the interval between completion of mitosis in the parent cell and completion of the next mitosis in one daughter cell or both (Mitchison, 1971). Conventionally, the cell cycle is divided into four unequal and biochemically distinguishable phases, each based on a temporal relationship to the period of DNA synthesis (Howard and Pelc, 1953). The cycle begins with the birth of the cell at cytokinesis or cell division. As the cell ages it goes through a gap phase, G_1, during which it prepares for replication of its chromosomes. This is followed by a DNA synthetic-phase, S, and a further gap phase, G_2 where it prepares for imminent mitosis. Finally the cell undergoes cytokinesis which ultimately gives rise to two daughter cells. (Diagram 1).

The length of the cell cycle is the sum of the lengths of its component processes, and the probability of a cell having reached a certain point in the cycle is entirely a function of its postmitotic age (Liskay and Prescott, 1978).

Recent evidence indicates that not all cycles are of the quadriphase (G1—S—G2—M) kind; indeed the G1 and G2 phases may not be essential. Measurement of the phases has shown that while variability exists in S and G2



R* - Restriction point

Diagram 1 A diagrammatic model of the cell cycle (see text for details)

Adapted from Perris et al (1983)

phases (Gelfant, 1977; Drewinko et al, 1985), G1 represents the most variable phase of the cell cycle. The length of G1 regulates the rate of cell proliferation. In some very rapidly proliferating cell lines (several transformed mammalian cell lines and tumour cells) there is no detectable G1 period (Prescott, 1976; Wynford Thomas et al, 1985).

The mechanisms controlling the length of G1 are still not fully understood but most current hypotheses suggest that much of the interval is accounted for by the time taken for growth to achieve a given cell size. (Cooper, 1979; Prescott et al, 1982; Wynford Thomas et al, 1985). In fact, Prescott and co-workers (1982) consider that the cell cycle comprises two interdependent, yet different subcycles; a deterministically programmed chromosome cycle (S + G2 + M) and a growth cycle (G1) subject to environmental conditions. Thus, if doubling in cell size is completed as rapidly as the chromosome cycle, the cell cycle lacks a G1 period. The slower the growth cycle in relation to the chromosome cycle, the longer the G1 period becomes. The hypothesis of Prescott and co-workers completely disregards the possibility of a unique event in G1 which might regulate the rate of proliferation and it does not account for most of the proliferative responses observed in different cell types under in vivo conditions.

Smith and Martin (1973) have proposed a different model for control of cell proliferation. In their simple random transition model, the cell cycle consists of a probabilistic A state (corresponding to G1) and a deterministic B state (S + G2 + M). The length of the cycle is a function of the probability of the random transition of the cell from the A to the B state governed by the unique transition point (probably attributed to certain biochemical differences) and the duration of the chain of events in the B state. Furthermore, the likelihood of a cell having reached a particular point in its cycle is a function of

its transition probability rather than postimitotic age (Smith and Martin, 1973).

Although the hypotheses for variability in the cell-cycle are controversial, it is evident that regulatory events occur during G1 that direct the progression of the cells through the cycle (Pardee et al, 1986). Pardee (1974) advanced the concept of a unique, precisely timed biochemical restriction point (R point) in the G1 phase, beyond which neither the mitogen nor rapid transcription and translation are imperative for commitment to cellular proliferation (Zetterberg and Larson, 1985; Yang and Pardee, 1986). This control point is perhaps related to the 'master-initiator' paradigm of the Smith and Martin model (1973) and to the early G1 serum-sensitive or labile protein (Rossow et al, 1979) which, in turn, may influence the assembly of enzymes or proteins into the so-called multiprotein 'replitase' complex associated with onset of DNA synthesis (Coppock and Pardee, 1985; Pardee et al, 1986). In addition, a multitude of other G1-phase associated events can also determine whether a cell becomes quiescent or proceeds into DNA synthesis. Under sub-optimal conditions of high cell density and nutrient deprivation, some cycling cells leave the cell cycle in G1 phase and enter a discrete quiescent compartment, 'Go' (Lajtha, 1963). 'Go' is a broad term encompassing a wide range of metabolic states. One such state includes terminally differentiated cells such as neuronal cells and erythrocytes incapable of entering the cycle, whilst a second cohort contains quiescent cells eg. peripheral blood lymphocytes and hepatocytes which can be slowly recruited in response to an appropriate stimulus such as antigen activation or partial hepatectomy (Whitfield et al, 1979; Klaus and Hawrylowicz, 1984). These proliferatively-inactivated cells have reversibly repressed proliferogenic genes and may be distinguished from the third population of rapidly recruitable 'Go' cells. Such dormant cells eg. thymic lymphoblasts and bone marrow cells may have accomplished certain

preludes to DNA synthesis but are restricted from entering G1 by temporary lack of an essential component such as growth factor/essential amino acid. The longer the duration of the quiescent phase the more likely is the degradation of labile protein and/or repression of appropriate genes pertinent for proliferogenic activity. Besides a G1 arrest, some cells may undergo quiescence in the S and G2 phases possibly due to their disparate requirements for transit through the cycle (Melchers and Lernhardt, 1985). Different restriction points in the cell cycle have certainly been observed in mammalian cells of different lineages (Gelfant, 1977; Cooper, 1979; Melchers and Lernhardt, 1985). Thus, the classic 'Go' compartment may be composed of quiescent cells (Q cells) arrested in all stages of the cell cycle - QG1, QS and QG2 (Rotenberg, 1982; Drewinko et al, 1985).

Regardless of the state of quiescence, appropriate chemical factors or stimuli are required at relevant positions for effective progression through the cell cycle. This progression may be mediated by growth factors and can occur in competent activated cells (Scher et al, 1979; O'Keefe and Pledger, 1983; Pardee et al, 1985).

1.2 GROWTH REGULATING AGENTS

Amongst the various factors involved in regulation of growth, one particular group collectively referred to as polypeptide growth factors (PGFs) and representing a large family of regulatory agents has gained particular prominence. Recently, major progress has occurred in the identification and isolation of about three dozen PGFs. The gene sequences, mRNA and amino acid sequences of these PGFs have now been determined along with the structural analysis of their receptor (James and Bradshaw, 1984).

No single description or definition encompasses these growth factors.

However, all are polypeptides and their actions are mediated by binding to specific high affinity cell membrane receptors. This leads to anabolic cellular metabolism and the modulation in expression of specific genes either at transcription or translation levels. Although second messenger generation as a result of the formation of the PGF receptor complex seems likely, no single universal messenger can exclusively account for the actions of these factors. After binding, PGFs and their receptors are internalised by receptor-mediated endocytosis ultimately resulting in degradation of the ligand and the receptor as well (James and Bradshaw, 1984; Wileman et al, 1985).

Some growth factors act in a classical endocrine manner via the blood stream, eg. insulin like growth factors (IGFs) (Froesch et al, 1985), others diffuse over shorter distances affecting adjacent cells in paracrine fashion, eg. lymphokines (Bocci, 1985) whilst others may exert an autocrine action and stimulate growth of the cell which actually produces it, eg. transforming growth factors (TGFs) found in culture fluids of transformed murine cells (DeLarco & Todaro, 1978; Kaplan et al, 1982; Sporn & Roberts, 1985). For maximum stimulation of cell division several different growth factors may be required (Pledger et al, 1978). Indeed, available data are consistent with a model of cell cycle control in which at least two signals are needed for proliferation of non-transformed cells. Exposure of a cell to one growth factor can lower the threshold for mitogenicity of a second growth factor (O'Keefe & Pledger, 1983). Moreover, growth factors operate at different points of the cell cycle (Olashaw & Pledger, 1985). Thus classified into competence and progression factors, the first set of factors render cells competent to divide in response to the second class of factors which allow progression into division (Scher et al, 1979). The latter are insulin-like and include the somatomedins which have a very broad target specificity and tissue distribution and are

present in plasma at concentrations sufficient to induce mitosis. The former, eg. platelet derived growth factors (PDGFs) however confer proliferative competence on cells rendering them responsive to IGFs. They have a restricted target specificity and act predominantly in the locality in which they are produced, unlike the systemically active insulin-like factors (Olashaw & Pledger, 1985). Thus, the multiplicity of growth factors in various tissues, the varying cell specificity of GFs, and the requirement for multiple GFs for stimulation of specific cell types presumably provides the fine tuning of relative proliferation rates necessary for co-ordinated growth of cells to form tissues during development and to maintain tissues in the adult state.

Those growth factors which have been most widely studied and characterised are described briefly below so that the studies on primary lymphoid cell proliferation which form the basis of this thesis may be better placed in context.

Epidermal growth factor (EGF) was first described by Cohen (1962) as a peptide which stimulates precocious eyelid opening and tooth eruption in new-born mice. As the name implies, it promotes growth and proliferation of a variety of epithelial and mesenchymal cells in culture (Cohen & Carpenter, 1975), although the potential to inhibit division and promote differention has also been ascribed to it (Carpenter & Cohen, 1976). The precise role in vivo is difficult to ascertain with certainty. Classical glandular extirpation is impossible since it is synthesised at several sites and acts in paracrine fashion over very short distances (Oka et al, 1983; Fallon et al, 1984). Antibodies against EGF also fail to affect EGF levels (Gregory et al, 1979) since it has a very high turnover rate (half-life 1.5 minutes) in vivo. Antisera directed against the EGF receptor may prove more useful in defining the consequences of a lack of an EGF signal in various tissues (Stoscheck & Carpenter, 1983).

EGF is synthesised as a large precursor in a number of locations including the submaxillary gland, Brunners gland and kidney (Rall et al, 1985; Scott et al, 1985). It is also to be found in various body fluids including urine; human urogastrone is probably identical to EGF (Carpenter, 1985; Gregory, 1985). The larger precursors yield mature EGF, a 6 KDa polypeptide of 53 amino acids displaying 3 internal disulphide bonds (Taylor et al, 1972).

The cellular receptor for EGF is the best understood and has served as a paradigm for the others. It is found in the plasma membrane of a large variety of cells as a 170 KDa protein. There is an extracellular binding domain, a transmembrane region, and an intracellular domain exhibiting the tyrosine kinase function and presumably binding sites for ATP phosphorylation substrates (Cohen et al, 1982). In response to EGF, the receptor is capable of autophosphorylation on tyrosine residues. Also exposure of cells to EGF results in a decrease in the cellular content of homologous receptors, a process termed 'down regulation' probably due to receptor-mediated endocytosis (Hopkins et al, 1985).

Transforming Growth Factors (TGFs) are a family of polypeptides that reversibly induce non-neoplastic cells to express the transformed phenotype (Todaro et al, 1980). Initially discovered in the culture fluids of Moloney sarcoma virus transformed mouse cells (DeLarco & Todaro, 1978) and termed 'sarcoma growth factor', these heat and acid stable low molecular weight (5-30 KDa) proteins have been characterised into two distinct molecular entities: TGF type α and TGF type ß.

TGFα: This is a single chain polypeptide (MW 5,700) which interacts with the EGF receptor probably due to a similarity in conformation, especially to the structural homology in the third disulphide loop which may represent the receptor binding domain (Tam et al, 1984; Nestor et al, 1985). These TGFs

mimic most of the actions of EGF. For instance, TGFα resembles EGF in the stimulation of cell growth and promotion of precocious tooth eruption and eyelid opening in new born mice in vivo (Smith et al, 1985). Some actions of these closely related peptides are however different. For example, in the induction of cell ruffling (Myrdal, 1985) and in the promotion of calcium release from foetal rat long bones in vitro TFG-α is more potent than EGF (Ibbotson et al, 1986; Stern et al, 1985). Moreoever, TGF-α is more effective than EGF in promoting angiogenesis and as it is secreted by a variety of human tumours, it may contribute to tumour-induced angiogenesis. Apart from its role as an autocine growth regulator (Sporn and Todaro, 1980), TGF-α may be important in malignancy-associated neovascularisation, contributing to the generation of a local microenvironment that is favourable for solid tumour growth (Schreiber et al, 1986). Despite such function, TGF-α is present in non-neoplastic tissues such as embryos and placenta and in adult urine (Twardzik et al, 1982; Lee et al, 1985). In fact TGF-α may represent the embryonic form of EGF that is inappropriately expressed in certain neoplastic cells.

TGFβ: This differs from the type α in molecular composition, receptor binding and thus not surprisingly in the biological responses it elicits. It has been purified as a 20 KDa disulphide-linked homodimer from both normal and transformed cells, and from normal and neoplastic tissues (Assoian et al, 1983; Roberts et al, 1983; Derynck et al, 1985). TGFβ is also present in foetal tissues (Proper et al, 1982; Centrello & Canalis, 1985) implying a probable role in tissue growth and maturation during mammalian development (Hill et

al, 1986). Originally characterised by its synergistic action with EGF (or TGFα) to cause anchorage-independent growth of normal rat kidney (NRK) cells in soft agar, TGFβ has now been shown to antagonise the mitogenic effects of these two peptides on NRK cells in monolayer culture (Roberts et al, 1985). In fact, TGFβ is the same molecular entity as the inhibitor released by confluent BSC-1 kidney cells (Holley et al, 1978; Tucker et al, 1984). A growth inhibitory effect has been observed in both neoplastic and normal cells including thymocytes, peripheral T-lymphocytes and embryonic rat fibroblasts (Roberts et al, 1985; Ristow, 1986; Kehrl et al, 1986). A natural inhibitory role for TGFβ in the control of lymphoid cell proliferation has been suggested. When T cells are activated with phytohaemagglutinin, PHA, TGFβ is produced in significant quantities between 2-4 days after stimulation and may be capable of inhibiting T cell proliferation. Exogenous addition of TGFβ certainly inhibits the usual IL2-induced upregulation of IL2 and transferrin receptors and blocks subsequent cell proliferation. Besides its potential as an autoregulatory lymphokine, the T cell produced TGFβ may be important in T cell interaction with other cell types, eg. fibroblasts for collagen synthesis during wound healing (Kehrl et al, 1986). Additionally, TGFβ regulates the nature of the cellular response to various differentiation signals including the differentiation of B lymphocytes to an immunoglobulin secreting state (Sporn & Roberts, 1985).

Platelet Derived Growth Factor (PDGF)

The well known growth-promoting effects of serum may be largely attributable to the presence in it of PDGF at a concentration of about 50 ng ml^{-1}

(Antoniades, 1984). This heterodimer (Waterfield et al, 1983) is synthesised in large vessel endothelial cells (Dicorleto & Bowen-Pope, 1983) and aortic smooth muscle cells of newborn but not adult rats (Siefert et al, 1984). Presumably megakaryocytes can also synthesise it since it is stored in α granules of platelets to be released during clotting when it promotes wound healing and exerts a chemotactic effect on fibroblasts and smooth muscle cells (Ross & Vogel, 1978; Deuel & Huang, 1984). Additionally, PDGF-related proteins have been detected in the placenta and transformed cell lines (Bowen-Pope et al, 1984; Niman, 1984) ascribing an important role for these proteins during embryonic development and in pathogenic processes (Stroobant, 1985). The mitogenic action on various cells of mesenchymal origin is of restricted duration because of the receptor down-regulation via internalisation (Heldin et al, 1985). Some authors maintain PDGF alone can induce division in target cells (Shipley et al, 1985) whereas others indicate PDGF only confers upon the cells the competence to respond to additional progression factors (Olashaw & Pledger, 1985; Pardee et al, 1985).

Nerve Growth Factor (NGF)

NGF, a polypeptide of 118 aa (Scott et al, 1983) has been detected in various normal and neoplastic cell lines (Young et al, 1975; Lindsay, 1979) and several exocrine fluids (Bradshaw, 1978; Harper et al, 1982). It is still the only protein to act as a trophic factor in vivo, where it supports the survival of sensory and sympathetic neurons (Calissano et al, 1984). Thus, administration of anti-NGF antibodies to embryonic or young animals results in massive destruction of sympathetic and sensory ganglia (Aloe et al, 1985). In fact, the density of sympathetic innervation is a function of the levels of this factor in its target tissues (Korsching & Theonen, 1983). In the adult, NGF along with

other proteins of the extracellular matrix is pertinent for maintenance of nerves (Edgar, 1985). Although these actions of NGF are mostly confined to neural-crest derived cells, proliferation in a non-neuronal cell type, the mast cell has been reported upon administration of NGF to the neonatal rat (Aloe & Montalcini, 1977). NGF is also mitogenic for cultured rat adrenal chromaffin cells (Lillien & Clauda, 1985). These effects are mediated by retrograde transportation of internalised NGF along axons to the neuronal cell bodies or perikaryons; interference in this process may cause death of young neurons implying that the retrograde transport is crucial, at least for the trophic action of NGF (Yanker & Shooter, 1982; Bradshaw et al, 1985).

Insulin-like Growth Factors I and II

The role of growth hormone (GH) as the principal regulator of balanced growth has long been recognised (Salmon & Daughaday, 1957). Considerable evidence indicates that the proliferogenic propensity of GH is attributable to an intermediary series of mitogenic factors, the somatomedins (Phillips & Vassipoulous, 1980). Two of these chemically related peptides which stimulate division in fibroblasts and chondrocytes are IGF I and IGF II (Salmon & Hosse, 1971; Humbel, 1985; Froesch et al, 1985). While IGF I is a basic peptide of 70 amino acids, IGF II is a neutral peptide composed of 67 amino acids. These molecules bear marked structural homology with insulin and thus exhibit anabolic insulin-like actions on adipose and muscle tissues which cannot be suppressed by anti-insulin antibodies (Clemmons & Vwyk, 1985). Additionally, infusion of recombinant human IGF I restores normal growth in diabetic rats (Scheiwiller et al, 1986). The major source of both IGFs is the liver (Rechler et al, 1979; Scott et al, 1985) and when released from this organ, IGFs circulate in the plasma and other extracellular fluids

reversibly bound to large carrier proteins (Nissley & Rechler, 1985). These carrier proteins apparently prolong the half-life of IGFs and constitute a delivery system for release of IGFs to the tissues. The relative abundance of IGF I in the liver and the plasma is determined by the GH secretory status of the animal (Roberts et al, 1986; D-Ercole & Underwood, 1986). IGF-I levels are certainly low in GH-deficient animals and raised in GH-secreting pituitary tumours (Scott et al, 1985). Injections of GH to GH-deficient animals may partially restore hepatic and circulatory levels of IGF-I and cause longitudinal bone growth (Zapf et al, 1981; Van Wyk, 1985; Roberts et al, 1986). In contrast to IGF I, IGF II is only partially GH-dependent and its role in postnatal growth is not clear. It appears that IGF I may be the predominant adult growth factor while IGF II may be its embryonic and neonatal counterpart at least in rodents (D'Ercole & Underwood, 1986). Contrary to the above evidence, a direct effect of GH on cartilage both in vivo and in vitro has also been reported (Isaksson et al, 1982; Russell & Spencer, 1985). These disparate observations can probably be accounted for by the dual effector theory in which the preferred target cells of IGF I action are created by the direct action of growth hormone (Zezulak & Green, 1986).

Both IGF I and IGF II appear to have their own receptor to which they preferentially bind, although cross-reaction is seen at high growth factor concentrations (Rechler & Nissley, 1985). The cellular receptors for IGF I (type 1 receptor) show homology to the insulin receptor, a heterotetrameric (450 KDa) complex consisting of 2 transmembrane β sub-units each disulphide bonded to one α subunit (Czech et al, 1983). The α subunit provides the insulin (or IGF) binding domains, whereas the β sub-units possess ATPase and tyrosine kinase activities (Van Obberghen et al, 1983).

The type II receptor (preferential for IGF II) is a single chain 250 KDa component (Czech et al, 1983) and may not undergo ligand-induced down regulation. The responsiveness of cells to insulin or IGFs therefore depends on the type of receptors, their affinities and the ability of the individual receptors to trigger the various biological responses in particular cell types (Czech et al, 1983; Van Wyk et al, 1985).

Fibroblast Growth Factors (FGFs)

Extracts of bovine neural and pituitary tissue contain a family of growth factors mitogenic for cultured fibroblasts and vascular endothelial cells (Gospodarowicz et al, 1976; Gospodarowicz, 1985). Termed FGFs, these factors are apparently single chain 14-18 KDa peptides. Until recently elusive, these peptides may be acidic or basic; however they may show similar features such as sequence and antigenic relatedness (Thomas et al, 1984; Esch et al, 1985). Pituitary FGF and brain FGF appear to be identical (Gospodarowicz et al, 1984). Interestingly, a slight homology between FGF and a macrophage derived factor, interleukin 1 has also been observed; the precise significance is not year clear (Thomas et al, 1985). Other members of the family include endothelial cell growth factor, chondrosarcoma growth factor and heparin-binding growth factor from bovine pituitary and hypothalamus. (Shipley et al, 1985). FGFs stimulate cell migration, cell proliferation, stabilisation of phenotypic expression and cellular senescence in its target cell types presumably by an increased synthesis of extracellular matrix and its components (Gospodarowicz, 1985). FGFs may cause cellular differentiation during blastema formation such as angiogenesis and formation of granulation tissue. A paracrine role for FGF in the stimulation of tumour angiogenesis has

also been suggested (Folksman, 1983).

Haemopoietic Growth Factors (HGFs)

The production of blood cells in mammalian organisms is regulated by complex cellular interactions between a set of progenitor cells and appropriate growth stimulatory factors (Burgess et al, 1977). These factors are present in conditioned media from a variety of normal and leukaemic cell lines and also from activated T-lymphocyte populations (Whetton & Dexter, 1986). Most of these regulatory factors have now been well characterised. HGFs have been categorised into multipotent growth factors, eg. Interleukin 3 (IL3) and granulocytic-macrophage colony stimulating factor (GM-CSF) and lineage restricted growth factor, eg. erythropoietin (EPO). HGFs are an absolute requirement for the survival, proliferation and development or self renewal of their target cells when cultured in vitro in the absence of stromal cells (Burgess, 1985). Lack of growth factor may lead to a rapid loss of progenitor cells presumably due to a decrease in the primary metabolism of the cell (Whetton & Dexter, 1986). Besides regulating proliferation and development, HGFs can activate mature cell functions in vivo. For example, GM-CSF can stimulate production of plasminogen activator and prostaglandin E2 by macrophages, increase parasite killing by macrophages and killing of neoplastic cells by granulocytes. Such activities may be of major importance in wound healing and combating infectious diseases during localised production of such agents at a site of inflammation or infection.

Both murine and human leukaemic cells require stimulatory activity to proliferate in vitro. A leukaemic murine cell WEHI-3B secretes IL3 which may be associated with the initial transformation of these cells (Ymer et al, 1985). Presumably, the leukaemic cell resembles a haemopoietic progenitor cell which

displays an imbalance between proliferation and development such that the facility to self-renew is inappropriately expressed (Dexter et al, 1985).

The HGFs also include the macrophage- and lymphocyte-derived lymphopoietic factors, termed as Interleukin-1 (IL-1) and Interleukin-2 (IL-2) respectively. These factors influence the proliferation and differentiation of lymphocytes and will be discussed while considering differentiation in lymphoid tissues in the next chapter as they form the major thrust of this thesis. In addition to the above growth factors, there are unquestionably classical endocrine hormones which also have characteristic growth-promoting effects. These include the steroid hormones, oestradiol and progesterone which are essential for mitotic activity in development of secondary sex organs. The effects of oestradiol in its target tissues may be exerted by certain inducible growth factors, termed 'oestromedins' that enter the circulation and promote the growth of distant target tissues. Potential sources for these factors are the uterus, kidney and pituitary for mammary cells, and uterus, kidney and hypothalamus for pituitary cells (Sirbasku et al, 1985). Other trophic hormones which may also act via secondary mediators are the ACTH, TSH and FSH/LH. Thus, circulating factors in the serum may mediate the growth-promoting activities of serum and hence contribute tremendously to growth.

While focussing attention on growth regulating agents, it is worthwhile considering the negative or inhibitory growth regulatory molecules that may function in cellular homeostasis possibly by counteracting the positive factors discussed above. The existence of endogenous inhibitors of cell division termed 'chalones' was first suggested by studies on epidermal wound healing and carcinogenesis (Bullough 1975; Iversen, 1981). Although, to date, a number of chalones have been shown to reversibly inhibit proliferation of their

native tissues, the concept of chalones has remained elusive. Produced continually by differentiated cells within an organ or tissue, these molecules inhibit tissue growth when their local extracellular level reaches a predetermined concentration. They cause a mitotic blockade arresting cells in a quiescent or Go state. Removal of the chalone producing cells automatically reduces the concentrations of the inhibitory factor and allows restoration of cell division until the crucial inhibitory level of their chalone is attained (Bullough, 1975). The very existence of chalones has however been challenged by several investigators. A major setback in developing the chalone concept experimentally has been the formidable problem of isolation and chemical characterisation of these molecules.

More recently, several endogenous inhibitors of cell proliferation have been purified from various sources including normal tissues (Assoian et al, 1983) and conditioned medium from epithelial (Holley et al, 1980) and fibroblast cell lines (Hsu & Wang, 1986). A growth inhibitor purified from kidney epithelial cell line BSC-1 derived from the African green monkey (Holley et al, 1980; Nilsen-hamilton & Holley, 1983) appears to be structurally and functionally identical to TFGβ (Tucker et al, 1984). A factor isolated from the rat liver reversibly inhibits the proliferation of non-malignant cells in cultures, whilst exerting no effect on the proliferation of hepatoma cells (McMahon et al, 1982). Similarly, a factor from bovine mammary glands inhibits the growth of Ehrlich ascites mammary carcinoma cells in vitro. The growth inhibitory effect on Ehrlich ascites cells can be abolished by antisera raised in mice and also by EGF (Bohmer et al, 1985). It seems likely that such cells although inhibited, are still in a competent activated state and can be easily recruited into the cell-cycle.

Density-inhibited 3T3 cells release a single soluble polypeptide, the fibroblast growth regulator (FGR) into the culture medium (Steck et al, 1982). If this is neutralised by monoclonal antibody directed against it growth resumes illustrating the autocrine role of FGR in growth arrest. (Hsu et al, 1984). Alternatively, membrane components may be involved in growth inhibition at high cell density. Addition of cell-surface derived fragments (isolated from 3T3 fibroblasts) to sparsely seeded proliferating cells leads to cessation of growth by arresting the cells in the G1 phase of the cycle in a manner identical to that at high cell density (Petersen & Larch, 1983; Weiser et al, 1985). Such contact inhibition of growth apparently occurs via plasma membrane glycoproteins (Weiser & Oesch, 1986). The physiological significance of such membrane-associated factors however remains unclear.

Other growth inhibitors may include (a) a glycopeptide from bovine cerebral cortex cells which inhibits growth of normal but not transformed cells (Kinders & Johnson, 1982); (b) a heparin-like molecule produced by cultured endothelial cells which inhibits growth of smooth muscle cells (Castellot et al, 1981) and (c) protein factors derived from lymphocytes, termed interferons (IFNs). βIFNs exert a transient inhibitory non-cytotoxic effect on haematopoietic cells by promoting the arrest of these cells in Go/G1 as part of their terminal differentiation via an autocrine mechanism (Yarden et al, 1984). Such antiproliferative effects of interferon are also evident on additions of IFN to lymphoid cells (Siegel et al, 1986) and non-lymphoid cells stimulated with serum or growth-factors (Taylor-Papadimitrious et al, 1985; Einat et al, 1985). Although the mechanism of action has not yet been identified (Taylor-Papadimitrious et al, 1985), it is plausible that IFN-activated genes/protein products (Revel & Chebath, 1986) may block the Go/G1 — S transition by inhibiting the expression of genes associated with progression

through the cell-cycle (Einat et al, 1985).

A lipid molecule on the plasma membrane of lymphoid cells may also suppress the growth of normal lymphocytes and lymphoid tumour cells (Stallcup et al, 1984). Gutowski and co-workers (1985) observed that while a cytoplasmic activator of DNA replication (ADR) may be present in mitogen-stimulated lymphocytes unstimulated lymphocytes contain a heat-stable protein capable of suppressing induction of DNA synthesis in isolated nuclei by ADR from both normal and neoplastic sources. It seems highly likely that this inhibitor protein is similar to the inhibitor of mitotic factors, (IMF) activated during telophase and throughout G1 (or Go in quiescent cells) in several fibroblast cell lines (Rao & Adlakha, 1985). As the name implies, IMF arrests cells by inactivating mitotic factors (Rao & Adlakha, 1985). It is thus conceivable that resting cells may maintain quiescence by a mechanism involving suppression of cytoplasmic mitogenic signals by intracellular inhibitors. Whether this mechanism represents a general property of cells alternating between proliferating and resting states has not yet been elucidated. Thus, if inhibitory or acceleratory GFs are involved in growth control, the majority of their actions may occur via some form of transmembrane signalling.

1.3 TRANSMEMBRANE SIGNALLING

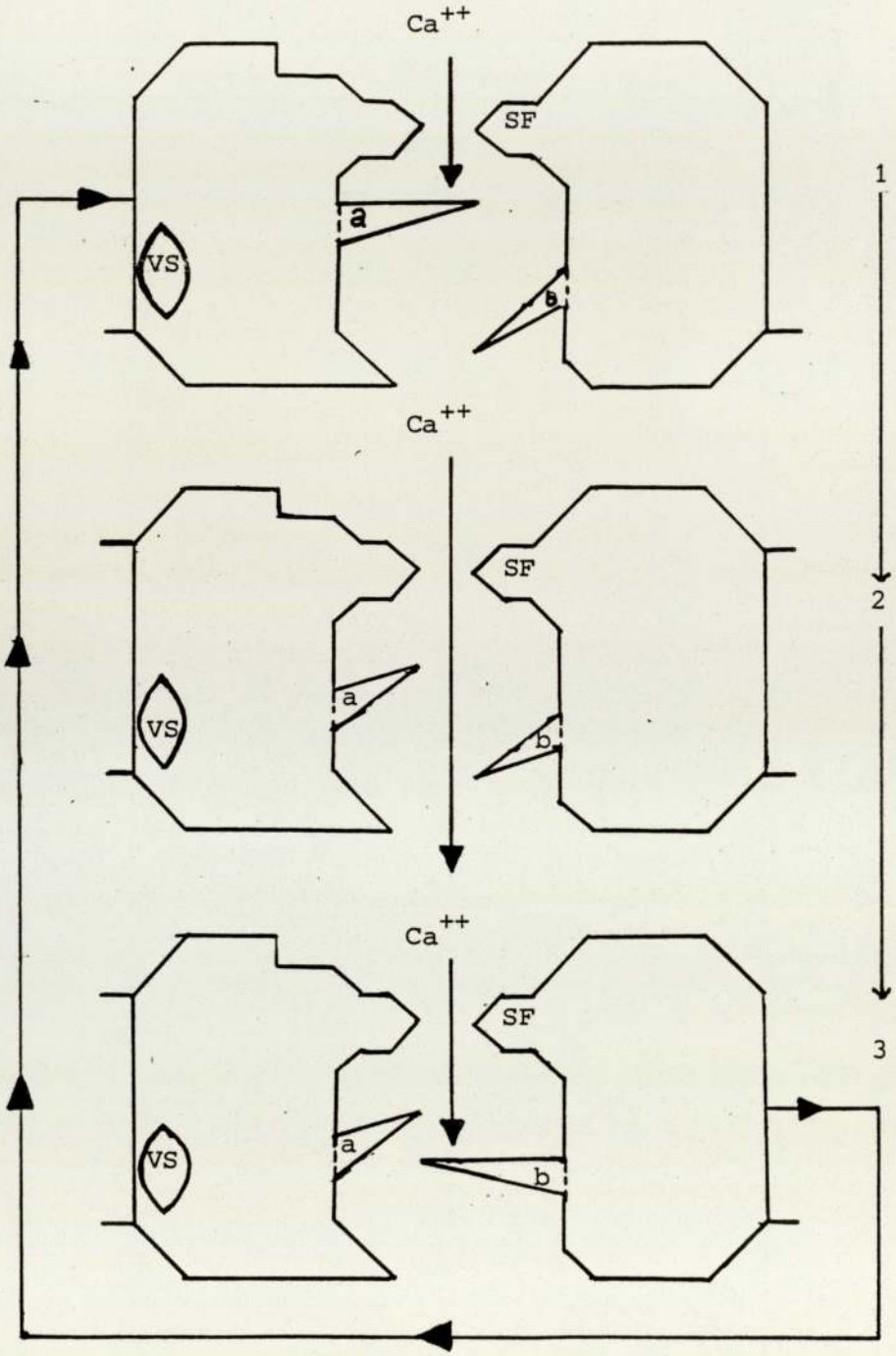
Ligand-receptor binding is followed by activation of transduction pathways leading to genesis of second messengers. This is frequently a consequence of transmembrane ion flux or membrane enzyme activation.

1.3.1 Calcium

The intracellular distribution of calcium and, in particular, the cytosolic free calcium concentration undoubtedly constitutes a major signal in many key biological processes (Rasmussen & Goodman, 1977; Whitfield, 1982; Rasmussen & Barrett, 1984; Whitfield et al, 1985). The unique role of calcium is attributed to its ionic properties that allow selective and reversible interactions with anionic groups in protein molecules (Carafoli & Penniston, 1985). Formation of cross linkages induces a conformational change thus transmitting allosteric information into cellular regulation (Levine & Williams, 1982). Calcium is asymmetrically distributed across the cell membrane, the intracellular calcium concentration being exceedingly low (10^{-7}M) compared to the extracellular milieu (2.5×10^{-3}M) (Rasmussen & Goodman, 1977; Cheung 1980, 1982). It is this large electrochemical gradient which provides for a rapid intracellular signalling system. Stimulation of cells causes a transient increase of calcium which binds with its receptor protein to evoke the appropriate response. (Means & Dedman, 1980; Klee et al, 1983).

Cellular Calcium Homeostasis

Although the plasma membrane is relatively impermeable to calcium, the ion can enter the cytosol via various pathways which serve to elevate the Ca^{2+} concentrations to the appropriate levels. The first mechanism is the passive



SF: selectivity filter
a: activation gate
VS: voltage sensor
b: inactivation gate

Diagram 2 A schematic diagram of the Ca^{2+} channel and its three kinetic states

Part 1 represents the resting state, Part 2 is the activated state and Part 3 is the inactivated state

influx of calcium ions. This steady state inflow occurs in resting cells and is a function of the extracellular calcium concentrations indicating a carrier-mediated facilitated diffusion pathway with a specificity for calcium (Borle, 1982).

The second pathway of calcium entry is represented by specific voltage dependent Ca^{2+} channels (VOC) (Reuter, 1983; Chandy et al, 1985). These channels are presumably constituted of multisubunit proteins which span the cell membrane and contain Ca^{2+} binding sites on both extracellular and intracellular sides (Johnson, 1984). Double occupancy of these sites apparently causes ionic interactions which helps to promote a quick influx of Ca^{2+} ions into the cell (Hess et al, 1986). The ionic conductance of these calcium channels is controlled by two distinct gates: activation gates which lie towards the extracellular side of the membrane and the inactivation gates which lie on the cytosolic side. In the resting or 'silent' state, the activation gates are closed while the inactivation gates are open. Reduction of membrane potential during depolarisation induces the opening of activation gates permitting the flow of Ca^{2+} current; this occurs only when both gates are in the open state. The restoration of transmembrane potential constitutes the channel deactivation process and is accompanied by a rapid closure of the activation gate (Fenwick et al, 1982; Reuter, 1983). In some tissues, depolarisation may cause a gradual inactivation of VOCs (Lee & Tsein, 1983). Furthermore, high cytoplasmic calcium concentrations may themselves initiate the inactivation sequence of calcium channel (Eckert & Tillotson, 1981).

Receptor occupancy by a ligand may also lead to the opening of specific ionic channels (Kuno et al, 1986). These so-called receptor operated channels (ROC) and ligand-gated channels can thus convey calcium ions into the cell and thus activate metabolic events therein (Bolton, 1979, 1985). In cardiac tissue, ROC activation by β-adrenoreceptor stimulation may lead to partial cellular

depolarisation automatically triggering the opening of VOCs. In this particular case, cAMP-dependent phosphorylation of specific membrane protein may induce the Ca^{2+} channels to enter an open state constantly stimulating Ca^{2+} influx for mediation of cellular responses (Cachelin et al, 1983; Reuter, 1983).

The stimulation of a receptor can open not only its closely associated channel (ROC) but also trigger the release of Ca^{2+} from stores located in the membrane increasing cytosolic Ca^{2+} levels (Bolton, 1979, 1985). The exact site of the membrane bound Ca^{2+} is as yet uncertain. It may be located on the endoplasmic face of the plasma membrane perhaps tightly bound to membrane proteins (Borle, 1981). Alternatively, substantial quantities of Ca^{2+} may be present on the exoplasmic face of the membrane associated with membrane phospholipids and negatively charged groups on the glycocalyx (Langer, 1978; Schulz, 1980).

The rise in intracellular calcium can release yet more calcium from sequestration sites within the cell such as sarcoplasmic reticulum and endoplasmic recticulum (Cavero & Spedding, 1983; Huggins & England, 1985). Such release may be mediated by a change in the properties of the surface membrane, most commonly depolarisation resulting in the opening of voltage-gated Ca^{2+} channels and/or by a diffusible intracellular second messenger, Inositol 1,4,5 phosphate (IP_3) generated by hydrolysis of membrane phosphoinositides (Henkart & Nelson, 1979; Gardiner & Grey, 1983; Streb et al, 1983; Joseph et al, 1984; Somlyo, 1984). A possible link between the guanine nucleotide regulatory mechanism and calcium release has also been suggested (Gill et al, 1986; Baker, 1986).

The mitochondria can also reverse its function of a cytoplasmic calcium sink and ultimately raise the free intracellular calcium levels. In this respect the existence of two different mitochondrial calcium efflux mechanisms has

been elucidated. In excitable cells, Ca^{2+} release may be mediated by a Ca^{++}/Na^{++} antiport system which appears to be coupled to a Na^{+}/H^{+} counter transport so that charge compensation for Ca^{++} efflux comes ultimately from the H^{+} influx. In non-excitable tissues however, an electroneutral $Ca^{++}/2H^{+}$ antiport in association with the H_2PO_4-H^{+} symport for maintenance of intra-mitochondrial pH is responsible for Ca^{2+} release. (Fiskum & Lehninger, 1982; Carafoli & Penniston, 1985).

Although raised $[Ca^{2+}]_i$ can serve as a triggering mechanism for a variety of biological actions, the duration of the response is counteracted by specific expulsion mechanisms. Indeed, calcium efflux is accomplished by two transporters associated with the plasma membrane namely, the calcium pump predominant in excitable tissues and the sodium-calcium exchanger in the non-excitable tissues. In cells containing both systems, the high calcium affinity ATPase is responsible for the fine tuning (low pumping capacity) and the Na/Ca exchanger for the bulk ejection of excess intracellular calcium (Carafoli, 1980). While the ATP-driven calcium pump has been well-characterised in erythrocytes (Schatzmann & Burgin, 1978; Vincenzi & Hinds, 1980), most other cells (Pershadsingh et al, 1980; Sarkadi et al, 1982; Shen et al, 1983, Lagast et al, 1984) may feature essentially the same Ca-Mg ATPase or a comparable extrusion system. The calcium pump is an integral membrane protein displaying relatively different affinity sites for ATP; one low and the other high (Zurini et al, 1984; Schatzman, 1985). Although a single molecule, the pump contains all the machinery required for Ca^{2+} transport; a part that splits ATP and a part that binds and transports the Ca^{2+} ion (Carafoli & Penniston, 1985). In fact, the protein can be reconstituted in liposomes where it pumps Ca^{2+} with a 1:1 stochiometry to ATP probably exchanging 2 or $3H^{+}$ for each Ca ion transported (Niggli et al, 1982).

Binding of intracellular Ca to the pump in the presence of intracellular magnesium apparently evokes an ATP-dependent phosphorylation of the transport enzyme resulting in transmembrane calcium movement (Sarkadi et al, 1982). Magnesium appears to increase the affinity of the enzyme for ATP and increases its maximal rate of activity (Schatzman & Burgin, 1978; Schatzman, 1985). At increasing magnesium concentrations however, the enzyme activity and affinity for Ca^{2+} decreases which suggests that Mg^{2+} may be involved in the enhancement of pump dephosphorylation and eventually continuous working of the pump. The enzyme can also be influenced by the calcium-dependent regulatory protein, calmodulin. In erythrocytes, calmodulin increases the maximum transport rate of the pump and also the affinity of the pump for Ca^{2+} ions as a result of hydrophobic interactions (Vincenzi & Hinds, 1980). In all probability one calmodulin molecule per enzyme molecule is sufficient for the realisation of this effect (Hinds & Andreason, 1981). A recent report, however, indicates that the free form of calmodulin can also participate in the regulation of the calcium pump (Orlov et al, 1985).

Interestingly, the enzyme can be shifted to the high affinity state by any of the numerous acidic phospholipids (phosphatidyl serine or phosphatidyl inositol) or unsaturated fatty acids even in the absence of calmodulin. This may have implications for the regulation of the pump in situ since the active site of the ATPase is surrounded by phospholipid environment of almost 30% phosphatidylserine; a concentration apparently adequate for the activation of ATPase. Thus phospholipids in addition to calmodulin may play a role in the modulation of the enzyme in the native membrane. (Niggli etal, 1981). Proteolytic enzymes can also influence the pump protein but the physiological relevance of this is unclear (Zurini et al, 1984).

In cardiac muscle the plasmalemmal pump is stimulated by an entirely different mechanism. The pump protein in this tissue appears to be phosphorylated by a cAMP dependent protein kinase (Caroni et al, 1982). This process however is prominent at only low intracellular calcium levels (Barry & Smith, 1982). At high intracellular calcium levels or under activated conditions when rapid Ca^{2+} influxes are apparent, the Na/Ca exchange process becomes the predominant extrusion process (Dipolo & Beague, 1980). This coupled exchange process is not entirely energy dependent since it utilises the electrochemical free energy of the transmembrane Na^{+} gradient. This mechanism is prominent in electrically excitable tissues such as nerve axon, heart and smooth muscle (Dipolo & Beague, 1980; Aickin et al, 1984; Morel & Godfraind, 1984) but has also been described in several non-excitable cells and tissues (Krieger & Tashjian, 1980; Kraus-Friedmann et al, 1982; Krieger-Brauer & Gratzl, 1982; Ghijsen et al, 1983, Ueda, 1983). Generally, the entry of 3 Na^{+} ions is coupled to the exit of 1 Ca^{2+} ion (Johnson & Kootsey, 1985). The overall rate of the exchange may be influenced by the membrane potential thus contributing to the complexity of the system (Johnson & Kootsey, 1985). It is highly likely that the membrane phospholipids particularly phosphatidic acid can activate this coupled process (Philipson & Nishimoto, 1984).

Along with these extrusion mechanisms featured within the plasma membrane, low cytosolic Ca^{2+} levels can also be maintained by regulatory processes operating within the mitochondria and endoplasmic recticular membranes. In fact, a critical role for mitochondria in the control of the cytoplasmic Ca^{2+} is now well-established (Joseph et al, 1983; Bygrave et al, 1985). While this buffering capacity is well-established for several tissues, in those tissues with a well developed sarcoplasmic reticulum mitochondrial

sequestration is of less consequence (Carafoli, 1979; Cavero & Spedding, 1983).

The translocation of cytosolic Ca^{2+} into the mitochondrial matrix may occur via an electrophoretic uniporter in response to the negative membrane potential generated internationally either by hydrolysis of ATP or electron transfer (Carafoli & Penniston, 1985). Despite the low affinity of the uniporter for calcium, the transport capacity is high. The charges on Ca^{2+} ions are electrically compensated by the respiration-dependent ejection of two hydrogen ions. An independent $H_2PO_4^-/H^+$ symporter may simultaneously transport PO_4^- in response to the alkaline-inside pH gradient (Fiskum & Lehninger, 1982). Small fluctuations in the calcium concentration within the mitochondrial matrix may act as a potent activator of oxidative metabolism (McCormack & Denton, 1986). Under certain circumstances, however, large quantities of calcium and phosphate or hydroxyapatite may be deposited enabling the mitochondria to act as true 'sinks' (Carafoli & Penniston, 1985). For instance, during prolonged stimulation of β-adrenoceptors the calcium content of cardiac mitochondria increases markedly. Such critical increases in cytoplasmic and mitochondrial calcium concentrations are associated with inhibition of ATP biosynthesis, and ultimately cell death.

The endoplasmic reticulum (ER) despite its relatively small binding capacity may also represent a high affinity, physiologically relevant intracellular calcium store (Borle, 1981; Somlyo, 1984; Baker, 1986). Such regulation may occur even under resting conditions when the mitochondrial Ca^{2+} uptake mechanisms are inhibited, suggesting that the ER rather than the mitochondria is the major site of cellular Ca^{2+} regulation (Burgess et al, 1983; Somlyo, 1984). A subcellular distribution of calcium by electron probe X-ray

microanalysis in liver snap frozen in vivo certainly demonstrates a high concentration of Ca^{2+} in the ER as compared to the low concentration in the mit ochondria (Somlyo et al, 1985). The identity of the subfraction responsible for Ca^{2+} accumulation is not known, although it is probably not inherent to the rough endoplasmic reticulum (Immelman & Soiling, 1983). In the ER, calcium accumulation is mediated by the ATP-driven calcium pump similar to that of the sarcoplasmic reticulum (SR). The activation of the pump may involve phosphorylation of proteins; a 118 KDa phosphorylated protein has been identified in ER from rat liver (Heilman et al, 1983). This protein is perhaps analogous to the phosphorylated intermediary protein of the Ca^{2+} pump in the SR (Heilman et al, 1983). Such protein phosphorylation may presumably occur via the calmodulin-dependent system operating in parallel with a regulatory process dependent on cAMP and a specific protein kinase as is the case for phosphorylation of phospholamban (Huggins & England, 1985; Carafoli, 1981).

Despite the multitude of extrusion and buffering systems, the opening of calcium channels and/or calcium mobilisation can temporarily overwhelm the cell's homeostatic mechanism and give a transient calcium signal and cell activation. Of the many events which calcium can trigger, the regulation of cell growth alone is considered here since it is the major concern of this thesis.

Calcium as a Signal for Cell Proliferation

A critical role of extracellular calcium in the initiation of cell division has been demonstrated in a variety of studies (Lichtman et al, 1983; Berridge, 1984; Whitfield et al, 1985). Whitfield and co-workers (1980) demonstrated that onset of hepatocyte proliferation in regenerating rat liver was preceded by an obligatory transient requirement for extracellular Ca^{2+} in the early stages of

DNA synthesis. Likewise omission of extracellular calcium from cultures of normal WI-38 human fibroblasts inhibited cell division (Tupper et al, 1980; Owen & Villereal, 1985). In contrast addition of supranormal concentrations of calcium or calcium-phosphate precipitates to the medium of density-inhibited cells induced mitogenic stimulation (Engstrom, 1982; Bowen-pope and Rubin, 1983).

Evidence has also been amassed linking intracellular calcium signals with mitogen-activated cells including eggs (Epel, 1982), fibroblasts (McNeil et al, 1985; Metcalfe et al, 1985), macrophages (Wright et al, 1985) and lymphocytes (Freedman, 1979; Pozzan et al, 1982; Lichtman et al, 1983). In fact, egg activation at fertilisation is associated with a progressive rise in intracellular calcium as measured by the photoprotein aequorin. This change is causal as manipulation of cytosolic Ca^{2+} by microinjection of the ionophore, A23187 or the calcium-mobilising agent, inositol triphosphate can activate unfertilised eggs. Such activation can be abolished, however, by simultaneous injection of the calcium chelator, EGTA (Whitaker & Irvine, 1984; Swann & Whitaker, 1986). An alteration in intracellular calcium distribution thus appears to be pertinent for cell activation. Such alterations in the cytosolic Ca^{2+} content may be attributed to calcium inflow, a release of calcium from intracellular stores or an impaired extrusion of Ca^{2+} through the Ca^{2+} efflux pumps in the plasma membrane.

An influx of calcium following mitogenic stimulation of quiescent cells may well be regulated by hydrolysis of phospholipids in the plasma membrane (Michell et al, 1982). Indeed, EGF stimulation of A431 cells is associated with enhanced phosphoinositide hydrolysis and Ca^{++} uptake which is impaired by addition of low density lipoproteins (Hui & Harmony, 1980; Sawyer & Cohen, 1981). Several authors claim that lectin stimulation of T-lymphocytes

is also accompanied by phospholipid breakdown and a rapid transmembrane Ca^{2+} influx as inferred from the $^{45}Ca^{2+}$ tracer flux measurements (Freedman et al, 1975). The magnitude and duration of the $^{45}Ca^{2+}$ influx and its relevance to mitogenic stimulation has however been questioned (Hesketh et al, 1977). These workers maintain that rapid Ca- Ca exchange systems may raise problems in the detection and interpretation of $^{45}Ca^{2+}$ flux changes. The use of Ca-sensitive fluorescent probes such as Quin 2 has enabled measurement of calcium transients in several cell types; T-cells treated with anti-T3 antibodies (Weiss et al, 1984a; O'Flynn et al, 1984), ß-cells stimulated with anti-immunoglobulin antibodies (Pozzan et al, 1982) and even growth-factor induced arousal of quiescent fibroblasts (Moolenaar et al, 1984; McNeil et al, 1985). Whether these transient increases are due to calcium influx or a calcium mobilisation is not clear since both channel and mobilisation blockers impair cell growth. (Birx et al, 1984; Grier & Mastro, 1985; Oettgen et al, 1985; Imboden & Stobo, 1985).

Although the calcium dependence of the proliferogenic response is clear, the precise timing and nature of this dependence is controversial. While a sustained or biphasic requirment during GO/G1 and late G1 or even S phase has been indicated by several workers (Bard et al, 1978; Hazelton et al, 1979; Komada et al, 1986), Whitfield has reported an obligatory requirement only in early G1 phase. (Whitfield, 1982; Whitfield et al, 1985). This may indeed be consistent with the hypothesis of Hesketh et al (1982) in which the transition from GO to G1 phase occurs during the early surge in cytosolic calcium within the range 0.1-1µm and is followed by a sustained dependence ensuring further progression through the cell-cycle. Not surprisingly, Ca^{2+} ions are also important during mitosis (Hepler & Wolniak, 1984; Hepler, 1985).

Recently the use of a new fluorescent calcium chelator, fura-2 for measurement of $[Ca]^{2+}_i$ has revealed a brief $[Ca]^{2+}_i$ surge at the metaphase-anaphase transition (Poenie et al, 1985). These small increases in cytosolic calcium concentration may initiate key physiological processes by activating Ca^{2+}-sensitive enzymes associated with cell growth.

Calmodulin

As in other calcium signalling systems, the calcium transients may activate the ubiquitous calmodulin molecule (Cheung, 1982). Calmodulin (CaM) is a small, highly acidic and heat-stable polypeptide with a molecular weight of 16,700. It has a tertiary configuration containing four Ca^{2+} binding domains with a homologous amino acid sequence and similar affinities for Ca^{2+} (Manalan & Klee, 1984; Means & Dedman, 1980). Upon binding Ca^{2+}, the calmodulin molecule undergoes a pronounced conformational change towards a more helical structure that exposes a hydrophobic domain believed to be a binding site for CaM acceptor proteins, termed response elements. The binding of Ca^{2+} is generally ordered or sequential as follows:

$$Ca^{2+} + CaM \rightleftharpoons Ca.\ CaM$$

$$Ca^{2+} + Ca.\ CaM \rightleftharpoons Ca_2.\ CaM$$

$$Ca_2.\ CaM + RE \rightleftharpoons Ca_2CaM.\ RE$$

$$Ca^{2+} + Ca_2\ CaM.\ RE \rightleftharpoons Ca_3\ CaM.\ RE$$

$$Ca^{2+} + Ca_3\ CaM.\ RE \rightleftharpoons Ca_4\ CaM.RE^*$$

(active)

The activated product complex* formed may interact with the enzyme substrate and cause protein phosphorylation essential for various physiological processes (Manalan & Klee, 1984).

The generally accepted stepwise Ca^{2+} binding as in the outline scheme usually forms the active Ca_3CaM or Ca_4CaM complexes. It has however been suggested that CaM containing different amounts of Ca^{2+} might also interact with different specific enzymes to produce qualitatively different biochemical responses (Klee et al, 1980; Manalan & Klee, 1984; Cox, 1984).

The relative effectiveness of the calci-calmodulin complexes depends on the amount of free calmodulin within the cell. This, in turn, is dependent on the balance of synthesis and degradation of the CaM molecule and the extent of its binding to non-specific binding proteins. (Means & Dedman, 1980). Furthermore, the magnitude of the Ca^{2}-CaM response can be limited by other tissue and species-specific Ca^{2+} binding proteins. One such protein, calcineurin (found in nerves and erythrocytes) binds calmodulin and inhibits the calmodulin-mediated activation of several Ca^{2+}-dependent enzymes (Klee et al, 1979). The actions of calmodulin can also be modulated by cAMP dependent phosphorylation of target proteins, eg. myosin and phosphorylase kinase. Regulation of the cell response by Ca^{2+}-CaM may also be achieved by redistribution of target proteins or calmodulin from one cellular compartment to another (Evain et al, 1979; Saitoh & Schwartz, 1983). Termination of the stimulatory response is, however, accompanied by the re-establishment of CaM distribution evident in non-activated cells. Finally, Ca^{2+}-CaM may limit the duration of the cellular response by itself activating the calcium extrusion pump (Vincenzi & Larsen, 1980; Carafoli, 1981; Manalan & Klee, 1984).

Role of Calmodulin in Cell Division

Given the array of CaM-dependent enzymes (Means & Dedman, 1980; Cheung, 1982) and the potential relevance of Ca^{2+} signals in mitosis, it is logical to assume a prominent role of calmodulin in control of cell growth. Calmodulin may be an ideal cell cycle 'trigger' as the cell needs to only regulate the levels of calmodulin at the critical time to set off the cascade of events pertinent for cell cycle progression. Chafouleas et al (1984) have observed a two-step action of calmodulin during the re-entry of Chinese hamster ovary (CHO) cells from the plateau (Go) phase into the cell-cycle. Such regulation may occur initially at the Go-G1 phase and subsequently at the G1/S transition in the cell-cycle. This two-step action would be compatible with the competence-progression model of cell growth (Pledger et al, 1977). Indeed, the concentration of calmodulin may increase prior to S as in CHO cells, regenerating rat liver or even rat liver T51B cells (Boynton et al, 1982; Boynton & Whitfield, 1983).

A surge in calmodulin synthesis is however not obligatory for initiation of DNA synthesis. Normal rat kidney cells infected with temperature sensitive transformation mutants of RNA sarcoma virus undergo growth at a permissive temperature without an increase in calmodulin (Durkin et al, 1983).

Irrespective of the controversy regarding changes in calmodulin levels with growth, studies employing calmodulin antagnoists provide circumstantial evidence for a specific role of CaM in cell division. Addition of W7, a naphthalene sulfonamide derivative arrests CHO cells at the G1/S boundary, late G2 and even M phase. Such inhibition may be due to disruption of microtubules as W7 permeates the cell membrane and is distributed primarily in the cytoplasm (Sasaki & Hidaka, 1982). Likewise, W13 delays the G1/S transition in CHO cells regardless of the increased calmodulin content probably

by interacting with the calmodulin molecule and hence inactivating the CaM.Ca^{2+} enzymes implicated in microtubule redistribution. (Chafouleas et al, 1982). The phenothiazine derivatives, trifluoperazine and chlorpromazine, similarly block mitogenic responses in lymphocytes (Cheung et al, 1983; Bachvaroff et al, 1984) and DNA synthesis in T51B rat liver cells stimulated with calcium (Boynton et al, 1980), or the phorbol ester, TPA (Jones et al, 1982). These results should however be interpreted with caution. The lack of specificity for calmodulin limits the usefulness of anti-calmodulins as markers of calmodulin-regulated enzymes in vitro or the involvement of CaM in cellular processes in vivo (Roufogalis, 1985). These drugs also inhibit other cellular functions by interaction with (a) the Ca^{2+}-dependent, calmodulin independent protein kinase C, PKC and/or (b) membrane-bound CaM-independent enzymes (Luthra, 1982; Schatzman et al, 1983). Such limitations occur at high concentrations; 40 μm and above for TFP; 50 μm and above for W7; concentrations far above those used to inhibit mitosis. These studies thus strongly support the regulatory role of calmodulin in cell cycle progression.

1.3.2 Magnesium

Magnesium, an abundant intracellular cation is important for many enzymatic systems and hormone-receptor interactions. In fact, magnesium is an essential co-factor involved in phosphorylation reactions and intermediary metabolism (Rubin, 1976; Sanui & Rubin, 1982).

Intracellular Regulation of Magnesium

In mammalian cells, intracellular magnesium is maintained at about 10 mm despite a wide range of fluctuations in the extracellular environment. Such uniformity may be due to an efficient homeostatic system operating to minimise

cell loss during conditions such as magnesium deprivation (Sanui & Rubin, 1982; Gunther et al, 1984; Corkey et al, 1986). As magnesium fluxes are too large to be accounted for by passive diffusion through the lipid bilayer, specific magnesium transport systems have been elucidated in several cell types (Flatman, 1984; Gunther et al, 1986). For example, in nerve and muscle cells, influx of magnesium occurs by the Na/Mg exchange (Mullins & Brinley, 1978). Such exchange at least in the squid axon may also be regulated by ATP (Flatman, 1984). A Mg/Mg exchange or even a separate Mg^{2+} channel has been indicated in the cardiac muscle (Flatman, 1984). An alternate mechanism for Mg^{2+} influx may be an inwardly directed Mg^{2+} pump or Mg^{2+} ATPase. However, it has not yet been characterised in mammalian cell membranes (Sanui & Rubin, 1982; Flatman, 1984). Efflux, in contrast, may be coupled to sodium uptake; the role of ATP or membrane-bound proteins in such extrusion mechanism is not known (Flatman, 1984; Gunther et al, 1984). Regardless of the transport systems within the plasma membrane, a large percentage (~ 90%) of the intracellular Mg^{2+} is complexed to internal membranes of the mitochondria and the endoplasmic or sarcoplasmic reticulum as well as nucleic acids (Somlyo et al, 1984; Corkey et al, 1986). In addition, the free Mg^{2+} ions are buffered by various metabolic products such as ATP, citrate, amino acids and many phosphorylated molecules. Not surprisingly, the measurments of cytosolic Mg^{2+} levels by several investigators have proved to be rather inconsistent (Rink et al, 1982). In spite of the discrepant values, most estimates of the free Mg^{2+} levels are nevertheless in the range of about 1 mM. This value apparently coincides or is just below the concentration required for maximal activity of many enzymes so that relatively small magnitude changes in intracellular levels can significantly affect metabolic control (Sanui & Rubin, 1982)

Despite the regulatory mechanisms, Mg^{2+} transport is very slow and major magnesium compartments are only slowly exchangeable. Indeed in the mouse lymphoma S49 cells only 2% of the total cellular Mg^{2+} which is localised in the subcytoplasmic pool is exchangeable. Alkalinization of the medium can, however, stimulate transport; perhaps by a proton-stimulated ATPase (Erdos & Maguire, 1983).

Alternatively, stimulation may be due to changes in cellular magnesium buffering. Some agonists can considerably alter Mg^{2+} transport. α-adrenergic agents (adrenalin; nor-adrenalin) and adrenocorticotropic hormone (ACTH) stimulate accumulation of Mg^{2+} in adipocytes (Eliott & Rizack, 1974). Similarly, insulin specifically provokes Mg^{2+} uptake in adipocytes and fibroblasts (Cech et al, 1980). Furthermore, glucose stimulation of insulin secretion in isolated pancreatic islets is also associated with an increase in Mg^{2+} transport (Henquin et al, 1983). ß-agonists in contrast, inhibit Mg^{2+} uptake in GM86 Friend erythroleukemia cells and G8 muscle cells and the much-studied S49 cells. In these cells, activation of adenylate cyclase and production of cAMP is not related to Mg^{2+} uptake (Erdos & Maguire, 1980). Alterations in Mg^{2+} transport and consequently in intracellular Mg^{2+} levels could affect metabolic responses directly or indirectly by variation of the Ca^{2+} status of the cell (Iseri & French, 1984).

Extracellular magnesium can act physiologically to control and regulate entry of calcium. In smooth-muscle cells, a reduction of extracellular Mg^{2+} concentration increases the calcium influx. In contrast an elevation of Mg^{2+} levels is associated with a dimunition in the influx rate as a result of competition with Ca^{2+} for non-specific binding sites at the membrane (Turlapathy & Altura, 1978).

Intracellular magnesium can also potentially modulate the regulatory mechanisms for calcium homeostasis. In skinned muscle fibres, a low intracellular magnesium concentration can potentiate calcium uptake in the sarcoplasmic reticulum. This increase may be an effect of the formation of the Mg-ATP complex, a true substrate of the ATPase. In fact, the ion is associated with several steps of the SR-ATPase cycle thereby facilitating cytosolic Ca^{2+} association and translocation (Chiesi & Inesi, 1981; Guillain et al, 1984). An elevated intracellular Mg^{2+} content however may inhibit sequestration of Ca^{2+} by the SR but increase the Ca^{2+} induced Ca^{2+} release thereby elevating the cytosolic Ca^{2+} content. A raised extra mitochondrial Mg^{2+} concentration may also inhibit Ca^{2+} uptake by mitochondria by competing for binding sites on the mitochondrial membrane. In liver where common binding sites for the two cations do not exist, Mg^{2+} may not have a significant effect on Ca^{2+} uptake or Ca^{2+} release from mitochondria (Carafoli, 1979; Corkey et al, 1986).

From the foregoing discussion, it is apparent that Mg^{2+} can modulate cell function by its effects on calcium handling. Since the participation of any magnesium dependent regulatory protein in the regulation of magnesium-dependent responses has not been elucidated, it seems that Mg^{2+} requires no intermediary to exert its co-ordinate control. The broad metabolic effects of Mg^{2+} may thus be due to its own intrinsic properties (Sanui & Rubin, 1982).

Role of Magnesium in Proliferation

The important and wide-ranging effects of magnesium within the cell have led to the suggestion that magnesium may be a plausible regulator of the co-ordinated array of reactions associated with initiation of DNA synthesis and

cell division (Rubin, 1975). The hypothesis is that small variations in free intracellular magnesium concentrations can effectively alter the rates of protein synthesis and kinetics of several enzymes which collectively determine the rate of cell proliferation (Sanui & Rubin, 1982; Terasaki & Rubin, 1986). The rate of cell division is certainly dependent on the availability of extracellular magnesium (McKeehan & Ham, 1978). Since the magnesium requirement for animal cells is itself strictly controlled by complex growth factors (McKeehan, 1984), it is difficult to critically define threshold magnesium concentrations necessary for growth. Considerable evidence however indicates that the Mg^{2+} requirement for animal cell proliferation is absolute (Walker, 1986).

The intracellular availability of Mg^{2+} may be directly correlated with rates of cell proliferation. Elevated intracellular Mg^{2+} levels are essential for maintenance of cell division in a variety of cell types (Cameron et al, 1980). Similarly, stimulation of DNA synthesis in mouse fibroblasts by an increase in serum concentrations is accompanied by a marked rise in intracellular Mg^{2+} concentration. Conversely, a decrease in cellular calcium content parallels the proliferogenic response (Sanui & Rubin, 1982). It has also been suggested that the effects of variations of external calcium concentrations on cell division is achieved indirectly by modulating intracellular Mg^{2+} availability. An increase in extracellular calcium may permit a rapid exchange of membrane bound Mg^{2+} at the cell surface liberating free intracellular Mg^{2+} for regulation of cell multiplication. Similarly, the effect of calcium deprivation on cell growth may be due to competition between Mg^{2+} and Ca^{2+} for intracellular binding sites. Ca^{2+} deprivation may cause drastic reductions or redistribution of cellular Ca^{2+} such that Mg^{2+} occupies those sites formerly occupied by Ca^{2+} reducing the availability of Mg^{2+} for critical proliferative processes (Rubin & Koide, 1976).

Cyclic fluctuations in intracellular magnesium during the cell-cycle may represent a fundamental means for co-ordinating growth and cell division (Walker & Duffus, 1983). A variability in cellular magnesium content as determined by atomic absorption spectrophotometry in lymphocytes cultured at different phases of growth certainly suggests that a rapid transit through the cell-cycle is associated with elevated levels of cytosolic free magnesium (Hosseini & Elin, 1985). Despite this indirect evidence indicating an elevation in cytosolic Mg^{2+} content, the various techniques employed by several workers have not been able to quantify any measurable alterations in Mg^{2+} levels. In contrast, rapid changes in $[Ca]_i$ have been reported for lectin-stimulated lymphocytes (Rink et al, 1982). Small but presently unmeasurable magnitude changes in Mg^{2+} may perhaps be sufficient for cellular regulation as magnesium dependent enzymes retain large amounts of intracellular magnesium (Abboud et al, 1985). Alternatively, changes in Mg^{2+} levels may be paralleled by alterations in cellular calcium content. Indeed magnesium deprivation alters intracellular Ca^{2+} binding and compartmentalisation and subsequently cellular calcium content (Vidair & Rubin, 1981). Likewise, where supernormal concentrations of Mg^{2+} promote cell proliferation it may not necessarily imply an initiating role for Mg^{2+} but may be due to Ca^{2+} displacement from the ER (Chiesi & Inesi, 1981), a decreased calcium sequestration or even a stimulation of Ca^{2+} efflux from mitochondria (Fiskum & Lehninger, 1980). Whether such variations in intracellular Mg^{2+} parallel regulation of protein synthesis during stimulation, or inhibition of cell growth is entirely speculative (Rubin, 1981; Sanui & Rubin, 1982; Terasaki & Rubin, 1986). It is however clear that intracellular Ca^{2+} content is sensitive to changes in Mg^{2+} levels. This lends evidence to the premise that Mg^{2+} is not actually a specific proliferogenic signal but is

essential as a permissive factor thoughout cell-cycle progression for modulation of calci-calmodulin activated magnesium dependent enzymes (Whitfield, 1982). If this is the case, then Ca^{2+} may be a primary mitogenic trigger for regulation of cell proliferation.

It is thus apparent that uncertainty still exists over whether or not a single cation activation 'axis' regulates cell proliferation. Nevertheless, it is obvious that both ions may mediate cell growth, perhaps they act in concert as in lectin induced lymphocyte DNA synthesis and in stimulation of RNA synthesis in 3T3 cells (Bowen-pope et al, 1979; Abboud et al, 1985) or in a sequential manner in response to serum growth factors. It may be that the enzymatic machinery and macromolecular rearrangments that govern mitosis are mediated by alterations in the free intracellular concentration ratio of the two main divalent cations; calcium and magnesium (Staron & Jerzmanowski, 1981).

1.3.3 Monovalent Cations

The cations sodium (Na^+), potassium (K^+) and hydrogen (H^+) may also function as messengers during cellular function. These cations may act directly to mediate their effects or act indirectly by effecting the cellular homeostasis of divalent cations or cyclic nucleotides.

Sodium and Potassium:

Sodium is asymmetrically distributed across the plasma membrane, the cytosolic Na^+ content approximately 30-fold lower than the extracellular fluid. Chemically or electrically induced alterations in the plasma membrane may cause a rapid inflow of Na^+ ions and hence raise the cytosolic Na^+ content. The former appears to be a consequence of specific membrane phosphorylation whereas the latter is a voltage-dependent gating most typically found in excitable tissues (Catterall, 1982; Kimmich & Randles, 1982; Cahalan et al, 1985). The channels are highly specialised glycoproteins and contain carboxyl groups within the ionic pore for selectivity. The mechanism of gate opening or closing remains unclear (Bezanilla, 1986). The specificity of these channels for Na^+ is however not absolute since Ca^{2+} can share the channel and, in turn, impede monovalent ion fluxes in several cell types presumably by competing with them for anionic binding sites on the cell-surface or within the pore (Quastel et al, 1981; Hohl et al, 1983; Yamomoto et al, 1984).

The consequences of sodium influx are manifold. There many be a direct mobilisation of Ca^{2+} from organelle-bound pools such as mitochondria and, as a consequence of hypopolarisation, an opening of voltage-operated calcium channels which separately or collectively increase cytosolic calcium content (Nicholls, 1978). This, in part, is compensated by the Na^+/Ca^{++} antiport exchange mechanism. Furthermore, both the elevated intracellular calcium and the

hypopolarisation caused by Na^+ influx will promote egress of K^+ from the cell via special K^+ channels (Petersen & Maruyama, 1984; Cahalan et al, 1985; Bregestovski et al, 1985; Pershadsingh et al, 1986).

The opening of K^+ channels may therefore affect the intracellular concentration of K^+. Such variations in intracellular K^+ content may serve as a signal for specific protein synthesis. The rapid inflow of Na^+ ions and efflux of K^+ ions may activate the Na^+/K^+ ATPase.

The pump operates to limit the duration of the response so that a relatively low Na^+, high K^+ is achieved in resting cells as compared to the high Na^+, low K^+ in the extracellular environment (Racker, 1976). Although the exact molecular mechanism of the active transport is not known, the enzyme may extrude 3 Na^+ ions for 2 K^+ ions translocated inwards by a cyclical process. Binding of Na^+ ions to specific high affinity Na^+ binding sites on the interior of the cell may induce a Mg^{2+} dependent phosphorylation and a consequent conformational change resulting in expulsion of Na^+ions. The binding of K^+ ions to their specific sites on the phosphoenzyme may transport these ions in the opposite direction. The phosphorylated intermediate may subsequently become hydrolysed to attain its original inactive configuration (Kaplan & Owens, 1982; Kaplan, 1985). The ions themselves may regulate pump expression and/or synthesis of pump protein. An increased membrane fluidity may also affect the pump presumably by enhancing the intracellular and extracellular concentrations of both its ionic substrates, Na^+, and K^+ (Hume et al, 1978). As a matter of surprise, Ca^{2+} may affect membrane elements to inhibit the ATP-ase (Turi & Torok, 1986). The physiological significance of such inhibitory effects is unclear. In addition to Na^+ and K^+ ions, H^+ ions may also participate in the signalling cascade leading to a cellular response.

Hydrogen Ions

The regulation of intracellular pH is predominantly achieved by an electroneutral transport system, the Na^+/H^+ antiporter (Aronson, 1985; Moolenaar, 1986). Depending on the size and direction of the transmembrane gradient for Na^+, the exchanger can mediate net transport of H^+ into or out of the cell with a stoichiometric ratio of 1:1 (Cassel et al, 1984; Grinstein et al, 1984; Aronson, 1985). A shift in the intracellular pH (pHi) activates the antiport. At a physiological pH (~ 7.2 at 37°C), the antiport is virtually quiescent. At a lower pH however, the protonation of the modifier site enhances the Na^+/H^+ counter transport. On the other hand, an alkaline pH causes deprotonation of the modifier resulting in inactivation of the antiport in spite of the prevailing combined Na^+ plus H^+ gradient (Grinstein & Rothstein, 1986).

The antiport system can also be activated by other factors. The Ca^{++}-CaM system has been said to cause activation of the antiport (Owen & Villereal, 1982). This deduction is largely based upon calmodulin antagonists which impair Na^+/H^+ exchange. However, it should be remembered that these drugs affect other systems including the enzyme, protein kinase-C. Some authors have indeed suggested that membrane phosphorylation as a result of PK-C activation may induce Na^+/H^+ exchange (Grinstein & Rothstein, 1986).

Despite the homeostatic control of H^+, deviations may trigger events by a modulation of the activity of several enzymes and the ionic status of the cell. For instance, H^+ may alter the activity of the Na^+/K^+ ATPase which in turn may exert a significant effect on the cytosolic calcium levels (Nicholls, 1978; Skou, 1982; Reuter, 1983). A rise in H^+ may also directly increase intracellular Ca^{2+} levels by inhibiting Ca^{2+} uptake by the mitochondria and Ca^{2+} efflux across the plasma membrane (Dipolo & Beague, 1980; Busa & Nuccitelli, 1984). This increase in cytosolic Ca^{2+} levels could act as a signal as outlined earlier. In contrast, a low

H^+ concentration seems to encourage Ca^{2+} sequestration by the endoplasmic reticulum thus buffering the free Ca^{2+} levels (Shoshan et al, 1981). Thus, the net algebraic effect of a pH change may depend upon the relative quantitative importance of the Ca^{2+}-transport system in the plasma membrane, mitochondria and endoplasmic reticulum.

It is quite clear that divalent or monovalent interactions can and do serve as real or potential signalling systems for DNA synthesis and division and indeed many other cell functions.

Role of Sodium and Potassium in Proliferation

An increase in Na^+ uptake in a variety of cells in response to diverse mitogenic stimuli is often associated with cell growth. Such mitogen-activated cells include the newly fertilised eggs (Epel, 1980), fibroblasts (Rozengurt, 1981, 1982; Owen & Villereal, 1982, 1983; Villereal & Owen 1982), neuroblastoma cells (Mummery et al, 1982, 1983; Boonstra et al, 1985), lymphocytes, thymocytes (Grinstein et al, 1985) and hepatocytes (Leffert & Koch, 1982; Fehlmann et al, 1982). The influxes are rapid 'burst-like' events occurring within seconds after mitogen treatment and persisting for at least 2-60 minutes in the presence of mitogen. The influx rates represent approximately 1.5-5-fold elevations over basal unstimulated levels (Smith & Rozengurt, 1978; Mummery et al, 1982). Significantly, the rates of Na^+ influx are usually directly proportional to the mitogenic dose (Leffert & Koch, 1982). Removal of extracellular sodium ions or blockade of these sodium influxes by addition of amiloride inhibits stimulated DNA synthesis in most cell types studied (Deutsch et al, 1981; Deutsch & Price, 1982; Moolenaar et al, 1981; Leffert & Koch, 1985). Such blockade by amiloride is usually effective at the onset of the synthetic phase S, but not once the cells are committed to replication (Koch & Leffert, 1979; Mummery et al, 1982). The interpretation of these results is, however, complicated as amiloride penetrates most cells and exerts non-specific effects like inhibition of protein synthesis, cAMP dependent protein kinase and mitochondrial functions which may all interfere with the initiation of DNA synthesis (Leffert, 1982; Holland et al, 1983; Stiernberg et al, 1983). In contrast, in growing fibroblasts, the retardation of cell growth due to an increase in cell density is associated with a decrease in rate of Na^+ influx (Adam et al, 1982). Such density-dependent inhibition of growth may

be a consequence of chalone release which in some way blocks the sodium uptake (Walsh-Reitz et al, 1984). Although it seems likely that Na^+ influxes may increase intracellular Na^+ concentrations and activate cell cycle specific genes associated with initiation of DNA synthesis, direct measurements of intracellular Na^+ activities in either nucleus or cytoplasm of mitogen-treated cells have not yet been made (Leffert & Koch, 1985). Thus, although x-ray microprobe analysis has revealed that rapidly proliferating cells have a higher total sodium content than their normal counterparts, it is not clear whether nuclear or cytoplasmic Na^+ concentrations are increased and serve as a positive signal for growth. Changes in total sodium content may merely be passive accompaniments of increased cytoplasmic volume.

The rapid increase in Na^+ influx is also followed by rapid and transient activation of the Na^+ pump which in turn, may somehow be associated with the proliferative process. An enhanced Na^+ and K^+ transport via the Na^+ K^+ATPase is certainly apparent in virally transformed or tumorigenic cell lines as compared to the confluent or density-inhibited cells (Kaplan, 1978). Moreover, mitogenic stimulation rapidly activates the Na^+K^+ pump in a variety of cell types (Deutsch & Price, 1982; Moolenaar et al, 1982; Heikkila et al, 1983; Leffert & Koch, 1985; Boonstra et al, 1985; Redondo et al, 1986). When pump driven flux is inhibited by addition of ouabain and DMSO differentiation rather than proliferation occurs. These inhibitors may thus induce hemoglobin synthesis in Friend erythroleukemia cells (Bernstein et al, 1976), increased surface IgM expression in a pre-B cell tumour line (Rosoff & Cantley, 1983) and granulocyte differentiation in human leukemia cell line (Gargus et al, 1983).

Despite the evidence that increased monovalent cation transport via the Na^+/K^+ pump is somehow related to cell growth, the function of this activity is unknown. Early stimulation of K^+ influxes may raise K^+ content, which, may be an essential signal for cell proliferation (Frantz et al, 1981; Wondergem, 1982; Rozengurt & Mendoza, 1980). Such changes in intracellular K^+ levels or K:Na ratio may constitute a growth signal by activation of specific cell-cycle associated genes (Kelly et al, 1983). A reduced K:Na ratio certainly retards growth of normal cells in comparison to the relatively insensitive tumour cells (Lubin, 1980; Jayme et al, 1981). Similarly, quiescent epithelial cells and density-inhibited 3T3 cells reveal a low K:Na ratio which on elevation by serum addition or reseeding of these confluent cells is adequate for initiation of mitosis. Perhaps serum addition or reseeding stimulates K^+ influx in these cells. (Cameron et al, 1980; Frantz et al, 1981; Adam et al, 1982). Moreover, mitogenic stimulation of 3T3 fibroblasts by several agents (Lopez-Rivas et al, 1982), epithelial cells by oestradiol (Cameron et al, 1980), hepatocytes by partial hepatectomy (Wondergem, 1982), human lymphocytes by lectins (Averdunk & Lauf, 1975) and T-cell lines by IL2 (Redondo et al, 1986) is accompanied by enhanced K^+ influx. Such increases in K^+ uptake by the pump are probably due to alteration in the physico-chemical properties of the membrane, de novo synthesis of new transporters or activation of the pre-existing transporters (Schenk et al, 1984; Boonstra et al, 1985).

High levels of cytosolic K^+ appear to be essential for polypeptide chain elongation as opposed to low K^+ levels. In reticulocytes, protein synthesis was inhibited when the intracellular K^+ concentration was decreased (Cahn & Lubin, 1978). As DNA synthesis is dependent on accumulation of a specific protein or enzyme, the role of K^+ in cell cycle progression is probably related

to its effect on protein synthesis. In fibroblasts, the surge of K^+ is associated with early G1 phase or the release of cells from Go/G1 arrest (Lopez-Rivas et al, 1982; Panet, 1986). This suggests that K^+ influx evoked by different stimuli is required to sustain protein synthesis at a level pertinent for optimal entry into S phase. In neuroblastoma cells however, the progression through G1/S is correlated with an inwardly directed K^+ flux which becomes more pronounced in late S phase (Mummery et al, 1981; Boonstra et al, 1985).

In spite of the evidence supporting a positive role for K^+ ion in cell growth, K^+ entry may just be a compensatory phenomenon. A heightened Na^+ influx may cause hypopolarisation which then allows more K^+ efflux so that the ATP-ase then extrudes Na^+ and recaptures K^+ to restore the status quo. Not surprisingly, some authors have concluded that this initial K^+ efflux may be a proliferative trigger. In lectin stimulated human T-cells voltage operated K^+ efflux occurs. When this is imparied by K^+ channel blockers such as quinine and citiedil, enhanced protein and DNA synthesis are certainly blocked (Chandy et al, 1984; DeCoursey et al, 1984). However K^+ efflux normally permits Ca^{2+} inflow (Tsien et al, 1982) which as indicated above could be the actual mitogenic trigger. Alternatively, the increased cytosolic Ca^{2+} content due to the Ca^{2+} entry following an influx of Na ions may stimulate the egress of K^+ ions from electrically excitable and non-excitable tissues. In fact, the activity of K^+ channels is regulated by intracellular calcium concentrations, K^+ may thus be indirectly linked with changes of cell metabolism and membrane potential by a Ca^{2+} dependent K^+ export (Petersen & Maruyama, 1984). Thus, it is most unclear whether these various monovalent cation changes are causative or passive accompaniments of the activation process.

Hydrogen Ions

As Na^+/H^+ exchange can be activated by mitogenic growth factors and lectins, it is plausible that activation of the Na^+/H^+ antiport is necessary for proliferation and represents the initial trigger of mitogenesis (Moolenaar et al, 1983; Hesketh et al, 1985; Macara et al, 1985; Moolenaar, 1986). Typically, activation of the antiport in the various cell types studied is associated with a simultaneous rise in intracellular pH levels (Grinstein & Rothstein, 1986; Moolenaar, 1986). Using mutant fibroblasts that lack functional Na^+/H^+ activity, Pouyseggur et al (1984) have shown that initiation of DNA synthesis is extremely sensitive to pH_i. An increase in cellular pH is an important event in the activation process of a variety of cell types including fertilised sea urchin eggs (Busa & Nuccitelli, 1984; Swann & Whitaker, 1986), neuroblastoma cells (Moolenaar et al, 1981, 1984), fibroblasts (Owen & Villereal, 1982; Moolenaar et al, 1984; Villereal & Owen, 1982), lymphocytes (Grinstein et al, 1983; Hesketh et al, 1985), thymocytes (Grinstein et al, 1985), HL60 cells (Besterman et al, 1985) and A431 cells (Whiteley et al, 1984; Cassel et al, 1984). In quiescent cells, an alkaline pH is crucial for the G1/S transit; a pH controlled-restriction point may perhaps occur in G1 in some cell types (Taylor & Hodson, 1984). It is significant therefore that proliferating lymphocytes have a more alkaline intracellular pH as compared to quiescent lymphocytes. In fact, there is a fairly close correlation between the timing of the pH_i rise and an increase in DNA synthesis (Gerson et al, 1982). In support of this evidence, cyclosporin A which blocks T-cell activation and the associated DNA synthesis inhibits the early pH_i increase (Gerson et al, 1984). Thus, an early rise in intracellular pH appears to be a common response of metabolically quiescent cells to various mitogenic stimuli.

The extrusion of H^+ ions in mitogenically activated cells occurs only until the new 'set point' (increased value by ~ 0.2-0.3 units) is attained (Moolenaar, 1986). Such intracellular pH increase could favour the rate of protein synthesis and operation of enzymatic pathways involved in cell division. In fact, several key regulatory enzymes including DNA polymerase show significant changes in their activity at a higher pH optima (Lad et al, 1984). This alkaline shift may also have profound effects on cytoskeletal regulation and may promote disassembly of microtubules, a necessary event for initiation of DNA synthesis.

It is plausible that the metabolic events provoked by alterations in pH are indirectly regulated by calmodulin which exhibits pronounced pH sensitivity to Ca^{2+} binding. Under appropriate conditions, therefore a pH_i increase of only 0.50 would have the same effect as a five fold increase in $[Ca^{2+}]_i$ at constant pH_i. Thus, CaM may conceivably serve as a sensor of both $[Ca^{2+}]$ and pH_i changes. This does not necessarily imply identical correspondence between the effects of pH_i and cytosolic Ca^{2+} on the activities of CaM-regulated enzymes, however, since these activities may themselves be pH dependent. Nonetheless pH_i and $[Ca^{2+}]_i$ changes might have interchangeable effects on calmodulin-mediated responses. Therefore, the pH dependence may constitute a universal mechanism of pH_i-mediated (Ca^{2+} dependent) metabolic regulation in cells expressing physiological pH_i changes. Although the pH changes seem to be important, whether they are permissive or causative however remains uncertain.

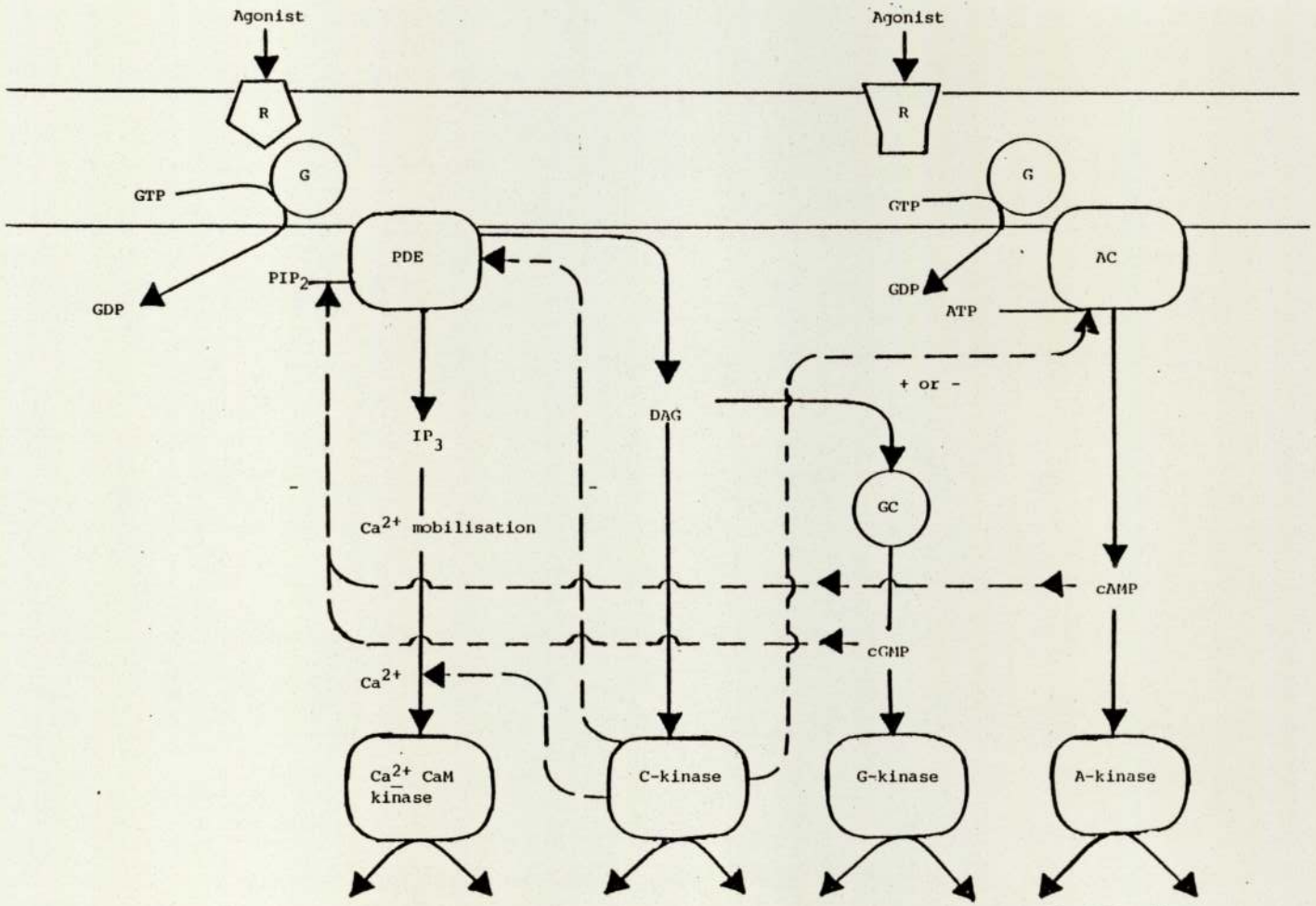


Diagram 3 Schematic outline of signal transduction through the inositol lipid and cyclic AMP pathways
In both pathways, a GTP binding protein (G) functions to couple surface receptors (R) to the enzymes phosphodiesterase (PDE) and adenylate cyclase (AC). PIP_2 is the lipid phosphatidyl inositol 4,5 biphosphate which generates inositol triphosphate (IP_3) and diacylglycerol (DAG). The latter by its specific lipases forms an intermediate which activates guanylate cyclase (GC).

In addition to the ionic fluxes, genesis of other second messengers via activation of membrane-associated enzymes could also transmit signals to the interior of the cell to evoke a cellular response.

1.3.4 Cyclic Nucleotides

The adenylate cyclase system constitutes a part of the complex regulatory system that mediates the actions of many hormones and neurotransmitters on their target cells. Structured within the lipid framework of the cellular membrane, the enzyme comprises at least three and distinct separable entities: the glycoprotein receptor (R) at the outer surface and on the inner surface, the guanine-nucleotide binding regulatory protein (G) and the catalytic unit (C), (Northrup et al, 1980; Sternweis et al, 1981).

A distinct receptor exists on the cell-surface for each hormone interacting with the cell. The regulatory protein has a crucial role in the coupling process by which the hormone-bound receptor conveys activation upon the catalytic unit for generation of cAMP from ATP. Such G proteins contain binding sites for GTP and are responsible for mediating the effects of GTP and the various hormones on the activity of C (Diagram 3). Two functional types of G-proteins have been distinguished in the adenylate cyclase system, the stimulatory protein (Gs) and the inhibitory protein (Gi). Separate classes of receptors are linked to the Gs and Gi nucleotide regulatory units; for example beta-receptors are Gs-linked and alpha-receptors are Gi-linked.

Role of Cyclic Nucleotides in Proliferation

The possibility that cyclic nucleotides (cAMP) and cyclic GMP (cGMP) may regulate the proliferative response of a variety of quiescent cells has been the subject of a large and controversial literature.

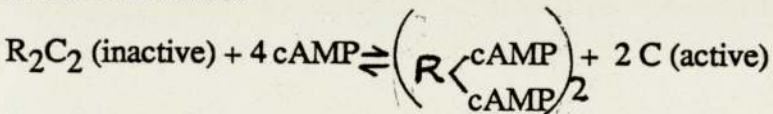
Following much experimentation, many authors consider cAMP as a negative regulator of cell division. Indeed, elevation of endogenous intracellular cAMP content by application of exogenous cAMP, its analogues or phosphodiesterase inhibitors (eg. methylxanthines) prevents cell growth in murine fibroblasts (Froehlich & Rachmeler, 1974), lymphoma cells (Coffino et al, 1975), lymphoid cells (Millis et al, 1974) several other cell types. The concentrations of cAMP or its derivatives employed in these studies were however pharmalogical rather than physiological. When cells are arrested in Go on attaining confluency or as a result of serum starvation cAMP levels are raised (Boynton & Whitfield, 1983). In contrast, low cAMP levels are found in actively growing cells (Moens et al, 1975; Boynton & Whitfield, 1983). Addition of fresh serum or other proliferative stimulants such as fibroblast growth factor, insulin (Rudland et al, 1974) and phorbol esters (Rochette-Elgy & Castagna, 1979) is often accompanied by a rapid decline in intracellular cAMP content (Seifert & Rudland, 1974). Finally, exposure of serum-starved chick embryo fibroblasts to exogenous cAMP phosphodiesterase (PDE) which rapidly degrades cAMP, may trigger metabolic events and reinitiate DNA synthesis (Lawrence et al, 1977). While some authors claim that the inhibition associated with cAMP occurs in Go/G1, others believe that it may occur during the G1/S transition or even in G2 (Sivak, 1977; Klimpel et al, 1979; Boynton & Whitfield, 1983). Since a continuous post stimulatory exposure inhibits DNA synthesis, it is possible that different facets of the cell-cycle may require different cAMP levels.

On the other hand, using the same cell line as Seifert & Rudland (1974) some workers have shown that intracellular cAMP levels decrease during growth arrest (Ory et al, 1974). Moreover, in parotid gland cells, the normal mitotic response to serum is accompanied by a decrease in cAMP content; this decrease is however not essential for DNA synthesis because when prevented by addition of

exogenous nucleotide, or phosphodiesterase (PDE) inhibitors, normal growth continues (Tsang et al, 1980). Indeed a positive role for exogenous nucleotide alone or in synergy with growth promoting agents has been described in Swiss 3T3 cells (Rozengurt, 1981), embryonic pancreatic epithelial cells (Filosa et al, 1975), epidermal keratinocytes (Green, 1978), Schwann cells (Roff et al, 1978) and human epithelial mammary cells (Taylor-Papadimitrious et al, 1980). In fact, cAMP surges are associated with mitotic activity in a variety of cell types (Sivak, 1977; Millis et al, 1974; Boynton & Whitfield, 1983; Whitfield et al, 1980). In the regenerating rat liver, cAMP surges are evident at both Go/G1 and G1/S transition steps (Whitfield et al, 1980). The first cAMP surge may not be essential since its elimination by adrenergic blockers does not affect the second surge or the initiation of DNA synthesis (Boynton & Whitfield, 1983). By contrast, the second peak is coupled to an increase in nuclear protein kinase activity and without it DNA synthesis is impaired (Laks et al, 1981). The cause of this critical surge in the proliferatively activated liver is unknown but may be a consequence of enhanced adenylate cyclase activity due to increased ß-adrenergic receptor expression or even an elevated calmodulin content (Bronstad & Christoffersen, 1980; Boynton & Whitfield, 1981). It is assumed that the increased cAMP surge at the G1/S boundary in some cells stimulates the transcription of cell cycle genes and directly activates some enzymes and proteins pertinent for cell growth (Whitfield et al, 1985).

It is conceivable that temporally distinct fluctuations in cAMP may trigger a cascade of G1 or G2 events by activation of protein kinase-A (A-kinase) which in turn may direct progression through the cell cycle by acting in concert with the calmodulin molecule. Although Coffino and co-workers (1975) dispute this premise, their evidence is based entirely on kinase negative mutants of S49 lymphoma cells but says nothing about other cell types. Nevertheless, it is

generally accepted that cAMP exerts its functions via the well-known intracellular receptors, namely, types I and II cyclic AMP-dependent protein kinase (PK) holoenzymes. These enzymes consist of different regulatory subunits RI or RII and the same catalytic or C subunit. Binding of cAMP to the regulatory subunits results in a conformational change and dissociation of the holoenzyme into cAMP, R and C subunits.



Regulatory subunit dimer cAMP complex catalytic unit

The liberated C subunits catalyse the phosphorylation of serine or threonine residues of various proteins. The type I kinase is activated at lower cAMP levels than type II kinase. The two types of kinases may be activated sequentially for distinct purposes. Some tissues may also have different type I/type II protein kinase ratios. Cell-cycle related changes in both type I and type II kinases may also occur. In synchronised CHO fibroblasts both kinases increased during the period G1-S, the identity of the kinases interacting with the DNA-synthesis determining mechanism is not clear (Haddox et al, 1980). The growth arrest of these cells by dibutyryl cAMP is associated with an increased type I/type II kinase ratio possible due to an increased type I synthesis or a decreased type II synthesis. In lectin-stimulated lymphocytes, an increase in type I kinase is associated with cell growth while an increase in type II is correlated with inhibition of growth (Russell, 1978). The cAMP type II kinase induced by high cAMP content has however been favoured (Boynton et al, 1981). The type II kinase may trigger certain critical events in late G1 cells, but may inhibit different vital events if generated in appreciable amounts at other stages of the cell cycle (Boynton & Whitfield, 1983). In fact, exogenous addition of type II kinase or of a specific inhibitor of the kinase to the cell culture promotes or prevents the

DNA synthetic response respectively (Boynton et al, 1981). Such a response may occur by phosphorylation of membrane-bound proteins as the kinase molecules are too large to penetrate the membrane. The phosphorylation products could open ion-specific gates for K^+ and Ca^{2+} and after cytosolic ionic levels. The opening of Ca^{2+} channels may be a prelude to increased cytosolic Ca^{2+} levels which perhaps in concert with cAMP might represent the ultimate mitogenic signal. Another possibility is that an increase in cAMP and its kinases may initiate nuclear events but this is as yet uncertain.

While much attention has been, and still is, focussed on the action and regulation of Ca^{2+} and cAMP, interest in the production and function of cGMP in proliferation has diminished greatly. Nevertheless a brief review of the relevant literature was felt appropriate.

The role of cyclic GMP in the control of cell division remains contentious. While some authors have assigned a negative role to cGMP (Miller et al, 1975; Macmanus et al, 1978), others have failed to detect any fluctuations in cGMP content following mitogenic stimulation (Parker, 1978; Boynton et al, 1978). These discrepant observations may perhaps be accounted by differences in culture conditions, mitogen concentrations and nucleotide assay methods. It is also possible that release or intracellular redistribution of sequestered cyclic nucleotides may be adequate for activation of enzymes pertinent for proliferogenic activity (Russell et al, 1976).

Many authors are of the opinion that cGMP has a positive role in cell growth. A variety of mitogen-activated cell types including BALBC 3T3 foetal mouse cells (Seifert & Rudland, 1974), rat hepatocytes (Tse et al, 1981) and Chinese hamster ovary cells (Millis et al, 1974) demonstrate a significant rise in cGMP levels. Exogenous cGMP, its derivatives or cGMP-elevating agents such as acetylcholine, noradrenaline, carbachol and phorbol esters similarly provoke

mitogenesis in several cell types including lymphocytes (Whitfield et al, 1974; Hadden et al, 1979; Coffey & Hadden, 1981). Lectin-stimulation of lymphocytes is also accompanied by an increase in cGMP content possibly by activation of both membrane and soluble forms of guanylate cyclase (Hadden et al, 1972; Whitfield et al, 1974; Hadden et al, 1979; Coffey et al, 1981). Such an increase may be associated with a transit through the G1/S phase of the cell cycle and subsequently cell division since both, mitosis and cGMP surges are mitogen concentration dependent, calcium dependent and can be induced by ionophore, A23187 (Coffey et al, 1978; Hadden & Coffey, 1982). The elevations in cGMP content may cause a progressive increase in cGMP-dependent protein kinases, the G-kinase. A PHA-induced increase in cGMP is indeed followed promptly by a 4-5 fold increase in G-kinase (Carpentieri et al, 1980). On binding of 2 molecules of cGMP, this G-kinase gets activated without dissociation of the holoenzyme.

$$\underset{\text{inactive}}{\text{cGMP dPK} + 2\ \text{cGMP}} \rightleftharpoons \underset{\text{active}}{\text{cGMP dPK (cGMP)}_2}$$

This active enzyme may subsequently phosphorylate substrate proteins imperative for cellular functions including cell division.

The proliferative effects of cGMP may thus be caused by phosphorylation of specific non-histone proteins in association with the pronounced early increase in RNA synthesis following mitogenic stimulation of lymphocytes (Hadden et al, 1979). In isolated nuclei from unstimulated human lymphocytes, cyclic GMP stimulates RNA polymerase activity and RNA synthesis (Ananthakrishnan et al, 1981). Studies of liver regeneration following partial hepatectomy also show quantitative increases in nuclear G-kinases. Such actions however require Ca^{2+} ions (Tse et al, 1981) suggesting that calcium and cGMP are involved in modulation of nuclear RNA synthesis and consequently cell division by some

intricate mechanism (Hadden & Coffey, 1982).

A unique inter-relationship is apparent between the cellular cGMP and cAMP levels. Exogenous application of cGMP-elevating agents can reverse the inhibitory effects of cAMP in both B and T lymphocytes (Diamanstein & Ulmer, 1975). In thymic lymphocytes however, a paradoxical situation is apparent as exogenous cGMP promotes an immediate surge in cAMP levels. The individual role of these cyclic nucleotides in cellular activation is thus clearly puzzling.

It is evident that the specific roles of cyclic nucleotides in the initiation of cell division are largely unknown. Whether the precise, temporally distinct fluctuations in the cyclic nucleotide content on cellular activation are indeed triggers for division or merely restorative homeostatic events is uncertain. It should be appreciated however that the cyclases are not the only enzymes that can be activated but that the phospholipases could also constitute a signalling mechanism for cell division.

1.3.5 Turnover of Phospholipids

The idea that phosphatidylinositol turnover is a primary event in hormonal signal generation has long been known (Michell, 1975). The initial proposition that activation of phospholipase C by agonist stimulation causes a breakdown of phosphatidylinositol (PI) has been replaced by reports indicating that phosphatidylinositol diphosphate PI (4,5)P_2 a PIP_2 is the key phospholipid; (Diagrams 3 & 4) The hydrolysis of the lipid upon phospholipase C attack apparently represents a bifurcation point in the signalling pathway forming diacylglycerol (DAG) and inositol triphosphate (IP_3) which function as potential second messengers (Berridge, 1984). The hydrolysis of PIP_2 is a common response to diverse external stimuli in a variety of cell types (Berridge & Irvine,

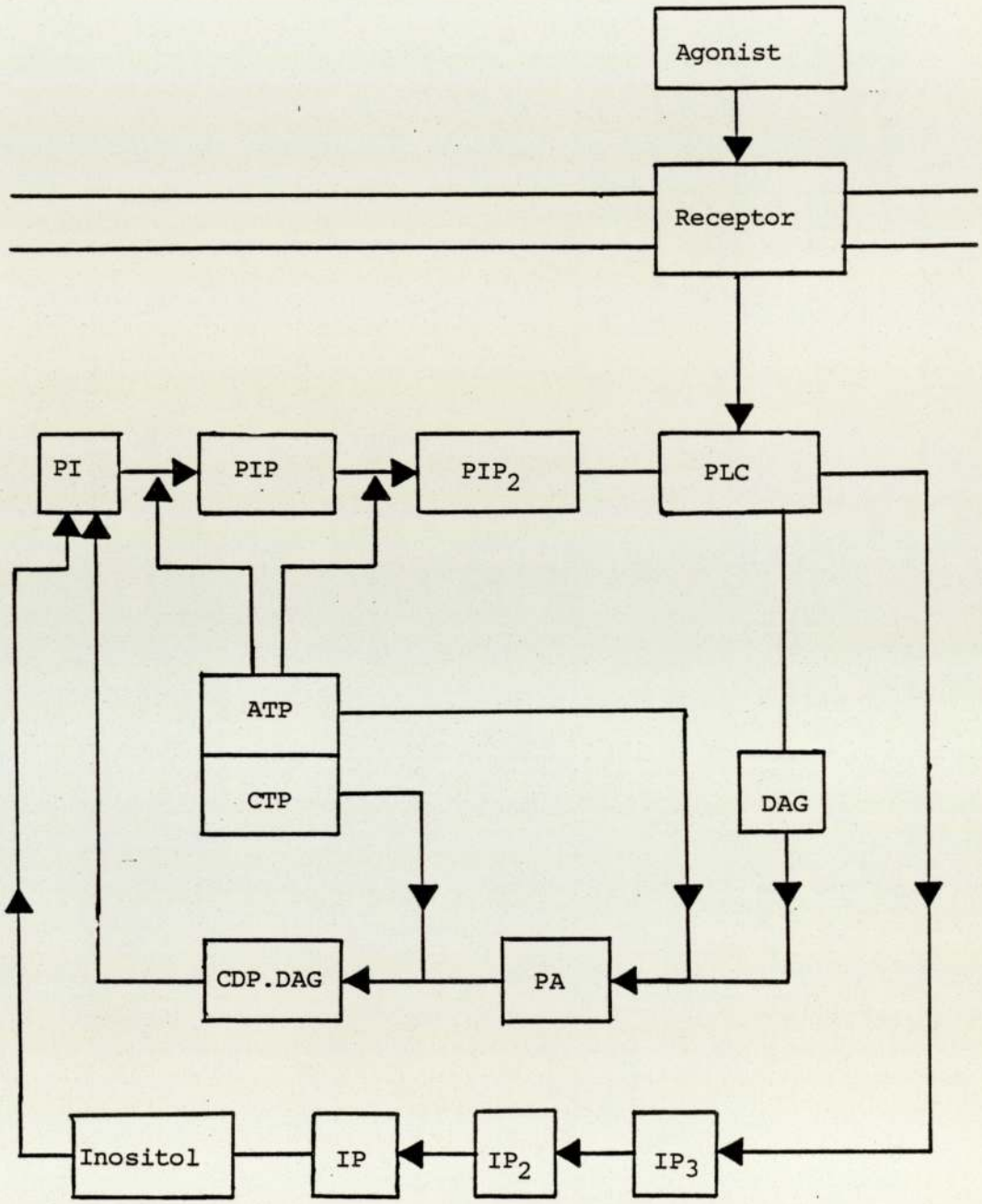


Diagram 4 Agonist-dependent phosphoinositide metabolism.

Phosphoinositol (PI) is phosphorylated to phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2). On occupancy of the receptor by the agonist, PIP_2 is hydrolysed by phospholipase-C (PLC) to yield diacylglycerol (DAG) and inositol triphosphate (IP_3). The latter is recycled back through an inositol phosphate cycle to free inositol for resynthesis to PI.DAG is phosphorylated to phosphatidic acid (PA) which on interaction with CTP forms the cytidine diphosphate diacylglycerol (CDP.DG) that recombines with inositol to replenish the PI pool.

1984). Most importantly, IP_3 has been described as a second messenger which mobilises Ca^{2+} from intracellular stores, in particular the endoplasmic reticulum (Berridge & Irvine, 1984). Although the precise concentration of IP_3 in stimulated tissues is unknown, the calcium-releasing property of IP_3 has been revealed by employing different permeabilization and Ca^{2+}-measuring techniques in several cell types (Burgess et al, 1984; Joseph et al, 1984; Berridge et al, 1984; Biden et al, 1984; Prentki et al, 1984).

The other major product, DAG, is only transiently produced in the membrane presumably both due to its conversion back to inositol phospholipids (Diagram 4) and to its further degradation to arachadonic acid for thromboxane and PG synthesis (Berridge, 1984). DAG can activate the membrane-bound enzyme, protein-kinase C (PKC) or C-kinase found in various tissues (Nishizuka, 1983, 1984). PKC acts on a wide range of substrate proteins and phosphorylates seryl and threonyl residues. The physiological significance of such protein phosphorylations is not clear. The activation of PKC by DAG usually occurs within a phospholipid environment and of ambient calcium concentrations. However, PKC can also be activated artificially by addition of phorbol esters (Gelfand et al, 1985). Some phorbol esters require normal phospholipid and calcium concentrations, while others may activate PKC regardless of the calcium concentration (Gelfand et al, 1985). The amount of DAG produced when PI turnover is stimulated by various agonists is perhaps sufficient to activate all the PKC within the cell.

Further features of PI turnover which must be considered are the production of phosphatidic acid, (PA) and arachidonic acid (AA) (Diagram 4). The former is a phosphorylation product of DAG and could function as a Ca^{2+} ionophore (Salmon & Honeyman, 1981). While this ionophoretic role has been supported by

studies using liposomes (Serhan et al, 1981), other workers have obtained negative results (Holmes & Yoss, 1983). Recently, exogenous PA has been shown to elicit a transient rise in $[Ca^{2+}]_i$ in cultured cells, not by stimulating Ca^{2+} influx, but by releasing Ca^{2+} from intracellular stores. Unlike an ionophore, PA acts by triggering PI hydrolysis probably by activation of the PDE or by rendering the PIP_2 substrate susceptible to PDE. This may, in turn, produce IP_3 and DAG which may signal their well known effects; mobilisation of Ca^{2+} and rise in pH_i which appear to be essential for mitogenesis (Moolenaar et al, 1986). A phosphatidic acid-specific lipase may degrade PA to yield arachidonic acid (Billah et al, 1983). Alternatively, AA may be released from DAG by its specific DAG lipase (Prescott & Majerus, 1983). The released arachidonic acid, after conversion to a peroxide may activate guanylate cyclase (Diagram 3) and thus stimulate the formation of yet another signalling messenger, cyclic GMP as indicated earlier (Berridge, 1984; Nishizuka, 1984).

It is of some interest that the signalling cascade triggered by phospholipase C activation requires GTP-binding proteins for transduction as does the adenylate cyclase discussed earlier (Cockroft and Gompertz, 1985). Non-hydrolysable GTP-analogues can stimulate phosphoinositide breakdown in isolated neutrophil membranes (Cockroft and Gompertz, 1985). In fact, GTP-analogues can greatly augment accumulation of inositol phosphate in membranes from blow fly salivary glands and DAG formation in permeabilized platelets and evoke the cellular response (Litosch et al, 1985; Haslam & Davidson, 1984). The identity of this G-protein however has not yet been established.

Role of Phosphoinositide Turnover in Cell Growth

PI turnover appears to be a characteristic feature of most cell types in response to a wide array of agonists and may rapidly yield the two degradative products, IP_3 and DAG which serve as intermediates in the activation of cell growth. A conspicuous example is provided by thymocytes where IP_3 levels increased within 30 seconds of addition of Con A; the levels were approximately 6-fold after 5-8 minutes and then declined slowly (Taylor et al, 1984). Such increases in IP_3 levels may account for the rapid rise in cytosolic Ca^{2+} levels so often observed in mitogen-stimulated cells (Tsien & Rink, 1982; Moolenaar et al, 1984; O'Flynn et al, 1984; Weiss et al, 1984). In fact, in fibroblasts and lymphocytes, Metcalfe et al (1985) have noted increases in $[Ca^{2+}]_i$ within 0.1-5 minutes of mitogen addition. On the other hand, the DAG limb of the signal pathway will activate PKC which in turn via a rapid membrane protein phosphorylation could activate a Na^+/H^+antiporter. Certainly a rise in intracellular pH occurs in mitogen-activated cells (Moolenaar et al, 1983; Hesketh et al, 1985). This can be prevented by the supposedly specific sodium-channel blocker amiloride. It is not clear whether the pH change is an obligatory trigger for mitogenesis or merely a facilitating permissive event. The activation of the Na^+/H^+ exchanger may concomitantly increase intracellular sodium levels. The significance of this rise in intracellular Na^+ levels and its possibility as a trigger has been reviewed earlier.

Despite the foregoing evidence indicative of potential role of ions and cyclic nucleotides and the several protein kinases in cell growth, the precise sequence of events preceding cell division remain elusive. This is mainly because studies on cell proliferation have mainly concentrated on the search for a common mechanism of action for the wide array of agonists that may arouse a variety of cell types from

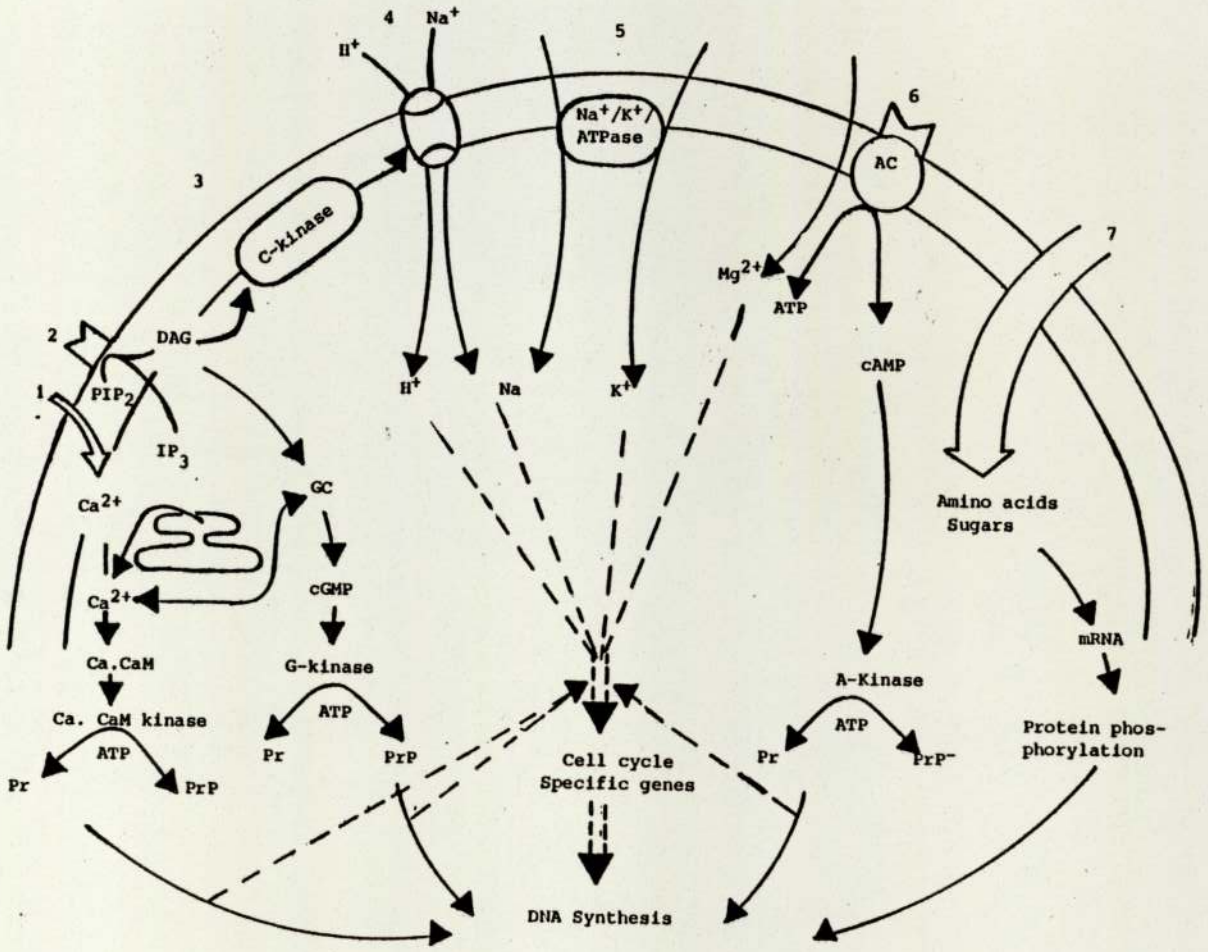


Diagram 5 The possible sequence of events during mitogenic activation. The signalling mechanisms are represented by numbers 1-7 (1 -> 2 -> 3 --> 7). For details refer to text (Section 1.3)

quiescence. A common mechanism however seems highly unlikely as different mitogens may generate disparate primary signals which in turn may generate a distinct pattern of secondary responses within an activated cell. Although it is not clear which signals are primary or secondary, permissive or obligatory, a generalised picture of the chronology of events is given in Diagram 5.

In addition to ionic signalling discussed previously, there is increasing evidence that some of the protooncogenes are directly linked to control of cell growth (Land et al, 1983). These genes which include the c-fos and c-myc are rapidly activated upon mitogenic stimulation of quiescent cells (Kelly et al, 1983; Campisi et al, 1984; Greenberg & Ziff, 1984; Muller et al, 1984). The amounts of c-myc mRNA and protein are elevated in normal proliferating cells including lymphocytes (Reed et al, 1985; Kaczmarek et al, 1985) and mouse thymocytes (Metcalfe et al, 1985). Although increased expression of these genes is evident, it is not known whether the genes are activated by ionic signals or whether their expression is obligatory for DNA synthesis.

The increased mRNA could increase protein synthesis. In stimulated cells, there is a marked rise in glycolysis and in the uptake of metabolites such as uridine (Hume et al, 1978; Brand et al, 1984). These biosynthetic responses late in G1 may be obligatory for progression of cells into S phase and ultimately cell division.

The cascade of events is however subject to a negative feedback system which may regulate the termination and duration of the response. The C-kinase can exert an inhibitory effect on the Ca^{2+} signal. In pituitary cells, the DAG/C-kinase pathway seems to reduce the Ca^{2+} signal by promoting the mechanisms that remove Ca^{2+} from the cytosol. PI hydrolysis may be inhibited by c-AMP as in platelets (Nishizuka, 1984). Cyclic AMP may perhaps interact with c-GMP, and inhibit the receptor-linked degradation of inositol phospholipid

in membranes, thereby blocking cellular functions and proliferation by counteracting the activation of C-kinase (Nishizuka, 1983). Cyclic GMP by itself may also act as a negative rather than a positive messenger providing an immediate feedback control that prevents over-response. Clearly, a multitude of signalling molecules can play a role in cell growth and clues to this might be found in the study of neoplastic cells where there may be accentuation or deletion of some of these supposedly specific signalling devices.

1.4 Neoplastic growth

Neoplasia or oncogenesis is a process by which the normal controlling mechanisms that regulate all growth and differentiation are impaired. Thus cells are freed from the constraints that preserve normal tissue cellularity and are transformed into uncontrolled progressively growing malignant cells. It is widely believed that these derangements are the result of abnormal expression of various genes. Such altered species have been termed 'oncogenes'. The protein products alternatively arising from such oncogenes, which differ only subtly from the normal or protoncogene, may differ in either quality or quantity from the normal counterpart. Several such oncogene products have been described which could interfere with the normal signalling mechanisms controlling cell growth (Weinberg, 1985). Thus, activation of autologous growth factor (GF) synthesis or autocrine activation, synthesis of altered GF receptor, activation of G-proteins or alteration of nuclear genes could lead to widespread signalling derangements and neoplastic growth.

An autocrine activation mechanism depends on the ability of malignant cells to synthesise and release growth factor-like peptides which bind to and activate autologous receptors. Cells transformed by oncogenic retroviruses and other tumour cell lines produce and respond to transforming growth factors (DeLarco &

Todaro, 1978; Marquardt et al, 1983). An example is provided by cells transformed by the Abelson sarcoma virus which spontaneously produces TGF provoking their own division. When these cells were infected by transformation-defective variants; TGF production was curtailed and transformation was no longer detectable (Twardizik et al, 1982). Abnormal production of growth factor may be a result of deregulation of genes coding for the particular factor. Indeed the p28 sis oncogene product is closely related to the platelet-derived growth factor and serves as an autocrine regulator of growth in cells transformed by sarcoma virus (Doolittle et al, 1983; Deuel & Huang, 1984). Many human sarcoma and glioma cell lines produce a PDGF-like mitogen (Heldin & Westermark, 1984). Other autostimulatory growth factors produced by melanoma cells, fibrosarcoma and mammary tumour cells are NGF and IGF (Sherwin et al, 19798; Knaver et al, 1980).

Receptor activation can sometimes occur in the absence of a growth-factor. In this instance, the receptors themselves become altered in a manner that allows them to continually bombard the cell with growth-stimulatory signals conferring GF automony. For example, the EGF receptor can itself become an oncogene if a viral promoter or activator is inserted into the cellular gene as in erythroblastosis. In fact, the erb B gene product and the EGF receptor illustrate considerable homology except for the absence of an EGF binding domain in the retroviral version (Downward et al, 1984). The erb B protein may thus function to relay a mitogenic signal even in the absence of EGF binding.

Many oncogenes encode proteins that phosphorylate the tyrosine-specific protein kinases. Functionally, both the EGF receptor and several transforming proteins, which are also protein kinases are able to phosphorylate proteins on tyrosine residues. Thus, the EGF receptor and pp 60^{src}, the protein encoded by the transforming gene of the Rous sarcoma virus, phosphorylate the same

regulatory 35kDa and 36 kDa proteins due to similar substrate specificities. Such phosphorylation could then perturb the regulatory network and, by mimicing the action of the physiological enzymes, cause uncontrolled activation of the cell cycle. However, such phosphorylation has not provided the key to growth control as 0.01% tyrosine kinases are present even in normal cells and there is no significant elevation in neoplasia. Some src-related protocogenes may however encode enzymes involved in the increased intracellular formation of IP_3 and DAG following GF stimulation (Berridge, 1984). These, in turn, provoke an ionic redistribution and activation of the C-kinase thereby effecting many of the pleiotropic changes associated with oncogene action.

The ability of cultured neoplastic cells but not normal cells to proliferate in media with a very low calcium concentration has been shown for a variety of cell types, eg. chicken fibroblasts (Balk et al, 1979), WI-38 fibroblasts (Hazelton & Tupper, 1981), rat liver hepatocytes (Swierenga et al, 1980), 3T3 cells (Paul & Ristow, 1979), human carcinoma cells (Swierenga et al, 1983). This deviant proliferative behaviour of transformed cells may perhaps be accounted by the disturbances in cellular Ca^{2+} homeostasis relative to their normal counterparts. Although this seems highly likely, it is not yet clear whether such disruption causes an increase in cytosolic free Ca^{2+} levels under basal or unstimulated conditions (Hazelton & Tupper, 1979; Hartmann et al, 1986). It is plausible that an elevated calcium content may lead to a loss or bypass of some Ca^{2+} dependent anchorage coupled mechanisms for regulation of cell growth (Durham & Walton, 1982; Hartmann et al, 1986). In fact, a diminished requirement for Ca^{2+} is certainly evident before onset of anchorage independent growth (Ribiero & Armelin, 1984). It should be noted however that not all tumour cells undergo proliferation in a Ca^{2+}-free medium, eg. adult T-cell leukemia cultures are highly dependent on calcium and calmodulin for growth (Shirakawa et al, 1986). Despite

such contrasting results, several authors propose that the characteristic abnormal proliferative behaviour with respect to calcium may be considered as an in vitro marker for neoplastic transformation (Swierenga et al, 1983).

An increase in calmodulin in conjunction with the de novo appearance of an apparently tumour specific calcium binding protein, oncomodulin, following neoplastic transformation may be responsible for the ability of tumour cells to become independent of extracellular calcium (Macmanus, 1982). Oncomodulin is a smaller calcium binding protein (MW 11,500) with only two calcium binding sites per molecule and may perhaps be activated by relatively low intracellular Ca^{2+} concentrations thereby usurping the role of calmodulin. Oncomodulin has not however been found in all tumours tested (Macmanus, 1981). Perhaps transformed cells which have lost the usual requirement for extracellular Ca^{2+} divide due to alterations in either calmodulin content or a redistribution of calmodulin and its target proteins. It is even possible that the target proteins may have an altered sensitivity to Ca^{++}-calmodulin. Some workers have indeed observed an increased calmodulin concentration while others have indicated no change at all in tumour cells relative to their normal counterparts. However, Viegl et al (1984) have pointed out that elevated levels of calmodulin do not necessarily correlate directly with growth rate. Such disparate results may perhaps be due to variations in culture conditions (McNeil et al, 1985; Veigl et al, 1984).

A marked change in intracellular calmodulin distribution has also been observed in some malignant cells. For instance, extracts of Morris liver hepatoma had 6-43 times more calmodulin in the cytosol than in the particulate fraction as compared to the distribution of 2:1 in normal cells. Such disturbances in the intracellular calmodulin content or distribution may alter CaM-regulated functions, eg. localisation of cytoskeletal elements involved in cell division or in maintenance of cell shape or even protein phosphorylation reactions causing unrestrained

growth in the absence of Ca^{2+}.

In addition to derangements in cellular calcium homeostasis some tumour cells lose their ability to regulate and to be regulated by Mg^{2+}. For example in neoplastic lymphocytes and transformed fibroblasts, intracellular magnesium is much higher than normal (Rubin, 1982). Perhaps structural or metabolic defects which alter the distribution of the free and bound magnesium cause malignant growth. Such cells may thus exhibit a reduced requirement for magnesium for division (McKeehan & McKeehan, 1980). This diminished requirement of Mg^{2+} for cell growth probably occurs after anchorage independent growth (Rubiero & Armelin, 1984). More remarkably, spontaneous or virally transformed fibroblasts when grown under severely magnesium-limited conditions quickly assume the non-transformed morphological and behavioural characteristics (Rubin et al, 1981; Rubin, 1982). This re-transformation by manipulating exogenous magnesium concentrations apparently restores calcium homeostasis to normal (Vidair & Rubin, 1982).

Alterations in the monovalent cation content also are evident in the transformed cells. In general, Na^+ levels are three- to four-fold higher in tumour cells than in normal quiescent tissue. It is possible that Na^+ influx is enhanced in transformed cells since the Na channel blocker amiloride reduced nuclear Na^+ levels (Sparks et al, 1983; Owen & Villereal, 1985). The increase in membrane Na^+ permeability is associated with an increase in Na^+K^+ ATPase activity and ultimately a rise in cytosolic K^+ (Shen et al, 1978). Thus K^+ concentrations are raised in tumour cell types. It is plausible to believe that such elevated K^+ levels along with the elevated Mg^{2+} levels may promote enhanced protein synthesis and thereby cause malignant growth. These studies clearly imply that the monovalent and divalent cation content is disrupted in transformed cells whether such disruption is related to changes in cell volume and hence to malignancy is

uncertain.

It was pointed out earlier that the role of cAMP in cell growth is controversial. Nevertheless, an increase in cAMP is often associated with cell division. Chemical induction of tumours is also related to an increase in adenylate cyclase activity and cAMP content. It is worth pointing out that in some cells Ca^{2+} and cAMP may act synergistically to promote mitosis; a defect in either of the two signalling mechanisms could then cause malignancy. Indeed, in fibroblasts transformed by the avian-sarcoma virus, there is a normal presynthetic cAMP surge but the dependence of cell growth on exogenous calcium supply is eliminated. In these cells, the content of the type II protein kinase pool is enlarged which disrupts the Ca^{2+} and cAMP controlled division (Durkin et al, 1981; Boynton & Whitfield, 1983). Thus, alterations in production of protein kinases or failure to produce regulatory and catalytic subunits could cause deregulation of growth (Lohmann & Walters, 1984). An enhanced cGMP concentration may also cause malignancy. The defective intracellular signalling mechanisms may perhaps be accounted by erroneous signal transduction by the p21 product of the ras gene. These p21 proteins exhibit GTP binding activity and hence may be indirectly linked to the activation of the adenylate cyclase and the phosphoinositide pathways crucial for cell growth. Microinjection of p21 induced DNA synthesis which can be blocked by antibodies specific to the p21 gene (Feramisco et al, 1984). On constitutive activation, the ability of p21 to hydrolyse GTP effectively is lost leading to a continual growth stimulus without any need for a growth factor or even its receptor.

The intracellular signalling mechanisms could induce the expression of a set of protooncogenes including c-fos, c-myc and p53 which code for DNA-binding proteins. These proteins prepare the cell for initiation of DNA replication (Muller et al, 1984; Olashaw & Pledger, 1983; Reich & Levine, 1984; Kaczmarek et al,

1985). Constitutive activation of these GF-regulated genes results in an apparently continuous stimulus to proliferate. This may be the case with Burkitt's lymphoma, the chromosomal rearrangements presumably transcriptionally activate the c-myc locus, resulting in elevated levels of c-myc mRNA which in turn, may lead to uncontrolled growth (Kelly & Siebenlist, 1985). The myc protein may also perturb the activity or specificity of the cellular transcription apparatus and perhaps mobilise the expression of a bank of cellular genes whose products are critical to growth and differentiation. The transcriptional deregulation of genes may thus relieve much of the normal GF requirement that must be satisfied in order for the cell to undertake a growth program.

It should be considered however that the conversion of a normal cell to a malignant one is a multifactorial process. Transfection of primary rat fibroblasts with an activated myc gene is not sufficient to cause a malignant phenotype. Full transformation requires a second co-operating oncogene from the ras family (Land et al, 1983). Thus, certain oncogene cell products may be involved in competence (eg. myc, myb, Ela, fos, sis), others in progression (eg. ras, raf/mil, B lym) and still others in both competence and progression (polyoma middle T). The involvement of multiple collaborating oncogenes in creating tumours however still requires extensive substantiation.

CHAPTER TWO

THYMIC LYMPHOCYTE PROLIFERATION

Having reviewed the several signalling devices which may serve to regulate growth in various cell types, the lymphoid cell in particular must now be examined since proliferation of thymic lymphocytes is the principal concern of this thesis.

Clonal expansion of T and B lymphocytes is vital in the mediation of an immune response. Since the generation of T cells depends in turn on development within a thymic environment, some properties of both early thymocytes and non-lymphoid thymic stroma along with a few comments on intrathymic events seem pertinent.

2.1 Colonisation of the Thymus

It is generally accepted that in the mouse primitive stem cells exist within the embryo by day 10 of the gestation period. Subsequently the first colonisers appear within the thymus in the form of large basophil cells on the 11th and 12th day of embryonic life (Stutman, 1985). This migration of primitive prethymic cells into the organ continues into adult life. The mechanism via which these wandering cells are attracted to and subsequently remain within the thymic environment is unknown; a membrane-bound component of the stromal cells has been suggested as a possible chemo attractant (Potworowski et al, 1985). In birds, the colonisation occurs in waves and it is possible that a similar episodic influx also exists in mammals (Le Douarin Jotereau et al, 1987). The influx of cells into the thymus is certainly low since an irradiated depopulated mouse thymus is reconstituted by addition of only a few bone marrow-derived thymic precursor cells (Ezine et al, 1984). Clearly the large basophilic colonisers have a high proliferative capacity (Scollay et al, 1984).

2.2 INTRATHYMIC T-CELL DIFFERENTIATION IN THE MOUSE

Colonisation by the basophils is followed by the generation of a population of blast cells (A) which in turn undergoes rapid and extensive proliferation to generate other thymocyte subpopulations. These subsets are characterised both by their topography and their cell surface antigens (Ceredig et al, 1983; Ceredig & MacDonald, 1985). The blast cells can be detected within the thymus by days 13-14 of foetal development and in the adult thymus about 4% of the total population is of this type (Ceredig et al, 1985; Raulet, 1985). They bear neither the Lyt-2 nor the L3T4 surface antigens typical of suppressor and helper cells respectively and thus exhibit no mature function. However, they can give rise to all the more mature sub-populations within the thymus since their administration to irradiated mice transiently reconstitutes the full spectrum of cell types within this organ. The type A blast cells are not a homogenous population of cells; various markers have been identified upon their surface such as transferrin and interleukin receptors, a glycoprotein (Pgp-1) typical of bone-marrow derived prothymocytes and even the MEL-14 marker which some claim typifies cells destined to migrate from the thymus (Reichert et al, 1984; Ceredig et al, 1985; Lesley et al, 1985; Ceredig & MacDonald, 1986).

Derived from the blast cells is another population of large cells (B) which appear in the cortex of the thymus at day 15-16 of gestation (Diagram 6). These cells now bear both the suppressor and helper antigen Ly2 and L3T4 (Ceredig & MacDonald, 1985). In the adult thymus 20% of the cells are large B cells. In the embryo these cells give rise to small cortical lymphocytes (C) which are also $Lyt2^+$ and $L3T4^+$. In the adult 60% of all thymocytes carry both of these markers a phenotype which intriguingly is never found amongst

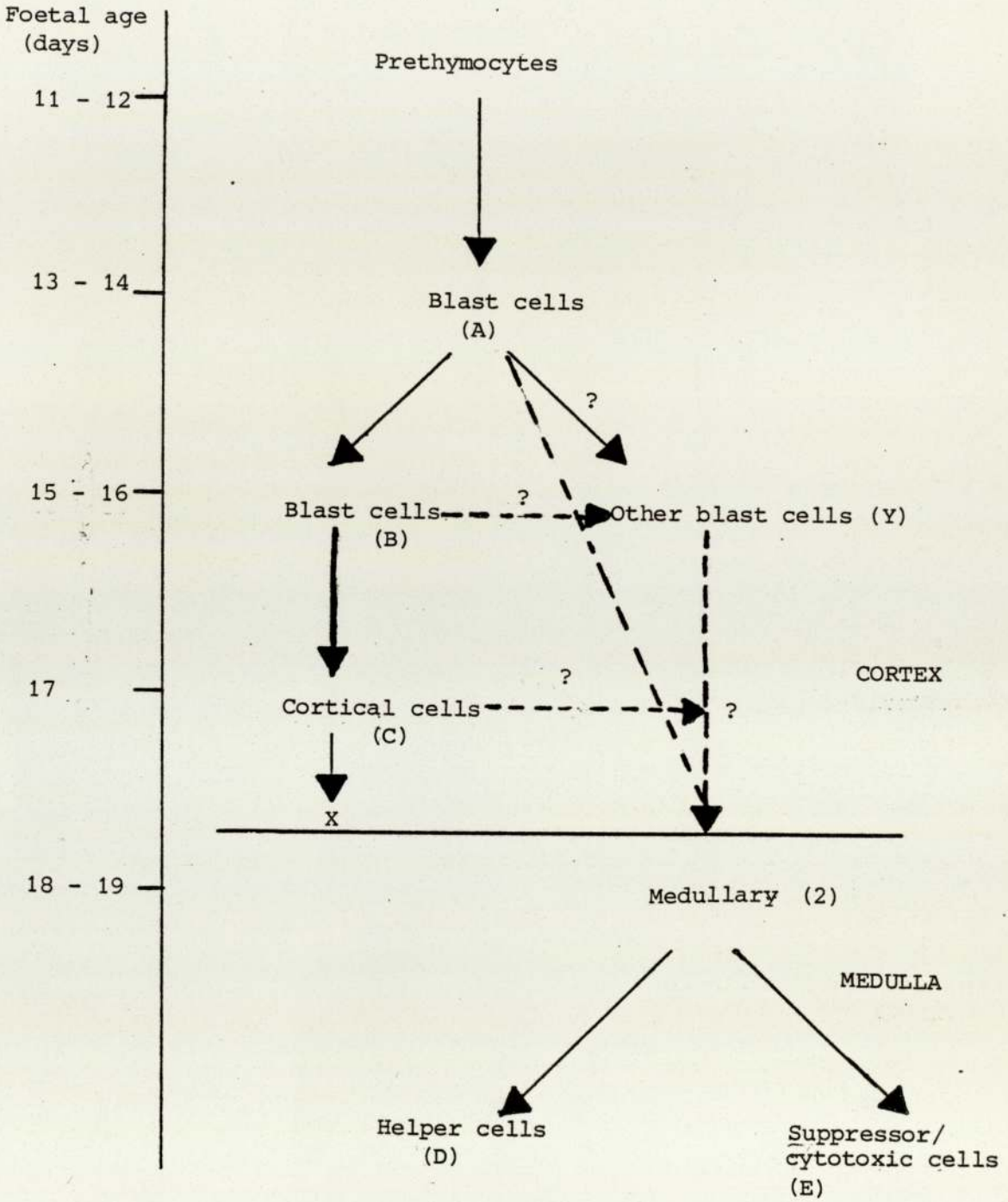


Diagram 6 An outline of thymocyte development and possible interrelationships between the cortical and medullary lineages within the fetal murine thymus

Key: (A) Lyt 2^- L3T4$^-$ phenotype, (B) and (C) Lyt^{2+} L3T4$^+$ cells; (D) Lyt2$^-$ L3T4$^+$ and (E) Lyt^{2+} L3T4$^-$ cells. (Y) and (Z) are Lyt2$^-$ L3T4$^-$ cells that may have arisen from population A . X denotes end stage and thymocytes

peripheral T cells (Ceredig & MacDonald, 1985).

On days 18 and 19 two further subpopulations D and E become apparent and are positive for only one or other of the two markers $Lyt2^+$ and $L3T4^+$ (Diagram 6). In addition both types have also acquired a receptor which binds the monoclonal antibody B2A2. These $Lyt2^+$ or $L3T4^+$ cells (comprising 5 and 10% all adult lymphocytes respectively) are largely found in the medulla and although destined to eventually become helper and suppressor or cytotoxic peripheral lymphocytes, they have not yet acquired the mature functions these markers typify. It is suggested that these $B2A2^+$, D and E cells are end stage thymocytes awaiting export from the thymus. When seeded in the periphery they lose the B2A2 marker and acquire their functional capabilities (Scollay et al, 1984; Ceredig & MacDonald, 1985).

Controversy surrounds the possible origins of the D and E cells. A simple progression from the small cortical lymphocyte ($Lyt2^+$ $L3T4^+$) to D or E medullary cells with a loss of one or other antigen seems an obvious possibility. However, some workers believe the C cells are a separate stream of non-functional rejected end cells (Ceredig et al, 1982). The only fate of these cells in vivo is intrathymic death (McPhee et al, 1979). Similarly, it has been claimed, the precursors of the small cortical lymphocyte, the large blasts (B), although capable of limited expansion, are on the same terminal pathway and so are unlikely to produce mature functional cells. Furthermore treatment of B and C cells with thymic hormones and IL2 failed to generate the D and E phenotypes (Chen et al, 1983; Andrews et al, 1985). Thus, it has been suggested that the early A-type blast cells may form two separate functionally distinct cortical and medullary lineages (Scollay et al, 1984; Scollay & Shortman, 1985). It has already been mentioned that although all type A blasts are Lyt2 and L3T4 negative it is a far from homogeneous population in terms

of the display of various other receptors and markers. Scollay and Shortman (1985) have described at least 8 minor but distinct subpopulations of each blasts. For example, two of these exhibit identical phenotypes with the exception of the Thy 1 marker. Thus within the cortex there are B2A2-, $Lyt2^-$, $L3T4^-$, $Lyt1^+$ and $Thy1^+$ cells and $B2A2^-$, $Lyt2^-$, $L3T4^-$, $Lyt1^+$ and $Thyl^-$ cells. These blast cells could represent a transition state between cortical and medullary thymocytes and may be the progenitors of cortical and medullary lineages.

Further differentiation of these putative precursors certainly occurs so that eventually cortical and medullary thymocytes are quite distinct. Thus, cortical cells bind avidly with the peanut agglutinin lectin, express high levels of the glycoprotein marker Thy1, low levels of the histocompatibility marker H-2 and are positive for Tdt (terminal deoxynucleotide transferase). In contrast medullary cells bind very little PNA, express low levels of Thy1 and high levels of H-2 and are Tdt negative. The various differentiation steps leading ultimately to the medullary phenotype can occur in either cortex or medulla depending on the timing of a cortico-medullary migration. Once mature and in the medullary environment, the cells are ready to migrate to the peripheral lymphoid tissues and assume their functional roles as helper or suppressor cells. This cellular export amounts to about 1% of the total thymocyte pool per day and seems uninfluenced by antigen-driven events in the peripheral lymphoid tissues (Scollay & Shortman, 1984).

2.3 THE THYMIC ENVIRONMENT

It is generally accepted that the non-lymphoid thymic cells constitute not only a simple framework for the organ but also provide the basis of a specific microenvironment capable of sustaining and directing the proliferation and

differentiation of immigrant T progenitors. This may be achieved by a cellular contact between the T-cells and the stromal cells, perhaps by a receptor on the T-cell and the major histo-compatibility complex determinants on the stromal cells. Alternatively, secretion of soluble factors such as thymic hormones and interleukins may direct the differentiation of the immature T-cell to a mature functional state.

2.3.1 The Thymus

The thymus originates as an epithelial rudiment from the third (in some species fourth) pharyngeal pouch by an outpushing of the endoderm (Le Douarin & Jotereau, 1981). Early during development, the endodermal bud interacts with the ectoderm (derived from the pharyngeal cleft), a necessary thymic constituent presumably associated with the formation of a lymphoid thymus (Owen & Jenkinson, 1984; Haynes, 1984). The thymus also contains mesenchymal cells of neural crest origin; the development of the thymic epithelium is apparently induced by the mesenchyme (Bockman & Kirby, 1984). A delicate balance between these embryonic germ layers seems critical for thymus development.

The thymus is surrounded by a mesenchyme-derived connective tissue capsule which intermittently invades the thymic anlage to form the septae. These septae divide each thymus lobe into multiple lobules. Within the capsule and the septae are all the cellular components of connective tissue including the adipocytes, macrophages, eosinophils, neutrophils, plasma cells and lymphocytes. This compartment is however devoid of epithelial cells and is demarcated from the cortico-epithelial compartment by a layer of epithelial cells and their basal laminae. The combination of these epithelial cells, cells of the connective tissue compartment and vascular endothelial cells with basal laminae

comprise the classic blood-thymic barrier. Despite the barrier, the capsule and the septae can conceivably constitute a major route for lymphocyte traffic probably via lymphatic vessels both into and out of the thymus. The septae, in addition may also carry the necessary vascular and nervous supplies which contribute to the cellular complexity of the system (Kendall, 1981; Haynes, 1984).

The epithelial cells are the dominant intrinsic component of the thymus. These cells form an extensive reticular meshwork within which the developing thmocytes mature and differentiate. They typically possess abundant tonofilaments and desmosomes in their cytoskeletal system and may demonstrate occasional thymic cysts which contain microvilli or even cilia (Kendall, 1981). The epithelial reticular cells can also be distinguished on the basis of morphological variation into dark cortical cells and pale medullary cells. While the dark cells are commonly associated with collagen fibres, the pale cells contain membrane bound vacuoles. These vacuoles in turn, probably have secretory granules and the appropriate synthetic machinery to produce them; thus supporting their potential for a humoral role in immune function (Haynes, 1984). The pale cells may also form Hassals or thymic corpuscles; these are distinct in man but not very well developed in rat and mouse (Haynes, 1984). The corpuscles presumably are a repository for dead thymocytes and possibly other peripheral blood cells (Kendall, 1981). Like the pale epithelial cells, they may also be associated with a detectable content of thymic humoral factors and conceivably related to thymic endocrine function (Haynes, 1984).

Along with epithelial cells, the two other non-lymphoid cell types evident in the medulla are the bone-marrow derived macrophages and the so-called interdigitating reticular cells (IDC) or the dendritic cells. The latter cell type can also occur in T-cell domains in peripheral lymphoid organs where they

probably act as stimulator cells in the education of T-helper cells (Ewijk et al, 1974). The former cell type form a minor non-lymphoid thymic population. The exact role of these macrophages in the process of T-cell differentiation is not clear at present. Their predominant localisation in the medulla suggests that together with IDC, they may assist in the antigen-dependent expansion of the T-helper subset (Beller & Unanue, 1980; Robinson & Jordan, 1983)

The non-lymphoid cell types of the thymic stroma are evident by day 13 of gestation and in fact, there is a division of stromal components into distinct cortical and medullary regions by this time (Van Vliet et al, 1985). Not surprisingly, the expression of the major histocompatability complex determinants on these stromal cell types is also developmentally regulated. While the Class II antigen expression initially appears in the medulla at day 13 and expands thoughout the lobe by day 16, the Class I antigen becomes barely detectable by day 16 (Owen & Jenkinson, 1984). Thymic antigen-presenting cell function is also initially apparent on day 13. The expression of these determinants on the stromal cells however precedes the expression of most T-cell markers and the antigen receptor on the lymphoid population, thus indicating that the cortical and medullary stromal environments are established before the development of the cortical and medullary thymocytes. Whether this early division of the stroma is pertinent for the maturation of lymphoid precursor types separately committed to cortical and medullary pathways (Ezine et al, 1984) or whether the progeny of a single precursor type move sequentially through the two stromal compartments remains a matter of speculation.

2.3.1a MHC Recognition and T-Cell Antigen Receptor

Besides acquiring specific differentiation antigens, T-cells also develop receptors for antigen/MHC during the process of differentiation. The antigen receptor in murine T-cells is a transmembrane, heterodimeric glycoprotein composed of α and ß chains (~ 43 Kd each) and four non-covalently associated polypeptide chains;$\gamma,\delta,\Sigma,\zeta_2$ (Samelson et al, 1985; Snodgrass et al, 1985). The expression of the T-cell receptor and its genes appears to be developmentally regulated (Raulet et al, 1985; Snodgrass et al, 1985). Indeed, in 15/16 day foetal thymocytes, the ß-chain genes become rearranged while the γ-chain genes attain a peak steady-state level and rapidly decline thereafter. At day 17 of gestation, the antigen-receptor becomes detectable coincident with the transcriptional activation of both α and ß-chain genes. As development proceeds, the ß-chain or RNA levels remain constant while the α-mRNA levels increase and reach a maximum at day 19 (Snodgrass et al, 1985). An increase in receptor expression is certainly observed with further maturation until a high receptor density is achieved in peripheral T-cells (Roehm et al, 1984). Thus, if the antigen receptor complex is required for repertoire selection, the selection cannot occur until day 17 of gestation. Incidentally, functional cells also appear in appreciable numbers from day 19 of development (Ceredig et al, 1983).

Despite the identification of the T-cell receptor and the genes encoding the receptor subunits, it is not clear whether MHC recognition requires a single receptor or two T-cell receptors. The bulk of the evidence strongly suggests that a single T-cell receptor or the α/ß heterodimer is adequate for the recognition of MHC and foreign antigen (Robertson, 1985). This view is also

supported by gene-transfection experiments which illustrate that α- and β-chain genes have the potential to transmit functional specificity from one cytotoxic T-cell to another (Dembic et al, 1986). The precise contribution of the α- and ß-chains to the recognition of MHC and antigen, however remains unclear.

A putative role for the δ-chain in the control of the T-cell repertoire within the thymus has recently been suggested (Raulet et al, 1985). Early in ontogeny, the γ-chain may be associated with the ß-chain, the γ/ß heterodimer may probably serve as a receptor allowing selection or suppression of clones. In mature thymocytes however the γ-ß dimer may be replaced with a much weaker affinity for self MHC, so that effective binding can occur only with the addition of the antigen. Although this theory has been questioned, it appears to be quite intriguing (Royer et al, 1985).

It is now appreciated that Class I-MHC-(cytotoxic) and Class II-MHC-specific T-cells (helper) may undergo different differentiation pathways. The self-MHC specificity of the cytotoxic cells is determined strictly intrathymically while the helper cells can recognise the MHC molecules expressed both in the thymic and in the extrathymic environment (Kruisbeek et al, 1984). The MHC pattern of the thymus therefore determines the repertoire of specificities in T-cells; the intrathymic events whereby restricted MHC recognition is generated are however largely unknown.

There are indications that interactions between the T-cell receptor on thymocytes and MHC determinants on thymic stromal cells may constitute the primary signal responsible for MHC-specificity. The isolation of thymic nurse cells which represent specific in vivo associations of thymocytes with epithelial cells certainly demonstrates an intimate relation (Kyewski & Kaplan, 1982). In

fact, such cells may sometimes even provide a secluded microenvironment in the outer cortex. These cells nurture immature thymocytes and prevent their terminal differentiation by providing a barrier to diffusion and ensuring a local concentration of thymic hormones or factors within the nurse cell itself (Andrews & Boyd, 1985). While a majority of the enclosed thymocytes are immature cortical cells some mature cells of the helper lineage may also be present. An association between cortical thymocytes and epithelial cells is perhaps provided by the T-cell antigen receptor mediated binding of MHC determinants on epithelial cells. Electron microscopy studies have revealed that the α/β receptor is capped on that part of the thymocyte which is in contact with the Class II bearing epithelial cells (Farr et al, 1985). Patching of receptor in areas of contact between thymocytes and the Class II bearing macrophages and dendritic cells was however not evident. The failure to observe thymocyte/epithelial interactions in the medulla may reflect the maturational state of the thymocytes or differences between cortical and medullary epithelial cells (Haynes et al, 1984).

Although an interaction between dendritic cells and thymocytes has not been observed, dendritic cells or thymic antigen-presenting cells (APC) may nevertheless play some role in T-cell differentiation. These cells may provide a stimulatory signal to self-class II specific thymocytes (Rock & Benacerraf, 1984). Indeed, injection of monoclonal anti-Ia antibodies into new born mice leads to a reduced expression of Class II antigens in the thymus; an abrogation of thymic APC function and complete lack of development of the mature helper phenotype (Class II-specific). The mature cytolytic phenotype, in contrast may develop normally in these injected mice (Kruisbeek et al, 1983, 1985). Thus the expression of Class-II antigens on APC may be essential for the generation of the helper T-cell subset. This significant role of APC does not discount the

possibility that APC may also be involved in delivering Class I-specific signals to the cytolytic phenotype and other non-specific signals (Kruisbeek, 1986).

Taken together, the foregoing evidence suggests that T-cell maturation via thymocyte receptors and MHC-recognition may occur in two stages:

(a) When immature double positive thymocytes develop into mature thymocytes and T-cells, and

(b) When cells that react with self-MHC or dendritic cells are eliminated and fully mature selected cells are allowed to enter the periphery.

Such T-cell maturation appears to be analogous to the antigen-independent differentiation of self-MHC restricted T-cells in the cortex and the antigen-dependent expansion of self-MHC-restricted T-cells in the thymic medulla and T-cell domains of peripheral lymphoid organs (Ewijk, 1984).

2.3.1b Thymic Hormones

In addition to regulation by MHC recognition, the T-cell repertoire is also subjected to the influence of growth factor -like hormones secreted by the nonlymphoid thymic stromal cells. Indeed, products of thymic epithelial cells can induce partial reversal of immune defects caused by neonatal thymectomy. These factors termed 'thymic hormones' form a heterogeneous group of peptides which have now been extensively characterised and sequenced. These factors are mainly secreted by epithelial reticular cells. One such hormone, thymosin fraction V, is a crude fraction isolated from the calf thymus gland. It contains a set of polypeptides with molecular weight ranging from 1,000-15,000 and categorised into α-thymosin and β-thymosin on the basis of

isoelectric focussing (Goldstein et al, 1977). Thymosin α, is an N-terminal blocked heat-stable peptide composed of 28 amino acids (MW 3,107) while thymosin β is composed of 43 amino acids. While the α–peptide induces differentiation and maturation of early thymocytes and immature T-cells by inducing the antigenic make-up, the ß-peptide appears to act selectively upon prethymic stem cells. A further heat-stable polypeptide isolated from the bovine thymus is the 49 amino acid residue, thymopoietin (TP). This molecule induces the differentiation of prothymocytes into thymocytes and promotes several T-cell functions (Low & Goldstein, 1984). Two forms of TP exist (I and II), their amino acid sequence varying by only 3 amino acid residues. A pentapeptide designated TP-5 corresponding to residues 32-36 in TPII has been found to display the full biological activity of the parent molecule (Goldstein et al, 1979).

Another factor, the thymic humoral factor (THF) is a N-terminal blocked heat-sensitive peptide, composed of 31 residues, and capable of inducing immunocompetence in lymphoid cells from neonatally thymectomized mice and patients suffering from primary or secondary immunodeficiencies possibly by mobilizing natural killer cells (Trainin et al, 1983). Facteur thymique serique (FTS) is a trypsin-sensitive N-terminal blocked, heat labile nonapeptide. Originally isolated from pig serum, FTS has now been detected in thymosin fraction V. Although initially localized in thymic epithelial cells, the thymic origin of FTS has been questioned by several workers, who demonstrate FTS-like immunoreactivity in epithelial cells of most tissues (Kato et al, 1981). Its effect on helper function is ambiguous; it clearly induces suppression of helper cells at high levels and inhibition at low levels. A thymocyte growth factor, distinct from thymosin and TP, and with a molecular weight of

1,000-2,000 has been identified and partially purified. It acts directly on the immunologically immature proliferating thymocytes of the thymic cortex whilst having no effect on the mature medullary thymic population (Soder & Ernstrom, 1983). The differentiating effects of thymic hormones are, however, not dependent on cell division and can be induced within a few hours even with blocked DNA synthesis. Although the fact that thymic hormones induce differentiation of pre-T cells is well-established, it is still not possible to demonstrate convincingly that intra-thymic differentiation depends upon thymic hormone. Furthermore, the fact that the cellular response of neonatally thymectomised mice cannot be reconstituted by humoral factors suggests that other factors are required for maturation. This seems likely as receptors for thymic factors have also not been identified or their target cells. Thymic hormones however appear to affect the activity of mature lymphocytes. For example, enhancement of lymphokine production, specific cytotoxic responses, mixed lymphocyte reactions, and autologous mixed lymphocyte reactions by thymic hormones have been described (Goldstein, 1984; Zatz et al, 1984). Since the adequate expression of IL2 receptors is an important requirement for the normal development of these responses, it is possible that the modulation of these receptors is one of the mechanisms through which thymic hormones contribute to maintaining normal immune response.

Table 1 - Cell sources of IL1 like activities

Cell type	Stimulants
Monocyte and macrophage lines	LPS, PMA, immune complexes, CSF, IFN-γ, A23187, MDP Activated T cells (Ia-restricted)
Dendritic cells	LPS
Langerhans cells	LPS
B lymphoblasts	LPS, anti Ig-M
Endothelial cells	LPS
Epithelial cells	PMA, LPS
Fibroblasts	MDP
Astrocytes	LPS
Microglial cells	LPS
Adult T cell leukemia (ATL) cell lines)	None

LPS = lipopolysaccharide; PMA = phorbol myristate acetate; MDP = muramyl dipeptide; CSF = colony stimulating factor; IFN-γ = interferon

In addition to these several thymic hormones, a complex signalling system trafficking between different members of the white cell series has now been revealed utilizing a number of additional secretory products termed interleukins which may act in both endocrine and paracrine fashion to affect cell division.

2.3.2 Interleukin I (IL1)

Interleukin 1 is a polypeptide manufactured and released predominantly by macrophages (M) although various other cell types also have this capacity (Table 1). Its major action appears to be on certain cells of the lymphoid series provoking some of them to synthesise another lymphokine, interleukin 2 (IL2) and stimulating others to express receptors for this material (Kaye et al, 1984; Mannel et al, 1985).

When macrophages are activated, IL1 is synthesised and released either onto the cell surface or into the extracellular fluid (Van Damme et al, 1985; March et al, 1985; Kurt-Jones et al, 1985; Oppenheim et al, 1986). Interleukin I seems to occur in two forms IL1α and IL1β but it is not clear whether they have identical receptors and biological activities (Dower et al, 1985; Killian et al, 1986). Various factors can elicit release of IL1 from the macrophage including activated T cells, colony stimulating factor, antigen-antibody-complement complexes and bacterial lipopolysaccharide (Hoffman et al, 1979). Whether the IL1 released is important for immune response is difficult to ascertain for most studies have been conducted with in vitro systems frequently divorced from physiological reality (Oppenheim et al, 1982; Kurt-Jones et al, 1985).

Despite a paradoxically low number of IL1 binding sites/cells on PNA^- thymocytes and peripheral T-cells, IL1 exerts a mitogenic effect on thymocytes and peripheral T-cells. Most studies indicate that the proliferative effects of IL1

Table 2 - Effects of IL1 or IL1-like factors on target cells and tissues

Cell/Tissue	Activity
T-lymphocytes	Lymphokine production, eg. IFN, CSF, BCGF, IL2, prolifertion
B-lymphocytes	Antibody-production; division
NK cells	Cytocidal activation
Neutrophils	Metabolic activation, chemotaxis
Macrophages	Prostaglandin formation Cytocidal activation Chemotaxis
Epithelial cells	Proliferation and collagen formation
Brain cells	Prostaglandin release, fever, anorexia
Synovial cells	Production of prostaglandins & collagenase, proliferation
Endothelial cells	Release of procoagulant activity, proliferation
Hepatocytes	Production of acute-phase proteins
Chondrocytes	Collagenase and prostaglandin formation
Bone	Proliferation of osteoblasts and alkaline phosphatase production; resorption of bone by stimulation of osteoblasts
Fibroblasts	Collagenase and prostaglandin formation; division

on thymocytes occur in conjunction with a lectin or antigen (Gery et al, 1972; Oppenheim et al, 1982; Chu et al, 1985). Indeed, a reference assay for IL1 is based on the ability of IL1 to costimulate (with PHA or Con A) the proliferation of the mouse thymocytes which are considerably more reactive to IL1 than are peripheral T-cells. A small proportion of thymic cells, the PNA^- subset presumably located in the medulla may divide directly in response to IL1 and produce IL2. The IL2 released may possibly activate the PNA^+ thymocytes in the presence of a lectin (Nishimura et al, 1984; Conlon et al, 1982,a,b). In fact even during costimulation of PNA^+ thymocytes IL2 may be released by a few contaminating PNA^- cells which may further induce PNA^+ cells to divide (Oppenheim et al, 1982; Gelin et al, 1984). The premise that IL2 may act on the PNA^+ of the cortical population is however disputed by several workers and there is at present no clear picture of the properties of IL2 producing and responding cells in the thymus. (Piantelli et al, 1982; Vatteroni & Papiernik, 1984).

The responsiveness of the thymic cells to IL1 may be attributed to the relative paucity of IL1 production in thymic cell suspensions compared to cells from other lymphoid organs with a higher macrophage content. In fact, when peripheral lymphoid cell suspensions are depleted of Ia-positive accessory cells, the T-cells display considerable reactivity to exogenous IL1 (Durum & Gershon, 1982; Durum et al, 1984). Alternatively, there may be qualitative differences in the capacity of thymic and peripheral T-cells to respond to IL1 (Durum et al, 1985).

The responsiveness of thymocytes to IL1 suggests that the interleukin may be involved in the expansion of T-cells in the thymus which can recognise self-Ia components. T-cells capable of self-recognition may bind to Ia

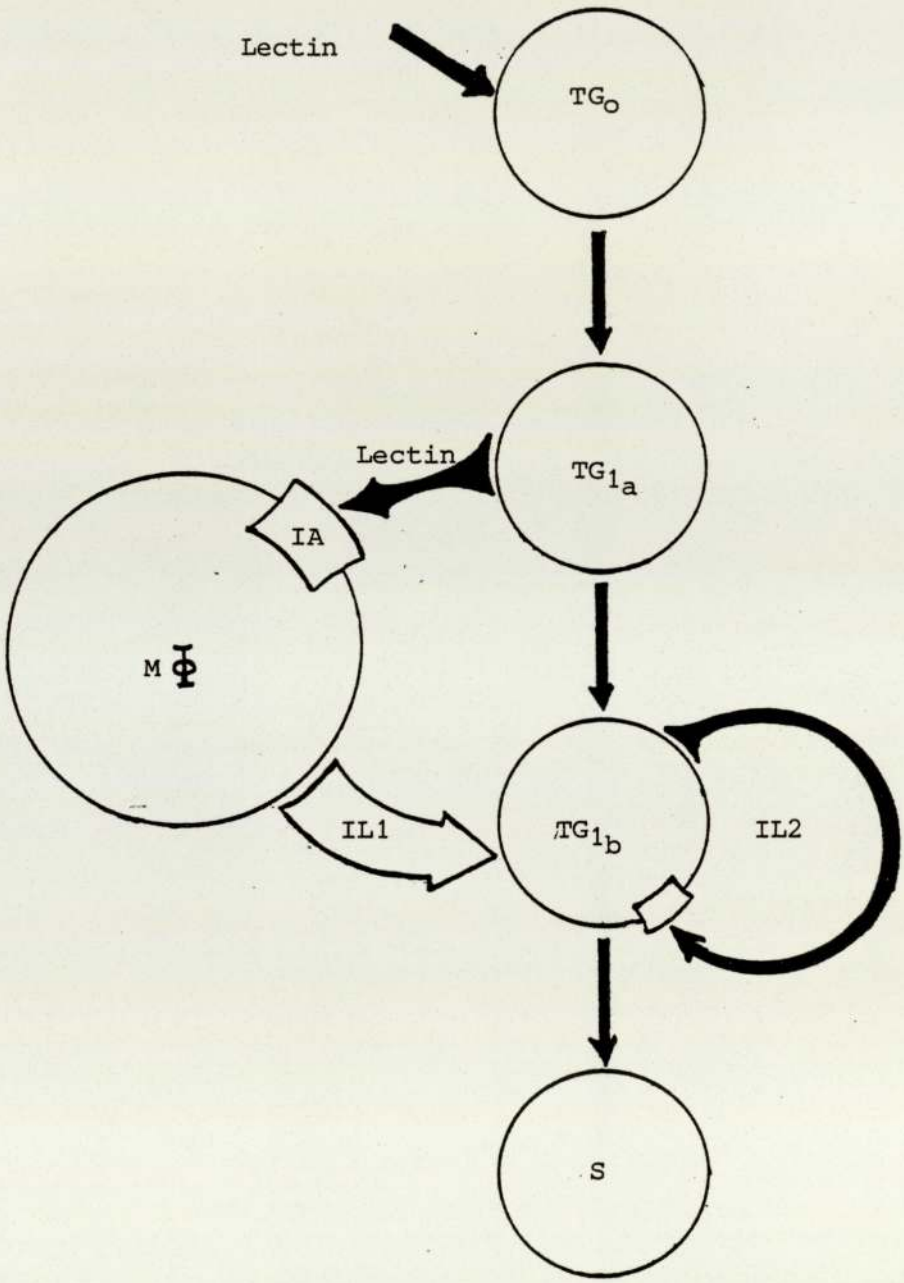


Diagram 7 A schematic diagram illustrating the role of IL1 in T cell mitogenesis.

molecules on macrophages or dendritic cells and thereby induce IL1 release perhaps via a receptor-like mechanism intrinsic to the Ia molecule. Such IL1 production and Ia expression on macrophages and/or dendritic cells could both be involved in self-recognition (Durum & Gershon, 1982). T-cells may subsequently undergo preferential expansion in response to IL1 relative to cells incapable of self-Ia recognition (Beller & Unanue, 1980; Longo & Schwartz, 1980; Gallily et al, 1985).

Cell-cycle studies indicate that IL1 is not required for the progression of lectin stimulated cells from Go into early G1a phase. Following entry into G1a however, T-cells respond to IL1 and proceed through G1b into S phase by an intermediate production of IL2 which induces G1b/S transit (Stadler et al, 1981; Kristensen et al, 1982). IL1 regulates IL2 both at level of production and receptor expression. Such regulation may perhaps be achieved by increasing the density of high affinity IL2 receptors or indirectly by synergising with IL2 (Kaye et al, 1984; Mannel et al, 1985; Oppenheim et al, 1986). The cellular basis of IL1's action on T-cells is shown in Diagram 7.

Various subsets of T-lymphocytes in the secondary lymphoid organs also respond to IL1. Thus, IL1 enhances the tumoricidal activity of cytotoxic lymphocytes, natural killer cells and monocytes. Such antitumour effects may be promoted by IL1 itself or in conjunction with IL2 generated by T-helper cells.

As IL1 clearly activates T-cells, it is logical to assume a role for IL1 during an immune response. IL1 can certainly enhance immune responses directly by stimulating T-helper cells or indirectly through immunoregulatory mechanisms. For example, Elevated temperatures elicited by IL1 during fever may strongly promote the T-helper cell (T_H) activation process although an additional contribution from an increased IL2 production cannot be ignored

(Duff & Durum, 1983). An effect of IL1 on T_H cells may probably be at the expense of T-suppressor (Ts) activities. IL1 can promote the generation of T_H cells and diminish the production of Ts cells. (Durum et al, 1985). Moreover, IL1 may protect T_H cells from Ts cells possibly by blocking the inhibitory effect of Ts cells upon T_H cells.

IL1 may also affect B cells inducing maturation of pre-B cells and a subsequent proliferation of mature B cells following activation by mitogens or antigens (Hoffman, 1980; Giri et al, 1984). Such clonal expansion may also require other B-cell derived factors such as B cell stimulatory factor and B cell growth factor. (Leibson et al, 1981; Noelle et al, 1984; Sahasrabuddhe et al, 1984). IL1 may also indirectly affect B cells by stimulating the production of a cascade of lymphokines from T-cells such as IL2, CSF, leukotrienes (LT), IFN-δ and lymphocyte differentiation and chemotactic factor (LDCF).

The pleiotropic effects of IL1 (Table 2) and also its production during an immune response are subject to negative regulation. Immuno suppressive agents such as cyclosporine (Bunjes et al, 1981), corticosteroids (Smith & Ruscetti, 1981; Snyder & Unanue, 1982) and prostaglandins (Dinarello, 1984) inhibit both IL1 production and some of the lymphocyte-dependent effects. As these agents also interfere with IL2 actions, they may be primarily interfering with the lymphocytic target cells. Suppressor T lymphocytes may also inhibit IL1 production by large granular lymphocytes (Scala et al, 1984). Some B-cell and glioma-cell lines may produce factors that inhibit IL1 and sometimes also IL2 activities (Fontana et al, 1984; Matsushima et al, 1985). Serum in addition contains factors that suppress both IL1 and IL2 mediated activities (Sandberg et al, 1985). Selective inhibitors of IL1 are also present in the urine

of pregnant or febrile individuals (Liao et al, 1984; Muchmore & Decker, 1985). Most recently, normal macrophages have also been reported to produce IL1 inhibitors in conjunction with IL1 on stimulation with immune complexes containing complement or infection by influenza virus (Arend et al, 1985; Roberts et al, 1985). The fact that specific modulators of IL1 are present in bodily fluids suggests that negative feedback mechanisms must regulate IL1 activities very closely and may be responsible for termination of immune responses.

Although IL1 in toto exhibits a complex array of properties as revealed by a host of in vitro techniques it is not entirely clear which of these has real immunological significance. Most authorities are of the opinion that the most significant function of IL1 is the synthesis of IL2, an important lymphokine for modulation of immune responses.

2.3.3 Interleukin 2

Originally termed T-cell growth factor, IL2 represents one element in a cascade of lymphokines released during an immune response. (Farrar et al, 1982). IL2 is released from T cells in response to antigen in the context of proteins of the major histocompatibility complex (MHC) and the macrophage-derived lymphokine, interleukin I (Smith, 1980). Although all subclasses of T cells can release IL2 under appropriate conditions, helper T cells appear to be the major source (Pfizen maier et al, 1984). Once released, IL2 can exert its biological effects by interaction with specific high affinity receptors. This is because the magnitude of the T-cell responses is closely correlated with the occupancy of these receptors rather than that of the low-affinity receptors (Robb et al, 1981; Robb et al, 1984; Cantrell & Smith, 1984).

Most resting T cells do not bear IL2 receptors (IL2-R) but they can be rapidly expressed on these cells following interaction with lectins, monoclonal antibodies to the T cell antigen receptor complex and alloantigen stimulation. Activation of resting human T cells with PHA results in maximal receptor expression at 48 to 72 hr and increased cell division (Depper et al, 1984b). However, with longer periods in culture, IL2 receptors decline and cellular proliferation diminishes (Cantrell & Smith, 1983; Depper et al, 1984b). Thus, the transient nature of IL2 receptor expression may determine the magnitude and duration of the T-cell immune response. The transient IL2 receptor expression is apparently regulated by alterations in the transcriptional activity of the IL2 R gene (Leonard et al, 1985).

Despite the loss of a majority of IL2-R, the cultured non-proliferating cells remain susceptible to signals which stimulate IL2 receptor expression and thus induce a second round of cellular proliferation (Cantrell & Smith, 1983; Depper et al, 1984; Smith & Cantrell, 1985). Three types of activation signals capable of

inducing IL2 receptor expression have been defined:

(a) Mitogenic lectin (Depper et al, 1984) or antigens (Hemler et al, 1984) upregulate IL2 R expression; such upregulation is dependent upon de novo RNA and protein synthesis.

(b) Stimulation of the protein kinase C by either phorbol esters or synthetic congeners of diacylglycerol (Depper et al, 1984).

(c) Addition of IL2 may itself augment IL2 receptor display (Depper et al, 1985; Smith & Cantrell, 1985). Such IL2 amplification of IL2 receptor expression has been described in freshly isolated, resting T lymphocytes (Reem & Yeh, 1984; Welte et al, 1984). IL2 probably induces both high and low affinity receptors but the effects of IL2 are produced at ligand concentrations which interact with the high affinity receptor (Weissman et al, 1986). Although the cascade of events, particularly intracellular events that include the promoting mechanism of the gene expression of IL2 and IL2-R is still fragmentary, both production of IL2 and expression of its receptor are pivotal events in the full expression of the immune response.

The interaction of IL2 with its receptors on the cell-surface is the crucial step for progression of T cells into the cell cycle as it is believed to promote and/or accompany other sequential signals for the G1/S transit (Smith, 1980; Robb et al, 1981) . In fact, IL2 may increase the expression of cell-cycle genes including IL2-R, ß-actin and c-myc. As the c-myc gene is also induced by several other growth factors it may have a role in cell division. Besides T cell proliferation IL2 may also enhance the production of IFN-α which may in turn affect NK cell activity, the generation of cytolytic cells, and the activation and modulation of the expression of histocompatibility antigens in macrophages (Farrar et al, 1982;

Welde & Mertelsman, 1985). IL2 may also directly enhance NK activity and lymphokine activated killer cell (LAK) activity beyond that attributable to induction of IFN-α (Henney et al, 1981). Moreover IL2 may induce the release of helper factors (Kaczmarek et al, 1985) for growth and differentiation of B cells to an antibody-secreting state (Howard et al, 1983; Jung et al, 1984; Miedema & Melief, 1985). In addition to the induction of B cell specific lymphokines, IL2 may also exert its influence directly by interacting with IL2 receptor on various activated B cells. (Ortega et al, 1984; Jung et al, 1984; Waldman et al, 1984; Zubler et al, 1984; Lowenthal et al, 1985; Mittler et al, 1985). IL2 can probably upregulate the number of these receptors and augment immunoglobulin production.

Despite the multitude of apparent properties of IL2 elucidated largely by in vitro experimentation, its physiological role in vivo remains uncertain. A major factor contributing to this uncertainty is the inability to quantify the IL2 liberated during immune reactions. This is because:

(a) It has a short serum half-life (Donohue & Rosenberg, 1983).

(b) Inhibitors to its function exist in normal sera (Hardt et al, 1981; Male et al, 1985).

(c) No reproducible radioimmunoassay or an enzyme linked immunoadsorbent assay (ELISA) is available.

Measurements of IL2 to date have thus largely been functional; such functional assays lack precision as the proliferative response measured does not reflect total IL2 production but rather indicates the net excess amount of IL2 available after its degradation or blockade by IL2 inhibitors. Nevertheless, in vivo studies with adoptively transferred cultured T cells provide compelling evidence that IL2 can function in vivo as a pharmacological modifier of T cell responses and may thus

be of therapeutic importance (Cheever et al, 1984, 1985; Cheever & Greenberg, 1985). Compared to the effects of IL2 on peripheral B and T cells, the role of IL2 in intrathymic differentiation is less clear. The recent identification of IL2 receptor expression on embryonic thymocytes may perhaps assist in the elucidation of the intrathymic differentiation pathways.

IL2 receptors have recently been detected on immature foetal thymocytes in situ despite the absence of antigen stimulation (Habu et al, 1985; Von Boehmer et al, 1985; Raulet, 1985). These IL2 R^+ cells constitute more than 50% of the total cells present in the thymus at 15 days of gestation and subsequently decline progressively to only about 2-3% in the adult thymus. These cells are apparently localised in the subcapsular area of the cortex and also the cortico-medullary junction, where the colonising lymphoid stem cell first appears (Habu et al, 1985; Takacs et al, 1984; Takacs et al, 1985). Not surprisingly, the majorityof IL2 R^+ cells are of the large blastlike Lyt 2^- L3T4$^-$ phenotype (Ceredig et al, 1985; Raulet, 1985). The IL2 receptor molecules are probably expressed on the immature cells immediately after their migration into the thymus from haematopoietic tissues. Indeed through all the embryonic stages, no IL2 R positive cells are found in the extrathymic tissues, including the liver (Habu et al, 1985). Moreover, IL2R^+ cells are not found in the spleen of athymic nude mice suggesting a thymic environment is required for the induction of the IL2-R (Habu et al, 1986). Monoclonal antibodies specific for IL2 binding sites have been employed by several workers (Osawa & Diamanstein, 1984). The expression patterns detected by immunofluorescent staining in the adult and embryonic thymus, ConA blasts and T-cell lines appears to be similar (Van Boehmer et al, 1985; Raulet, 1985). In fact, binding of recombinant human IL2 to embryonic thymocytes is blocked by pre-incubation with the anti-IL2 receptor antibody in agreement with other results on activated T-cells (Habu, 1986). In accordance with the disparate

affinities of the IL2 receptors in activated T-cells (Robb et al, 1984), the $Ly2^-$ $L3T4^-$ thymocytes in the adult and foetal thymus (16 d) express both high and low affinity receptors (Ceredig, 1985).

Although the mechanism for triggering the expression of IL2-R on embryonic thymocytes is unknown, the spontaneous expression of IL2 receptors reflects some transient 'antigen-like' differentiative stimulus (cellular or humoral) intrinsic to the thymus. It is plausible that lymphoid cells after migration to the thymus recognise MHC antigens on the thymic epithelial and/or dendritic cells and consequently on activation with MHC antigens express IL2 receptors. Such self-recognising activated cells appear to be closely associated with the selection of MHC restricted T cell generation in the thymus (Honjo & Habu, 1985). For such a mechanism to occur, thymic lymphocytes must possess the molecules that recognise MHC antigens. Indeed, several workers have indicated the sequential expression of such T cell receptor genes during ontogeny (Raulet et al, 1985; Snodgrass et al, 1985). As the α-chain gene is expressed before 15 days around the time when 50% thymocytes bear IL2-R, the products of the α-gene may perhaps be involved; the function of the gene products is however not clear. Alternatively, the thymic stem cells may express certain molecules which may unselectively recognise thymic elements and be induced to express IL2 receptors. This event may perhaps be required as the first step in the differentiation pathway. No supportive evidence for such speculation has however been obtained. The biological significance of IL2-R expression by foetal thymocytes is not yet clear. It could perhaps transduce the signal provided by IL2 for intrathymic differentiation. The evidence for production of IL2 by these immature cells is highly controversial and as such their nature if functional is not yet known.

In the later stages of thymic ontogeny, however, IL2 production by the T-helper cells may trigger the resultant expansion of the early thymocyte pool bearing the IL2-R and thus cause thymocyte differentiation (Lattimer & Stutman, 1986). Such thymocyte growth may be controlled by a unique repressive mechanism or gene which abrogates IL2-R gene activity (Ramarli et al, 1986).

The action of IL2 in the clonal expansion of lymphocytes during an immune response is also regulated by suppressive mechanisms. Interest has focussed on soluble mediators which function by limiting IL2 production or which neutralise the biological activity of IL2 (Hardt et al, 1981; Fontana et al, 1984; Honda et al, 1985). These factors could severely limit the effective concentration of IL2, expression of IL2-R and lymphocyte proliferation and perhaps cause immuno-suppression during ageing.

2.3.4 Ageing: A decrease in immuno competence with age could be due to several possibilities:

(a) Reduction in the number of responding cells (Miller, 1984)

(b) Reduction in the ability of cells to respond to various stimuli (Miller, 1984; Miller & Harrison, 1985)

(c) Change in the balance between lymphocyte subpopulations, eg. helper/suppressor cells (Smith, 1984).

The function of both T and B cells could thus be altered during ageing. Since the thymic environment is crucial for proper manifestation of both T and B cells (indirectly via helper and suppressor cells), it seems that the changes in thymic form and function could well be implicated for the decline in immune response for it is very well established that the thymic involutes with age (Dougherty, 1952). Differentiation of bone-marrow derived stem cells within the ageing thymus gland is impaired (Hirokawa & Makinodan, 1975; Hirokawa et al, 1986). Whether this

is a direct consequence of changes in thymic hormone production or an indirect effect of other exogenous trophic or suppressive hormones has been a matter for extensive debate.

It is well known that thymic involution commences at puberty so it is not surprising that sex steroids have been implicated (Corpechot et al, 1981). Both oestrogens and androgens in excess can cause thymic regression whereas their removal leads to hypertrophy and restoration of normal functions (Fitzpatrick et al, 1985). The dramatic effects of these steroids suggests that receptors for these hormones exist within the thymic environment. Several authors have argued that the lymphocytes themselves are receptor-negative whereas the reticulo-epithelial cells do bear these receptors (Grossman et al, 1979a,b; Pearce et al, 1983). In this case, altered immune responsiveness must be attributable to hormones or factors derived from these accessory cells. Whatever the locus of the steroid receptors, these hormones certainly can affect the levels of thymic hormones such as thymosin α_1 (Allan et al, 1984). Interestingly, some thymic factors can also affect gonadal structure and function. For example, injection of FTS causes a decrease in ovarian weight possibly by an action upon hypothalamic/pituitary gonadotrophin production (Allan et al, 1984; Grossman et al, 1985). Subtle interactions between gonads, thymus, hypothalamus and pituitary certainly exist with functional consequences for all the tissues concerned.

Pituitary hormones can certainly affect thymic activity. Growth hormone (GH) enhances mitotic activity in the thymus both in vivo and in vitro whereas anti GH antibody administration induced thymic atrophy (Whitfield et al, 1969; Pierpaoli et al, 1969). The posterior pituitary hormones may also have a role to play since oxytocin and neurophysin have been detected in high concentrations within the thymus. It seems that these peptides are produced by cells of neural

crest origin within the thymus itself (Geenen et al, 1986). Several reports testify the possible mitogenic role of GH, oxytocin and vasopressin (Hunt et al, 1977; Hanley, 1985). Both the latter hormones can substitute for IL2 in the production of ɣ-interferon by mature T helper cells (Johnson & Torres, 1985).

In contrast, a material apparently identical to α-thymosin has been detected in nerve cells within the brain suggesting perhaps a neurotransmitter or neuromodulatory role for this peptide affecting hypothalamic and pituitary function (Blalock, 1984; Besedovsky et al, 1985; Hall et al, 1985). Whether such an interaction could affect the production of thyroid -stimulating hormone is unknown but administration of thyroid hormones certainly influences growth and secretory activity of the thymic epithelium (Savino et al, 1984; Fabris & Mocchegiani, 1985).

Many other hormones (with or without hypothalamo/hypophyseal associations) have also been shown to affect the proliferation of lymphoid elements within the thymus. Parathyroid hormone can positively influence intrathymic cell production. Surgical removal of parathyroid glands induces hypocalcemia and subsequently thymic atrophy (Perris, 1971). Moreover, parathyroidectomy prevents the normal mitotic increment in primary lymphoid tissues on antigenic challenge and affects cell-mediated responses (Perris et al, 1984). Insulin may also act as a growth promoting factor. In diabetic rats, the cellularity of the thymus is reduced and the cell-mediated responses are diminished. Hence, the mitotic potential and the functioning of the thymus gland in vivo is clearly subject to modulation by both intrinsic and extrinsic hormones.

2.4 CONTROL OF THYMIC LYMPHOCYTE PROLIFERATION

Although it is evident that the hormonal environment can regulate cell growth in the thymus, most of the studies have been in vivo so direct mitogenic

effects cannot be ascribed with certainty. In the present work, the majority of the studies have been with short-term cultures of thymic lymphocytes themselves suggesting that these cells bear a multitude of different receptors on their surface which upon activation can lead to division. The purpose of this study is to investigate how the ultimate mitotic events are coupled to the initial association of mitogen with its receptor. It will attempt to demonstrate the role of putative mediators and any interplay amongst them during the activation process.

As stated earlier, the lymphoid population of the thymus is divisible into three distinct categories. The proliferatively inactivated small lymphocytes which form the bulk of the population comprise approximately 70% of the total thymic lymphoid pool. These are the end products of intrathymic division and differentiation of appropriate stem cells. These cells in fact die in situ (McPhee et al, 1979); the massive cortical death probably has intracellular biochemical causes and may represent the elimination of self-reactive clones of developing T lymphocytes. Despite the high proportion of non-dividing cells, the entire thymic cell population is renewed every two to four days (Metcalf & Wiadrowski, 1966). This self-renewal is due to the actively cycling large and medium lymphoblasts. These cells constitute 10-15% of all thymocytes and are often referred to as the thymus precursor pool (Scollay & Shortman, 1983). The remaining 10-15% are the rapidly responding lymphocytes, which cannot exogenously initiate DNA synthesis but are enzymatically equipped to do so very shortly after exposure to mitogenic agents. Perhaps these cells are found in the deep cortex, either scattered or concentrated near the cortico-medullary boundary. They may also be located in the medulla and may display a typical blast phenotype (Scollay & Shortman, 1983). These cells are thought to occupy a 'Go' compartment and once activated have a particularly short G1 duration being poised at the G1/S boundary (Whitfield et al, 1979).

During the oestrous cycle, when oestradiol levels decline a marked increase in the plasma calcium concentrations and thymic activity is evident in the female (Smith et al, 1975). Likewise, addition of high concentrations of ß oestradiol ($0.1\mu gml^{-1}$) to cultured thymocytes completely inhibits the mitotic response to elevated extracellular calcium concentrations. In an analogous manner, magnesium-dependent mitosis is compromised by high levels of testosterone in vitro (Morgan & Perris, 1974). In addition to the mitogenic abilities of raised calcium and magnesium concentrations, a whole series of stimulatory hormones and neurotransmitters which can activate quiescent thymic lymphocytes in culture have been identified. In fact, these hormonal agents or mitogens have been categorised into 2 distinct classes depending on their ionic requirements (Table 3). Type 1 mitogenic agents were effective in the 10^{-4} to 10^{-7}M range, were magnesium dependent and could be inhibited by testosterone. In contrast, type 2 mitogenic agents appeared to function in the 10^{-9} to 10^{-13}M range, were calcium dependent and oestradiol blockable (Morgan et al, 1975). It has been suggested that type 1 mitogens after associating with their receptors may stimulate adenylate cyclase. The resultant cAMP surge may cause a magnesium influx or activate a magnesium-dependent event which ultimately triggers DNA synthesis. Testosterone may inhibit this magnesium entry and thus block the action of type 1 mitogens (Perris & Morgan, 1975). The precise interplay between the divalent cation and cyclic nucleotide remains to be clarified. On the other hand, type 2 mitogens were thought to promote cGMP formation after receptor occupancy as a prelude to some calcium-dependent oestradiol-blockable processes. However, this part of the hypothesis has now proved to be untenable since no increase in cGMP has been detected after addition of any of the type 2 mitogens. In fact, Macmanus and co-workers have shown that acetylcholine only increases cGMP at non-mitogenic concentrations (Macmanus et al, 1975). In addition, oestradiol

Table 3 - Mitogenic compounds for thymic lymphocytes

Type 1 mitogens		Type 2 mitogens	
Cycic AMP	10^{-7}M	Cyclic AMP	10^{-12}M
Cyclic GMP	10^{-6}M	Cyclic GMP	10^{-11}M
Glucagon	10^{-4}M	Insulin	10^{-10}M
Adrenaline	5×10^{-6}M	Noradrenaline	10^{-12}M
Isoprenaline	10^{-6}M	Histamine	10^{-13}M
Dopamine	10^{-6}M	Parathyroid hormone	10^{-9}M

All the type 1 mitogens had an obligatory requirement for extracellular magnesium and were inhibited by testosterone (0.1 μgml^{-1}). All type 2 mitogens could be inhibited by oestradiol (0.1 μgml^{-1}) and had an obligatory requirement for extracellular calcium (Peris, Cade and Atkinson, 1983).

does not inhibit calcium influx (Perris et al, 1983).

Although type 1 mitogens can stimulate adenylate cyclase and increase cAMP levels, the role of the guanylate cyclase and cGMP is still obscure. The picture is further confused by the findings that the exogenous application of either of the two cyclic nucleotides exerts a biphasic effect. Thus, high concentrations of cAMP and cGMP (~ 10^{-7}M) exert magnesium dependent effects whereas very low concentrations (~ 10^{-12}M) stimulate mitosis in a calcium-dependent oestradiol blockable manner. Such observations suggest that there is some interplay between the putative mediators of the mitogenic response despite their distinct cationic requirements. The precise interrelationship between the divalent cations and cyclic nucleotides however, remains to be clarified.

The present studies were undertaken to investigate in more detail, the effects of mitogen-receptor binding on ion metabolism and the interaction of the sex-steroids with these events, thus aiming for a better understanding of the rather intricate and puzzling phenomena of stimulus-mitosis coupling in rat thymic lymphocytes. The mitogens that have been employed to study this phenomenon include acetylcholine (ACh), parathyroid hormone (PTH) and the lymphokines, IL1 & IL2.

Acetylcholine

The reports of a neural stimulation of mitosis in the crypts of Lieberkuhn (Tutton, 1973) and of increased DNA-synthesis in bone-marrow stem cells following exposure to cholinergic agents (Byron, 1973) is suggestive of a neural regulation of cell proliferation. Such neural regulation may be extended to other cell types, eg. lymphocytes. The possibility that neurotransmitters may play a physiologic role in lymphocyte proliferation and maintenance of immuno-competence is supported by the extensive innervation of the lymphoid

tissue and the presence of receptors for certain of these transmitters. For instance, ACh receptors on both rat and human lymphocytes, ß adrenergic receptors on B and T cells. An alteration in neurotransmitter secretion may thus influence the lymphoid elements of the immune systems. This seems feasible as ACh has been shown to stimulate mitosis in rat thymic lymphocytes in vitro (Morgan et al, 1975). It has been postulated that ligand receptor interaction may influence the metabolic activity of the cell by regulating intracellular cyclic nucleotide content, thereby affecting the early events of lymphocyte activation and eventually mitosis. The mechanism by which neurotransmitters exert their mitogenic potential however remains to be explored and is the subject of this study.

Parathyroid hormone (PTH)

This peptide hormone initiates a variety of biological activities by binding to specific cell-surface receptors on its major target tissues. Characterisation of binding sites in these systems indicate that they function as hormone receptors coupled to adenylate cyclase, consistent with the well-established response to PTH, ie. production of cAMP. In osteoblasts, a two-receptor model for PTH action has been indicated which utilises both cAMP and Ca^{2+} (Lowik et al, 1985). The intermediary role of Ca^{2+} is not surprising. In fact, the hypercalcemic episodes associated with PTH injections and increase in thymic activity demonstrate clearly that Ca^{2+} may be involved. As PTH is capable of employing both cAMP and Ca^{2+} in its actions, the nature of the second-messenger employed in its mitogenic action in thymocytes has been investigated in this study.

Interleukins 1 & 2

It is evident that interleukins play a crucial role in T-cell and B-cell proliferation. Although receptors for these factors have been characterised on

lymphocytes, the events which couple the initial association of interleukins with their specific receptors remains to be determined. There are conflicting reports as to whether IL2 acts by way of a calcium flux. Weiss et al (1984) have suggested that IL2 can stimulate T-cell proliferation in medium depleted of Ca^{2+} ions by chelation with EGTA, whereas Birx et al (1984) have shown that calcium-channel blockers block Ca^{2+} influx into cells and inhibit the proliferative response to IL2. Other authors have reported that a rise in $[Ca^{2+}]_i$ is imperative for IL2 production but not for IL2 action (Mills et al, 1985). Taking into account such controversies and the inhibitory action of IL2 on adenylate cyclase (Beckner & Farrar, 1985), the involvement of Ca^{2+} ions in the action of IL2 on thymic lymphocytes has been investigated.

CHAPTER THREE

METHODS AND MATERIALS

3.1 Experimental animals

Male albino rats of the Wistar strain (Bantin & Kingman Ltd) or Lou strain and about 6-10 weeks old (approximately 150g in weight) have been used throughout unless otherwise specified. Such age restriction limitations were imposed to avoid fluctuations in basal mitogenic activity due to age-related thymic involution. The animals were maintained under constant laboratory conditions; a standard Heygate's rat and mouse breeding diet (Heygate and Sons Ltd) and tap water ad libitum.

The animals were routinely sacrificed between 08.30 and 09.30 hours, thereby minimising changes due to inherent circadian rhythms in the rat thymocyte mitotic activity (Hunt & Perris, 1974). In spite of such precautionary measures a slight seasonal variation in basal mitotic activity was evident.

The animals were anaesthetised with diethylether; bartiburates were not employed as they have an antimitotic action (Baserga & Weiss, 1967). Additionally, ether is rapidly inducted and eliminated from the system and has no short-term effects on total red and white blood cell levels or plasma corticosteroid concentrations (Besch & Chou, 1971).

3.2 MITOTIC ACTIVITY IN THE THYMOCYTE

Measurements of mitotic activity in the thymocyte have mainly been conducted in short-term suspension cultures in medium 199 (Wellcome Diagnostics Ltd). Occasionally in vivo studies have also been undertaken.

3.2.1 Cultured Thymocytes

A distinct feature of the short-term suspension cultures is that the mitotic behaviour of thymic lymphocytes resembles closely that observed in an intact animal (Perris, 1971). As parallel activity is observed in cells in response to a series of mitogens both in vivo and in vitro, an understanding of the in vitro mitotic events provides an unique insight into the proliferative activity of the native tissue (Morgan & Perris, 1974). The studies are thus physiologically significant and have an advantage over many tissue culture adapted cell lines used in mitotic studies (Chlapowski et al, 1975).

The technique of short-term suspension cultures employed is ideally suited to the study of in vitro mitotic activity of thymic lymphocytes as several potential problems have been overcome:

1 As the available cell population is responsive and naturally quiescent, the necessity of inducing synchrony or quiescence by employing artificial procedures is eliminated.

2 The short-term incubations abrogates the need for serum-supplementation thereby preventing any interference from complex interactions between serum factors and added mitogens. In addition, the simple medium permits experimental manipulation of the ionic or hormonal composition of the extracellular environment or provides uniform conditions for cell growth.

3 As thymic lymphocytes are non-adherent, maximum cell surface area is exposed to added mitogens.

4 Incubation for a short period excludes the possibility of density-dependent growth inhibition and nutrient depletion. Constant agitation (culture tubes containing cells are rotated on a roller for 6 hours) prevents accumulation of toxic metabolites.

5 The preparation of cell suspension is rapid and simple; this prevents the metabolic stress encountered during the preparation of cell cultures from established cell lines.

3.2.1a Preparation of Thymocyte Cultures

Cultures of thymic lymphocytes were prepared using a modification of the technique developed by Whitfield and co-workers (1974). Thymus glands were rapidly removed from lightly anaesthetised animals. The glands were subjected to a rapid rinse in two changes of culture medium to remove erythrocytes and any other debris. The entire organ was minced thoroughly in medium to release the lymphocytes from the reticulum. The mince was subsequently filtered through moistened four-ply cheesecloth to eliminate residual cell aggregates and reticular fragments and the cell concentration adjusted to approximately 5×10^7 cells ml^{-1} by diluting with medium. The cell suspension was finally dispersed among the sterile plastic culture tubes (Sterilin Ltd) in 1 ml aliquots.

Medium 199 (Wellcome Ltd), a complete pre-prepared culture medium manufactured free of calcium and magnesium ions was employed throughout for culturing thymocytes. As the mitotic activity along with other cellular functions is pH sensitive, an optimal pH of approximately 7.2 was maintained in the plastic-stoppered culture tubes. Such adequate buffering was provided by the addition of sodium bicarbonate (3 mM) and Hepes Buffer (20 mM)

(Flow laboratories Ltd). Where appropriate, colchicine (Ciba Ltd), a metaphase arresting agent was added at a final concentration of 0.6×10^{-4} M; a concentration sufficient for spindle disruption without any effect on mitotic activity (Whitfield et al, 1974). Moreover colchicine at this concentration does not influence glucose, leucine and calcium transport across the plasma membrane. Where required calcium and/or magnesium ions were added as ten microlitre aliquots from stock solutions of their respective chloride and sulphate salts to give a basal concentration of about 0.6 mM Ca^{2+} and 1.0 mM Mg^{2+}; these ionic concentrations correspond to the ambient levels of the free ions in normal rat blood (Perris, 1971). EGTA buffers were not employed for adjustment of Ca^{2+} concentration as the chelator may perturb plasma membrane function and also chelate other vital ions. Putative mitogenic or antimitogenic agents were added in accordance with their requirements in ten microlite aliquots from their respective stock solutions to the cell suspension. The stock solutions were prepared in 0.9% saline or 0.9% saline-ethanol/dimethylsulphoxide. Cell viability was only slightly reduced (approximately 2-4%) over a six hour incubation period as assessed by the trypan blue exclusion method. Since the decrease is due to a reduction in the mitotically incompetent, small lymphocyte population, the technique is regarded as satisfactory.

3.2.1b Estimation of Mitotic Activity

After the incubation period, three drops of each culture were removed and placed on microscope slides with one drop of fetal calf serum, a binding agent. A smear was prepared and dried in a warm air-stream. The slides were subsequently fixed in neutral phosphate-buffered formalin (pH 7.4) and stained with Delafield's haematoxylin (Whitfield et al, 1964). Slides were prepared

permanently for subsequent scoring by mounting in DPX.

As colchicine arrests cells progressing through mitosis, the thymocytes are arrested in a quasi-metaphase configuration. Comparison of the effects of various potential mitogens on mitotic activity can thus be made by scoring percentage of nucleated cells in colchicine-metaphase. Two thousand cells per culture/slide were examined under oil immersion at 1250X magnification by two independent observers to reduce subjective error. Where differences occurred between the two observers, the slides were recounted until agreement within 0.5% difference was obtained.

When divalent cationic concentrations were adjusted to reflect ambient levels of rat blood (0.6 mM Ca^{2+}; 1.0 mM Mg^{2+}) approximately 3.5% of the cells acquired a C-metaphase configuration after 6 hours. Increasing the calcium concentration to 1.8mM increased the number of cells reaching metaphase over the same time period to approximately 6% confirming previous observations (Morgan et al, 1977). A 'basal' and a 'high calcium' culture were thus routinely included as internal controls in each of the experiments done in this study.

In addition to short term suspension cultures, proliferative activity of thymocytes and bone marrow cells was also assessed by in vivo administration of supposed mitogens. Animals were injected intraperitoneally with different doses of the mitogen in 0.5 ml of pyrogen-free normal saline. Control animals were injected with saline only. As the mitotic activity was again determined by employing colchicine, the rats were injected intraperitoneally with colchicine at the same time as the mitogen and again 3 hours later. Colchicine was administered as a .2 mg/ml saline solution; the dose at each injection being 0.2 mg/100 g of rat. Six hours after the initial colchicine injection, thymus were removed and slides prepared as described previously for cultured thymocytes

and subsequently scored for C-metaphase figures. Bone marrow was removed from the femurs of these colchicine-injected rats and the cell suspension prepared by passage through a hypodermic needle. Subsequently, the cell suspensions were smeared and stained in a similar manner to the thymus and ultimately scored for cells with metaphase configurations.

Despite being subjective and relatively imprecise in measuring small changes in mitotic activity, this technique has proved to be superior to other techniques:

(a) Measurements by progressive increase in cell numbers. Since only a relatively small proportion of the thymocyte population is mitotically active, the assessment of mitotic activity over a six hour period is precluded.

(b) Scoring of mitotic index by measuring percentage of cells in mitosis in the total population. Accordingly, if a mitogen recruits new cells into the cell-cycle then the proportion of cells in different phases of the cell cycle would increase correspondingly, thus elevating the mitotic index. An increased mitotic index may however be due to a prolonged mitosis, a shortening of the cycling time without a decrease in the duration of the mitotic phase or a reduction of the population size by destruction of the mitotically incompetent cells. Additionally as thymocytes are recruited in a semi-synchronous manner, the transient appearance of cells in mitosis may be missed resulting in erroneous values.

(c) Autoradiographic analysis of the incorporation of radiolabelled precursor ^{3}H- thymidine for measuring the proportion of cells synthesising DNA has been used by various investigators. This method is based on the assumption

that DNA synthesis is directly correlated with mitotic activity. There are however disadvantages as DNA synthesis by non-mitotic cells, tracer reutilization and alterations in the length of cell-cycle may drastically alter the number of labelled cells. These problems have been overcome by scoring the percentage of labelled cells in recognisable mitotic configuration; this, however makes the technique subjective and tedious.

Although the radiometric quantification of labelled precursor incorporation into newly synthesized DNA has proved to be successful for measurement of proliferation in different cell types, it has proved to be inappropriate for thymic lymphocytes. In fact, ^{3}H-thymidine incorporation appears to decrease after mitogenic stimulation presumably due to the endogenous thymidylate synthetase and thymidine kinase. These enzymes produce de novo thymidine in the rat thymocyte. The endogenous-thymidine may thus dilute the isotopic pool and specific activity within the cell. Thus besides being convenient and simple, the colchicine-metaphase technique thus appears to be the most suitable method for use in this study.

Mitogenic stimulation provokes various transcellular ion fluxes which are thought to cause changes in cytosolic calcium levels. Most of the evidence for the regulatory role of Ca^{2+} in the proliferative response is based indirectly on experiments involving manipulation of extracellular Ca^{2+}, measurements of calcium fluxes and studies of Ca^{2+} on isolated enzymes and systems. As direct evidence is lacking, techniques are required for monitoring free Ca^{2+} levels in intact cells. The measurement of cytoplasmic free Ca^{2+} may be of an advantage for the following reasons:

(a) To provide definitive evidence for intracellular Ca^{2+} as the mediator of the cell's response to external stimuli and distinguish this role from other passive roles of Ca^{2+} within the cell.

(b) To define the range of free Ca^{2+} pertinent for Ca^{2+} binding protein ultimately responsible for cell activation.

(c) To enable the identification of the source of Ca^{2+} for stimulus-mitosis coupling; intracellular or extracellular.

(d) To enable $^{45}Ca^{2+}$ fluxes to be interpreted accurately.

3.3 MEASUREMENT OF INTRACELLULAR Ca^{2+} LEVELS

There are certain criteria which define an indicator capable of measuring cytosolic Ca^{2+} levels. An ideal indicator should be sensitive within a range of 10 mM - 10μm and capable of generating a large but measurable signal for only a small fraction of calcium bound. It should be highly selective for Ca^{2+} in relation to other cations and should not be perturbed by alterations in cytosolic pH. The response of the indicator should be linear so that quantitation of free calcium concentrations is simple. Ideally, the response of the indicator should be faster than the changes in calcium concentration being measured. In addition, the indicator should be incorporated into the cytosol in sufficient amounts to be able to detect the free Ca^{2+} levels without disrupting the cell and the Ca^{2+} equilibrium being measured. Once incorporated, the indicator must not leak out nor be sequestered by intracellular organelles. The indicator should clearly not be toxic by binding to other molecules and interfering with important cell functions.

On the basis of the above criteria, the indicators which are currently used to measure cytosolic free Ca^{2+} may be categorised into distinct groups:

(a) Chemiluminescent Indicators

The Ca^{2+}-activated photoproteins such as aequorin and obelin contain a covalently bound chromophore which emits light when Ca^{2+} binds to the protein. Despite their wide applicability in monitoring free Ca^{2+} in large cells, these indicators have proved inappropriate for small cells, eg. thymocytes because of the difficulty of incorporation without impairment of cell function. In fact, most of the techniques adopted for their incorporation are associated with a decrease in cellular ATP and a loss of cytoplasmic contents (Snowdowne & Borle, 1984; Campbell et al, 1985).

(b) Metallochromic Indicators

These indicators namely Arsenazo III and Antipyrylazo III change colour upon binding Ca^{2+} and are required in considerable amounts for detection of cytosolic free Ca^{2+}. Moreover, they are highly susceptible to variations in pH and ionic content and hence unsuitable (Dormer et al, 1985).

(c) Ion-selective Microelectrodes

This technique gives a continuous readout with a time response capable of measuring changes in Ca^{2+}. This technique is however limited to large cells. Moreover, the potentiometric signal represents an extremely local measurement at the tip of the electrode (~ 1% of a cell) and can easily miss out fast Ca^{2+} transients which could be functionally very important (Tsien & Rink, 1980; Ammann, 1985).

(d) Nuclear Magnetic Resonance (NMR) Indicators

These indicators, 4-fluoro BAPTA and 5 fluoro BAPTA show a shift in resonance on binding to Ca^{2+} but as the spectrum accumulates over a certain time duration, they are not suitable for measuring Ca^{2+} transients (Metcalfe et al, 1985).

(e) Fluorescent Probes

Because of the disadvantages of the aforementioned techniques, a fluorescent, highly selective Ca^{2+} indicator, 'Quin 2' has been used extensively for measurement of cytosolic free Ca^{2+} concentration. Upon binding Ca^{2+}, fluorescence is enhanced at an emission wavelength of 492 nm and an excitation wavelength of 339 nm. The monitoring of free Ca^{2+} levels by Quin 2 particularly in small mammalian cells is partly due to the relative ease of incorporation and partly because of its straightforward calibration. Since Quin 2 appears suitable for measuring transient changes in Ca^{2+} in murine thymocytes as reported by other workers, it was selected for use in this study with rat thymocytes.

Measurement of intracellular Ca^{++} by the fluorescent indicator, Quin 2

Quin 2, whose structural formula is shown in Diagram 8, is one of a new series of fluorescent calcium indicators purpose-built by R.Y. Tsein for measuring and manipulating cytosolic free Ca^{2+}. Quin 2 demonstrates a high affinity, 1:1 stochiometry for Ca^{2+}, low affinity for Mg^{2+} and H^{+}, large absorbance and fluorescence changes due to calcium binding and insignificant binding to membranes. The tetracarboxylate anions are hydrophilic in nature and hence, membrane impermeant. Such substances can however be trapped in intact cells by incubating cells with the Quin 2 acetoxymethyl ester. This ester

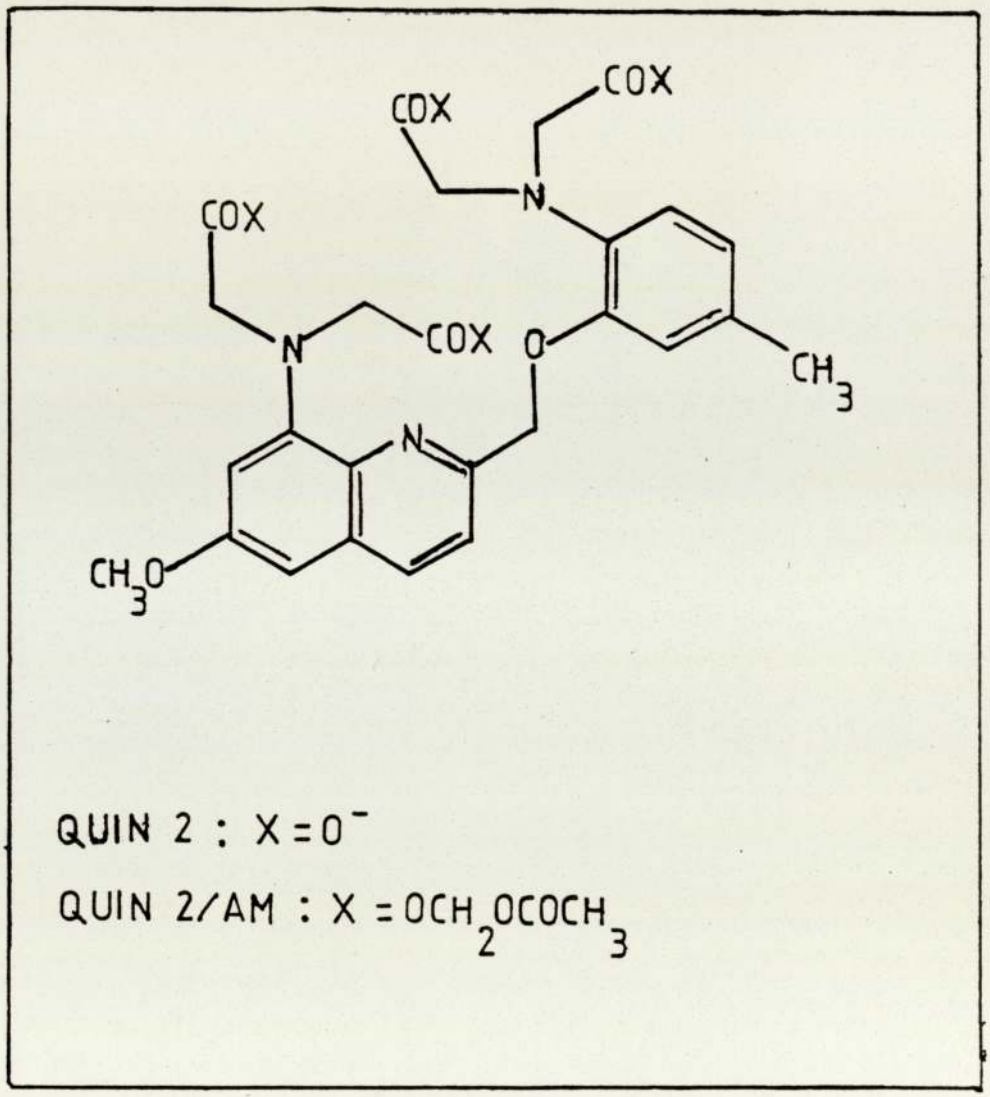


Diagram 8 Chemical structure of the Ca^{2+} indicator Quin 2 and its acetoxymethyl ester Quin 2/AM.

readily permeates the membrane and is hydrolysed intracellularly by the cytoplasmic esterase thus effectively trapping the impermeant parent tetranion. Accordingly, Quin 2 tetracetoxylmethylester (Quin 2/AM) supplied as a freeze-dried oil (Amersham International plc) was dissolved in dry dimethylsuphoxide (DMSO) and desiccated at -20°C between use (Tsien et al, 1982).

Quin 2 which has an effective dissociation constant of 115 nM in a cationic background mimicking cytoplasm shows a six-fold fluorescence enhancement on full saturation with Ca^{2+}, at an emission wavelength peak of 492 nm using excitation at 339 nm. Tsein & co-workers (1982) indicate that intracellular Quin 2 is freely distributed in the cytoplasm and nucleus and is not sequestered inside subcellular organelles. Moreover, the loading of Quin 2 does not perturb steady state [Ca^{2+}] and is without any serious toxic effects although high levels seem to lower cellular ATP. The fluorescence signal was influenced only slightly with variation in pH. Thus, this seems to be a simple and effective method for measuring changes in cytoplasmic free Ca^{2+} levels.

Loading of Quin 2

Thymic lymphocytes were isolated as described previously and their final concentration adjusted to 2×10^8 cells ml^{-1}. Loading is much more effective at 37°C than at room temperature; a lower temperature than 37°C can lead to intracellular accumulation of hydrolysed Quin 2/AM which cannot be rescued by subsequent exposure of the cells to 37°C. The cell suspension was loaded with Quin 2/AM in Hepes and sodium bicarbonate buffered medium 199 at a final concentration of 0.2 mM and incubated in stoppered plastic tubes in a roller drum for 20 minutes at 37°C. The suspension was further diluted tenfold with basal medium and incubated again for 60-90 minutes (the final

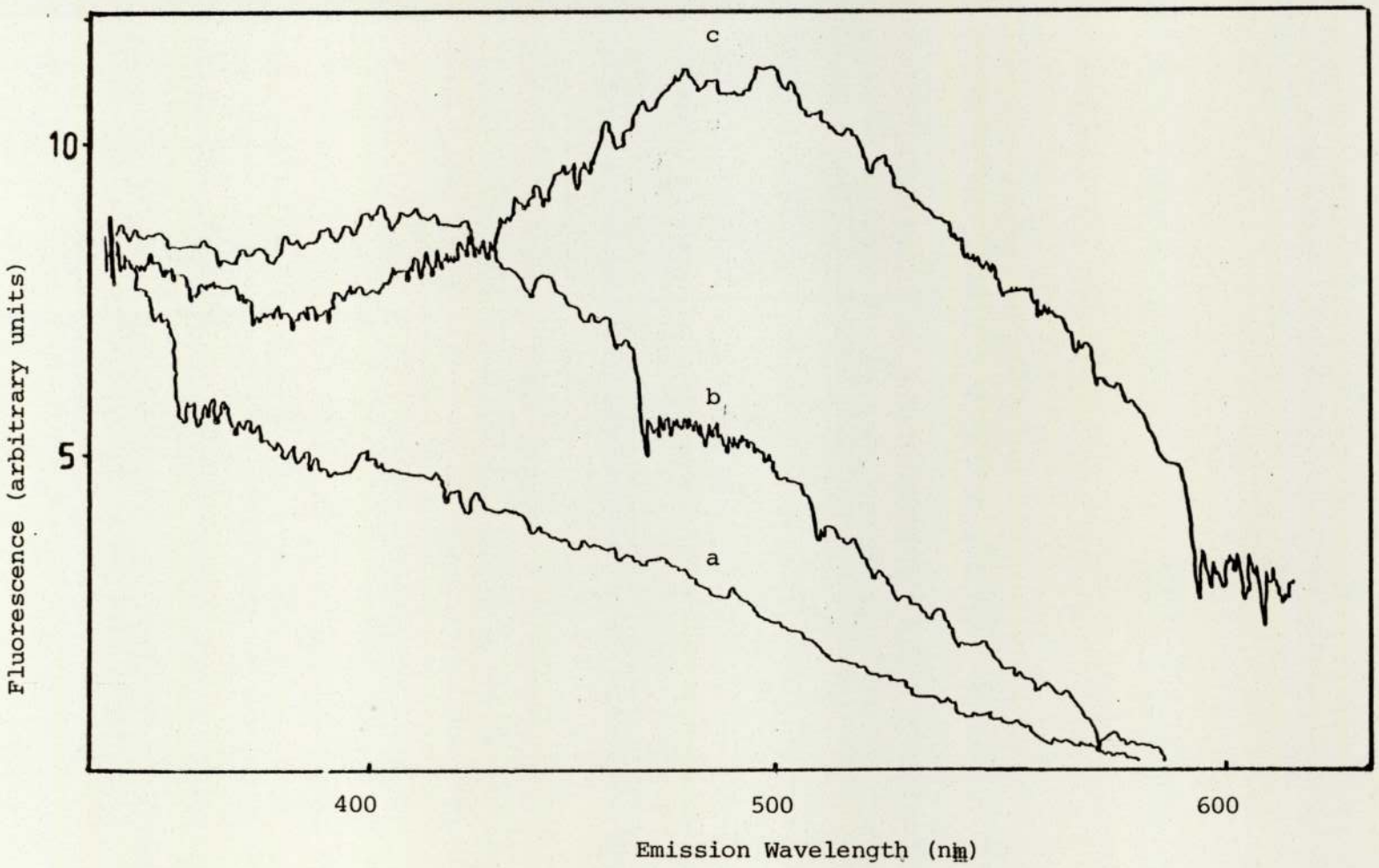


Diagram 9 Incorporation of Quin 2/A M and conversion to Quin 2

The diagram shows the uncorrected emission spectra from cell suspensions incubated with Quin 2 A/M, immediately after ester addition (b) and after 60 min incubation (c). The signal from unloaded cells is also shown (a). Excitation was at 339nM throughout.

concentration of DMSO from the Quin 2 stock did not exceed 0.02% vol/vol). After loading, cells were centrifuged (at 1,000g for 3 minutes) and resuspended in fresh media at approximately 2 x 10^7 cells ml^{-1}. The cell suspension was rotated in roller-drum assembly at 37°C until further measurements were to be made.

For measurement of fluorescence, one millilitre of stock cell suspension was centrifuged for 30 seconds in a microfuge. The cells were further resuspended in two millilitres of physiological saline (final concentration 10^7 cells ml^{-1}) and transferred to a square-quartz cuvette. The final step minimises any Quin 2 carryover. Simplified saline is preferred to Medium 199 as this medium may contain substances (such as phenol red) which may filter excitation and emission wavelengths and may also contribute their own fluorescence. The composition of physiological saline in mM was: NaCl, 145; KCl, 5; Na_2HPO_4, 1; Glucose, 5; Hepes buffer, 10; pH ~ 7.4. Mg^{2+} and Ca^{2+} were added from stock solutions of their respective sulphate and chloride as required.

Before proceeding with the fluorescence measurements, it is important to check whether effective hydrolysis has occurred within the cell resulting in the Quin 2 loading of cells. Thus, loading check was done in cells suspended in physiological saline. Quin 2/Am uptake and conversion to the free acid were followed fluorometically by exciting at 339 nm and observing a shift in the emission spectrum from a peak at 430 nm for the ester to a peak at 492 nm for the acid (Tsein et al, 1982). The peak shift is shown in Diagram 9. The rapid disappearance of the 430 nm peak was accompanied by the appearance of the 492 nm peak characteristics of free acid, although initially the ester emission spectrum overlaps and augments that of the acid. As this loading check was done routinely, it was however soon apparent that the rate-limiting step was

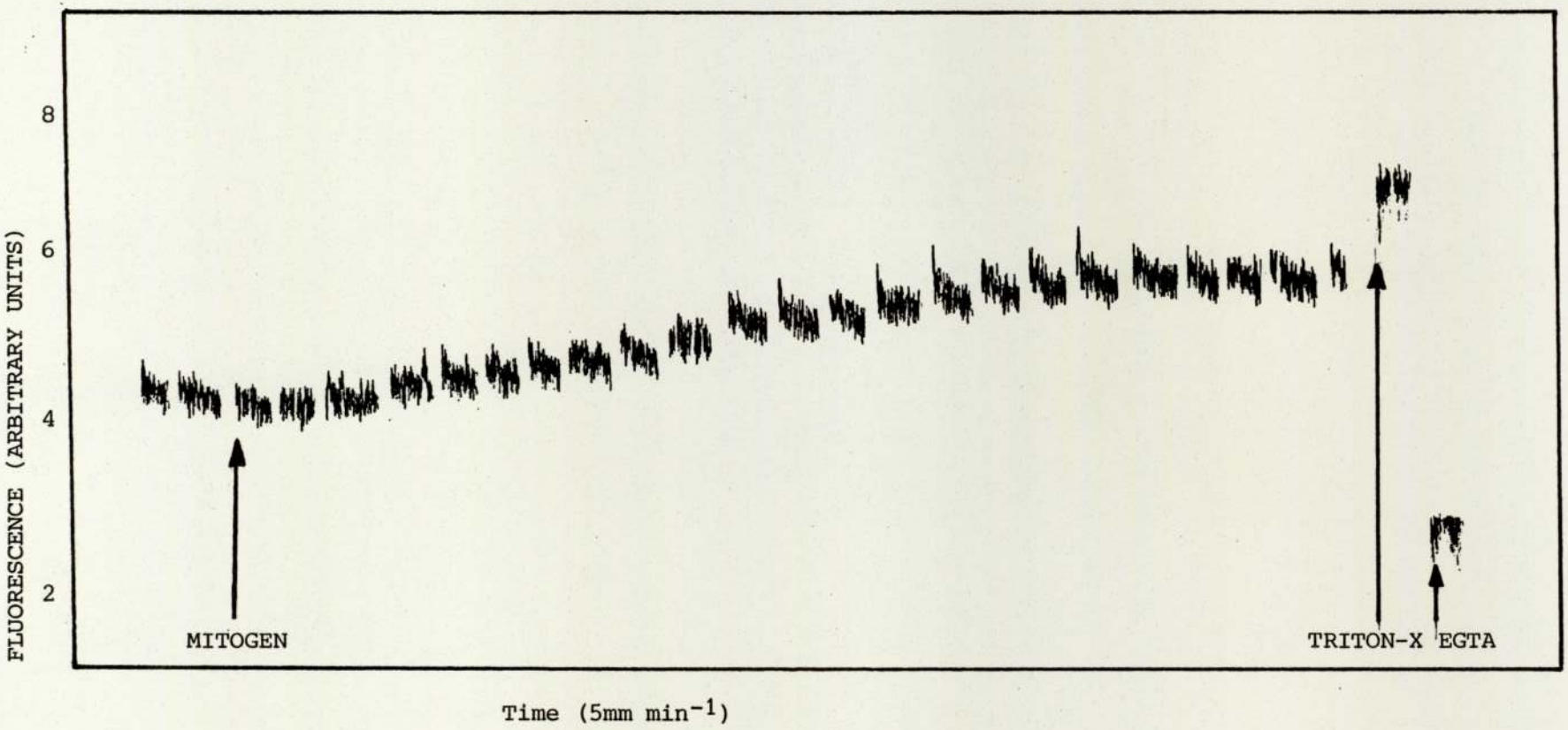


Diagram 10 Measurement of cytosolic calcium levels in Quin-2 loaded rat thymocytes.

hydrolysis as a shift was not always observed in cells loaded by the foregoing procedure. In certain instances, a routine check of the loaded cells and supernatant for the 492 peak revealed that extensive extracellular hydrolysis may have occurred possibly due to cytoplasmic esterases released by dead or dying cells. In an attempt to eradicate this problem, cells were extensively washed to remove any residual dead cells which may cause interference with the Quin 2 loading.

For fluorescence measurments, an Aminco-Bowman spectrofluorimeter was used; the monochromator settings were 339 nm excitation and 492 nm emission. 339 nm was chosen as excitation because Quin 2 and its calcium and magnesium complexes adsorb equally there. Moreover, longer wavelengths decrease sensitivity to Ca^{2+} while shorter wavelengths cause cell autofluorescence. The fluorescence signal was recorded on a flat bed recorder (JJ 'XX' plotter). As the cuvette was not thermostatted at 37°C, the suspensions were allowed to equilibriate for 5-10 minutes to attain a constant temperature of 30°C before recording basal fluorescence. The temperature was maintained throughout the determinations. The putative mitogenic agents were subsequently added as twenty microlitre aliquots and any change in fluorescence monitored.

Typically, traces obtained by following the technique of Tsien and co-workers are presented as in the Diagram 10. For measurment of $[Ca^{2+}]_i$, these recordings are usually calibrated according to the equation:

$$[Ca^{2+}] = Kd \frac{(F - Fmin)}{(Fmax - F)}$$

where the Kd or effective dissociation constant is 115 nM. F represents the fluorescence intensity of resting cells in basal medium. Fmax denotes the maximum

signal in the presence of excess Ca^{2+} (~ 1 mM) and Fmin the minimum signal in the presence of very low Ca^{2+} (< 1 nM) (Tsein et al, 1982). Following this procedure, however, the fmin value could not be obtained as the Triton-X signal could not be quenched despite addition of large amounts of EGTA. Thus, to obtain fmax the cells were lysed by a probe sonicator (Hallam et al, 1984) and for Fmin, EGTA was added at a final concentration of 1 nM. Although initially sonication appeared to be appropriate, repetitive experimentation revealed that the sonication seemed to give very inconsistent values for fmax and autofluorescence and hence, tremendous variation in resting $[Ca^{2+}]_i$ levels.

The mean value for basal calcium levels at n = 12 was 207.9± 31 with a range from 35 nM to 437 nM. Occasionally, the resting $[Ca^{2+}]_i$ levels even approached values > 1 μm. These values were clearly unsatisfactory and hence sonication did not seem appropriate for further calibration.

Subsequent systematic approaches to the calibration method revealed that Triton-X was highly autofluorescent and thus may be masking any Quin 2-Ca^{2+} signals obtained by EGTA addition. The concentration of Triton X-100 for Fmax determination was therefore reduced to 40 μl of a 10% solution. The signal obtained at this concentration could be quenched with 50 μl of 0.5 M EGTA in the presence of 25 μl of 10% NaOH for a pH value < 8.3.

Having established an effective calibration method, the results still appeared to be rather worrying; in particular the resting $[Ca^{2+}]_i$ concentrations seemed to be highly variable. The longer the loaded cells were maintained at 37°C before being transferred to the cuvette, the greater was the cytosolic $[Ca^{2+}]_i$ concentration. For instance, mean initial resting levels for $[Ca^{2+}]_i$ for n = 4 was 144.5 ± 40 as compared to the $[Ca]^{2+}_i$ of 214.5 ± 20 after a time period of approximately 3 ahours. The magnitude of rise in

Table 4 - Effect of different concentrations of Quin 2 on viability of rat thymocytes

Treatment (μM)	Viability (%) Time (hrs) ——> 1	2	3	4	5
Basal	97	97	-	-	96
Quin 2 200	94	93	93	87	74
Quin 2 100	94	93	-	-	88
Quin 2 50	94	-	-	-	92

Table 5 - Determination of the acid peak for Quin 2-loaded thymocytes

Incubation time after initial dilution at t = 10 (mins)	Fluorescence intensity 430 nM	492 nM
10	27.5	37.5
25	31.5	57.0
30	33.0	76.0
35	36.0	85.0**
45	34.0	81.0
60	34.0	78.0
85	33.5	73.0
95	29.0	61.5
135	29.0	60.0
150	28.0	52.0

** Represents the minimum time for wich the cells should be incubated for efficient hydrolysis subsequent to the initial incubation period of ten minutes.

$[Ca^{2+}]_i$ in response to a mitogen was also variable and sometimes even non-existent. When the cell viability was monitored using the Trypan bue exclusion method, a large proportion of cells were found to be non-viable thus accounting for the discrepant results (Table 4). In certain instances, only about 50% of the total cell population was viable.

Further experiments were therefore performed to establish optimal experimental conditions. Two possibilities were considered for an optimal fluorescence signal.

(a) A lower loading concentration of Quin 2 which may reduce toxic effects and/or

(b) A decrease in the initial incubation period (before dilution) and the total time (before and after dilution) prior to resuspension in fresh medium.

Since the detrimental effects of a high loading concentration of Quin 2 could be ignored by adopting the latter solution the technique was modified by considering both the possibilities simultaneously. After loading cells with different concentrations of Quin 2 (50, 75, 100, 150, 200 μm), aliquots of cells were analysed for effective hydrolysis. Employing this step, it was evident that the ester permeated the cell or conceivably was bound to the plasma membrane. In addition, Quin 2 peaks at 492 nm following the hydrolysis of Quin 2/AM were recorded for each of the loading concentrations and initial incubation times (0, 5, 10, 15, 20 mins) over a total incubation period of approximately 80 minutes. Provided the sensitivity of the meter multiplier is kept constant, the height of the peaks for the different concentrations may determine the shortest initial incubation period and the lowest possible loading of Quin 2/AM required for a satisfactory signal. Thus, an initial incubation of 10 minutes and a subsequent incubation of 35 to 45 minutes appeared to be appropriate for a Quin 2 concentration of 100 μm (Table 5).

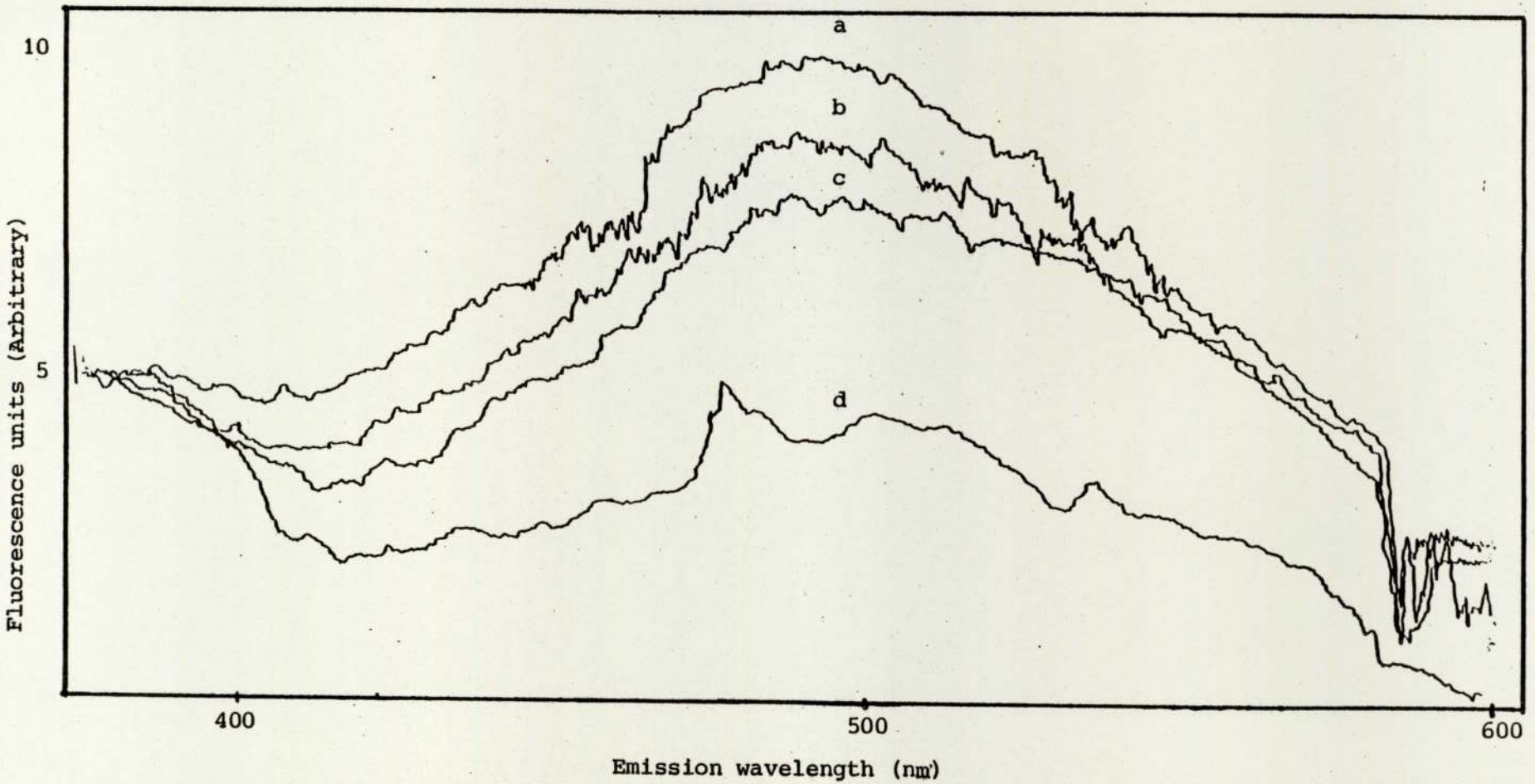


Diagram 11 The recorded illustration of fluorescence intensity of the quin 2 acid in thymocytes loaded with different concentrations of Quin 2/A M

a : 200 µm, b : 150 µm, c : 100 µm and d : 50 µm Quin 2/A M respectively. The excitation wavelength was maintained at 339 nM.

Following such exhaustive experimentation for adequate signalling it was evident that the optimal Quin 2 concentration was 100 μm as compared to the previously determined concentration of 200 μm (Cade, 1983). Although 100 μm Quin 2 demonstrated a smaller acid peak in comparison to the equivalent peaks of 150 and 200 μm in thymocytes the peak was nevertheless substantial indicative of sufficient amounts of intracellular Quin 2 (Diagram 11). This concentration was not cytotoxic. In fact, the viability was comparable to controls even after 5 hours (Table 4).

Besides the careful analysis of the hydrolysis as discussed above, the response to A23187 was also recorded as A23187 has been shown to raise the cytosolic Ca content by promotion of Ca^{2+} specific ionophoresis. An increase in fluorescence shortly after application of A23187 may demonstrate the effectiveness of the procedure (Rogers et al, 1983). The Quin 2 concentration of 100 μm provided a good response to A23187; albeit the 200 μm showed a still higher fluorescence signal possibly due to significant Ca^{2+} buffering (Table 6).

Table 6 - The effect of ionophore, A23187 on the intracellular Ca^{2+} concentration of rat thymic lymphocytes loaded with different concentrations of Quin 2

Quin 2 concentration (μm)	$[Ca^{2+}]_i$ in resting thymocytes (nM)	Treatment in stimulated	$[Ca^{2+}]_i$ thymocytes (nM)
100	132	A23187 (5×10^{-7}M)	278
200	131	A23187 (5×10^{-7}M)	670

Despite modifications in the technique, the results were variable because of disparate Fmax and Fmin values. The cells were subsequently checked for leakage of Quin 2 by determining the acid peak with duration of time (Table 5). On considering the data in the table, it was evident that Quin 2 was leaking out of the cells. The determination of the $[Ca^{2+}]_i$ in response to mitogens was therefore restricted only to a limited time period of approximately 30 minutes so that the leaked extracellular Quin 2 does not interfere with the effective fluorescence causing an artefact. As leakiness of Quin 2 has previously been assessed by addition of manganous (II) chloride to cell suspensions (Hesketh et al, 1982; Rink & Pozzan, 1985), it was employed to ascertain the effective leakage from the Quin 2 loaded cells. A considerable drop in fluorescence was obtained, on occasions even amounting to ~14% but enough to indicate that Quin 2 was definitely leaking. Nevertheless, one cannot ignore the possiblity that $MnCl_2$ itself may cause some Quin 2 leakage. It should be noted however that irreproducible results may also be attributed to the cuvette temperature of ~ 30°C in the Aminco-Bowman spectrofluorimeter.

Because of the uncertainty surrounding the intracellular calcium measurements with Quin 2, it was felt appropriate to combine these studies with an indirect technique which quantifies the transient transmembrane fluxes of radiolabelled calcium during mitogenic stimulation.

3.4 CALCIUM UPTAKE

One of the common technical problems in studies of this nature is the difficulty in separating the cells and excluding the residual unincorporated isotope from the extracellular fluid. The procedure of extensively washing the cell pellet to remove any unincorporated isotope is inadequate as it can allow tracer exchange between cells and the washing media. Not surprisingly, the reported values of $^{45}Ca^{2+}$ uptake in mitogen-stimulated cells using such methods vary widely. This problem has however

been surmounted by using a modification of the technique developed by Freedman, Raff and Gompertz (1975). In this technique, the cell associated $^{45}Ca^{2+}$ can be distinguished from the unincorporated extracellular tracer by centrifugation through a chemically inert and water impermeable oil barrier into a layer of formic acid which lyses the cells releasing $^{45}Ca^{2+}$ to be counted. Thus, polypropylene microcentrifuge tubes (Beckman PRO-22) were prepared by addition of 125 µl MS 550 silicone oil (BDH Ltd) over a layer of 50 µl 98% formic acid. These tubes when spun revealed a clearly defined two-tier system ready for the addition of the cell suspension.

Thymocyte cultures were prepared as described above except cell density was increased to $10^8 ml^{-1}$. Preliminary studies demonstrated that this increase in cellularity had no effect on mitosis. One millilitre cultures were preincubated at 37°C for at least 30 minutes before addition of 1 ml of a prewarmed mixture of isotope and other agents of interest. Triplicate 200 µl samples were removed at appropriate intervals thereafter and placed above the formic acid/oil layers and immediately centrifuged at 8,500 g for two minutes in a Beckman Microcentrifuge; maximum force is generated within 5 seconds.

Tubes were then frozen in liquid nitrogen and the bottom (approximately 1cm) containing the lysed cells was amputated by a lateral cut through the oil layer and placed in vials containing NE-260 scintillation fluid (Nuclear Enterprises Ltd). Preliminary studies revealed that carry over of isotope from the aqueous phase (presumably trapped within the interstitial spaces between the cells) was 0.13% or less of the total supernatant activity. Such amounts were trivial compared to the cell-associated $^{45}Ca^{2+}$ and were therefore ignored. Radioactive calcium was measured using liquid scintillation spectroscopy in a Packard tri-carb counter, model No 2660. An internal standard was used to correct for decay and the efficiency of the ß-detector corrected using an on-line handling system.

Cell-associated $^{45}Ca^{2+}$ consists of the transmembrane influx of the ion plus non-specific adsorption to the plasma membrane. The latter component was taken to be cell-associated calcium at zero time, ie. immediately after mixing of cells with isotope followed by rapid centrifugation. The Ca^{2+} taken up into the cells was then derived from the following equation; all values were precorrected for counting efficiency and background activity.

$$\% \, Ca^{2+} \text{ uptake at time (t)} = \frac{\text{Sample dpm at time (t)} - \text{Membrane associated dpm at time } (t_o)}{\text{Total dpm}} \times 100 \quad \ldots\ldots 1$$

where the total dpm represents counts of a 200 µl aliquot of isotope-laden cell suspension without spinning through the oil layer.

For calculation of the actual amount of Ca^{2+} translocated from the product of calculated Ca^{2+} from (1), the total calcium concentration of the culture must be considered.

Thus, the µ moles of Ca^{2+} taken up by cells present in 1 ml of culture is calculated as:

$$Ca^{2+} \text{ influx} = \frac{\text{Percent Ca uptake} \times \mu \text{ moles } Ca^{2+} \text{ per ml culture}}{100}$$

Other authors using peripheral blood lymphocytes have chilled cells to eliminate transmembrane Ca^{2+} transport and used cell-associated $^{45}Ca^{2+}$ values at 4°C to represent non-specific membrane binding. We found that such procedures gave highly irreproducible and sometimes non-sensical results with thymocytes. The procedure outlined above using "zero time" binding at 37° eliminated inconsistencies and was thus preferred. Membrane bound Ca^{2+} by this technique was about 80% of the total uptake over a 20 minute period comparing

Table 7 - The effect of the ionophore, A23187 on calcium uptake in rat thymocytes

	pm/10^6 cells/20 mins
Basal	8.9
A23187 10^{-5}M	514.7
10^{-6}	317.5
10^{-7}	28.2
10^{-8}	4.2
10^{-9}	3.7
10^{-10}	6.5
10^{-11}	4.3
10^{-12}	4.9

Table 8 - The effect of β-oestradiol on $^{45}Ca^{2+}$ influx in rat thymocytes in basal media

Treatment concentration	pm/10^6 cells/20mins
Basal	3.8
β-oestradiol 1 $pgml^{-1}$	8.6
β-oestradiol 10 $pgml^{-1}$	8.4
β-oestradiol 100 $pgml^{-1}$	2.4
β-oestradiol 100 μgml^{-1}	4.8

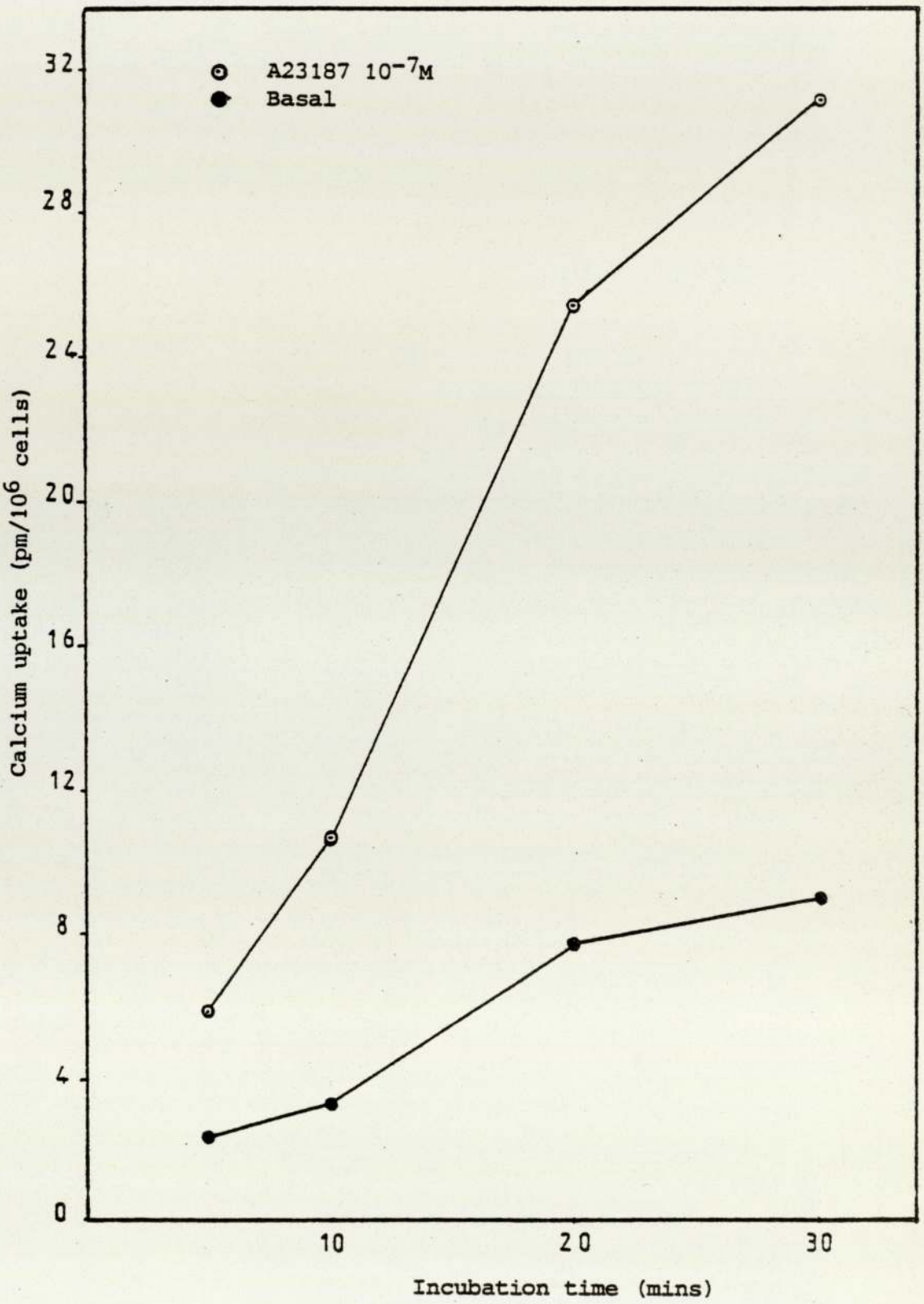


Figure 1 The effect of ionophore, A23187 (10^{-7}M) on calcium uptake in thymocytes in basal media.

Basal media contains 0.6mM Ca^{2+} and 1.0mM Mg^{2+}.

favourably with values found by Freedman et al (1975).

Employing the technique outlined above enhanced Ca^{2+} uptake after application of the ionophore A23187 was readily detected. A dose-dependent reponse was evident with maximal uptake at a concentration of 5×10^{-5} M (Table 7). Such enhanced Ca^{2+} influx could be seen as early as five minutes after addition of ionophore (Figure 1). However, as the ionophore is non-physiological, it seemed relevant to check the utility of the technique by employing the physiological mitogen oestradiol which has been claimed to enhance Ca^{2+} uptake (Cade, 1983). Cells were therefore incubated with different concentrations of oestradiol and the ^{45}Ca uptake measured. An enhanced Ca^{2+} uptake was observed at the mitogenic concentrations of 1 and 10 μg per ml (Table 8) which confirms results of Perris and co-workers (1983).

3.5 STATISTICAL ANALYSIS

Groups of data have been compared using Anovar one way analysis of variance. The least significant difference (LSD) was determined by substituting the value of 't' at a given probability level of $p = 0.05$. Thus, in each of the figures and tables the asterisk denotes the degree of significance at $p \leq 0.05$. For details of Anovar and LSD refer to Appendix E.

CHAPTER FOUR

EXPERIMENTAL RESULTS

In accordance with previous studies, rat thymic lymphocytes progressed into the mitotic phase and accumulated in a quasi-metaphase configuration when suspended in colchicine-supplemented medium. At divalent cationic concentrations adjusted to reflect the ambient levels of normal rat blood (0.6 nM Ca^{2+}; 1.0mM Mg^{2+}) approximately 4% of the cells entered mitosis over the 6 hour incubation period (Figure 2). Elevation of the extracellular Ca^{2+} or Mg^{2+} concentration to 1.8 mM or 2.5 mM respectively raised the proportion of cells entering mitosis to ~ 6%. High divalent cation concentrations apparently initiate the recruitment of normally quiescent 'Go' thymic cells into the cell-cycle. Consistent with the evidence that gonadal steroids cause thymic involution, high concentrations of testosterone and oestradiol had a marked effect on thymocyte proliferation (Figure 2). Oestradiol inhibited mitosis provoked by high Ca^{2+}, whereas testosterone exerted a restraining effect on mitogenesis induced by high Mg^{2+}. The enhanced extracellular divalent ionic concentrations employed for stimulating cell division would probably increase internal cationic concentration. An increase in the cytosolic content of Ca^{++} or Mg^{++} could therefore be responsible for initiating or triggering the mitogenic response.

In an attempt to block putative Ca^{2+} entry consequent to raised external ionic levels, a calcium-channel blocker, Nifedipine, was employed (Fleckenstein, 1977; Spedding & Cavero, 1984). The proliferogenic response normally induced by 1.8 mM Ca^{2+} was certainly impaired by the antagonist in a dose-dependent manner with maximal inhibition at 10^{-5}M (Figure 3). The 10^{-4}M concentration proved to be cytotoxic. Therefore, Ca^{2+} influx appears to be pertinent for Ca^{2+} induced mitosis in thymic lymphocytes. Nifedipine at 10^{-5}M concentration did

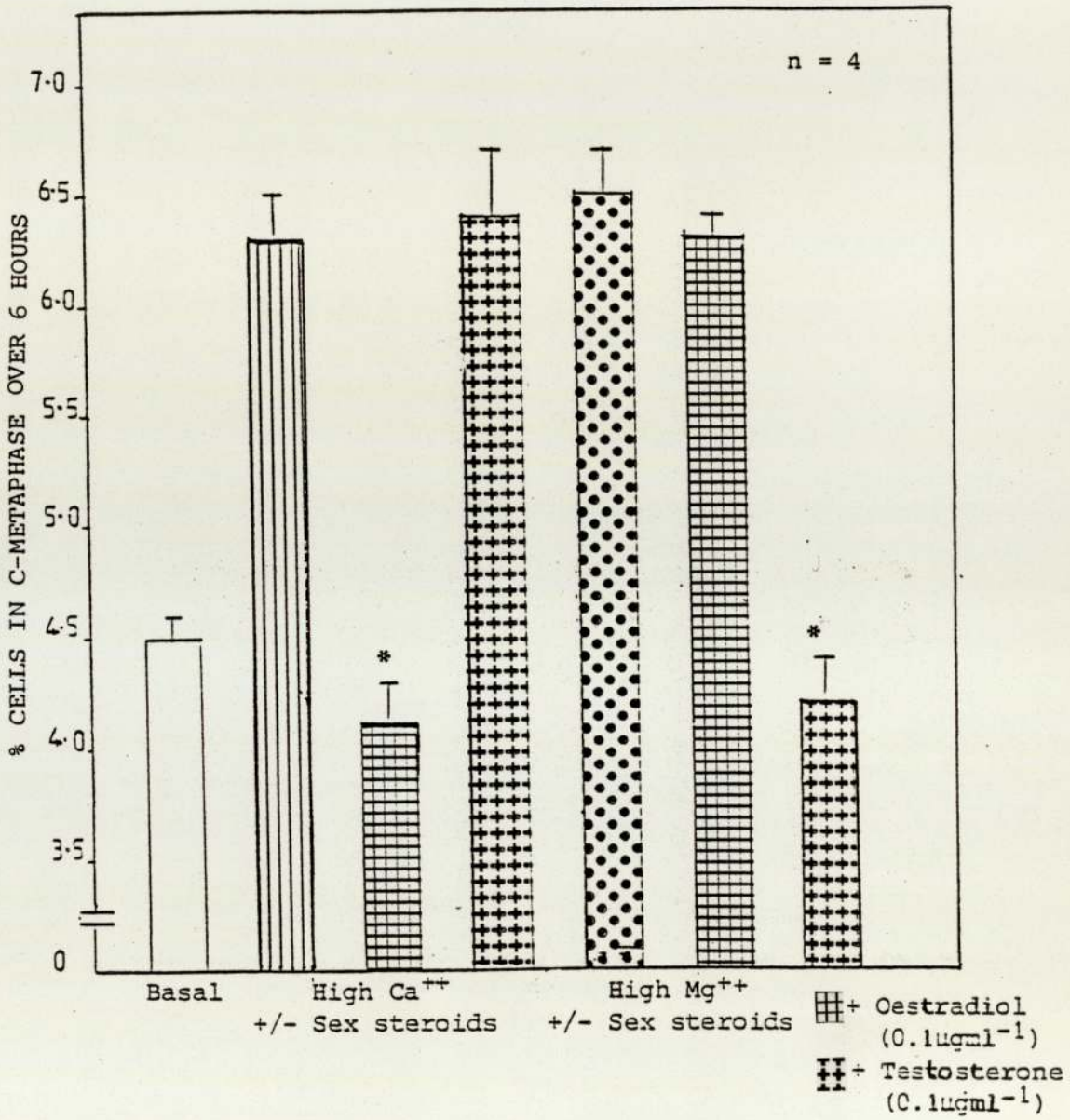


Figure 2 Effects of sex steroids on calcium and magnesium induced mitogenesis

The basal medium contains 0.6mM Ca^{++} and 1.0mM Mg^{++}. High Ca^{++} and high Mg^{++} indicate where these concentrations were increased to 1.8mM and 2.5mM respectively. Columns represent mean values ± s.e.m, and n = the number of separate experiments in each group. Values significantly different from those of corresponding cultures without steroids are indicated by asterisk.

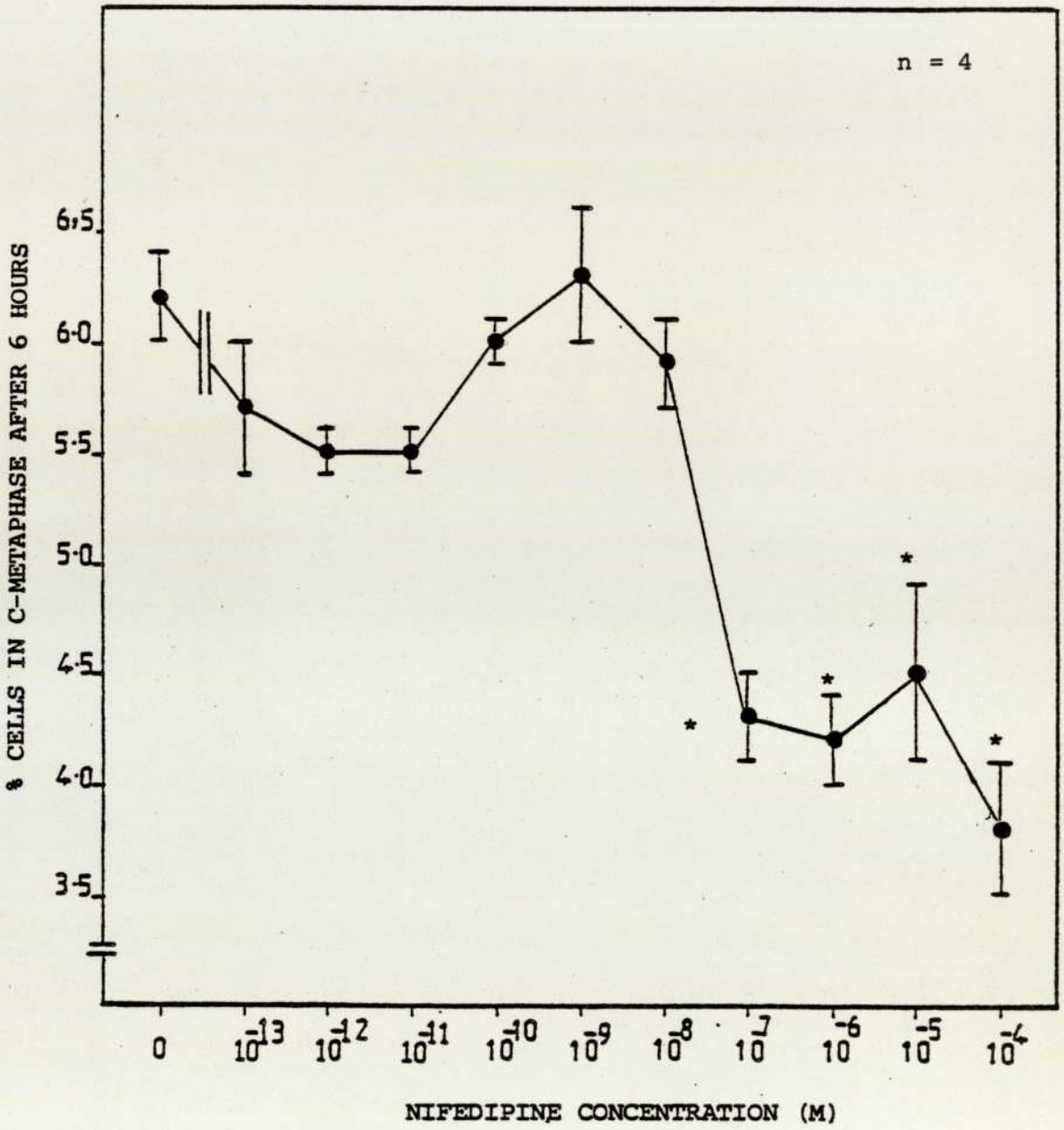


Figure 3 Effect of nifedipine on mitogenesis induced by high Ca^{++}.

High Ca^{++} represents calcium concentration in culture medium of 1.8 mM. Values significantly different from those of cultures incubated without nifedipine are indicated by asterisk.

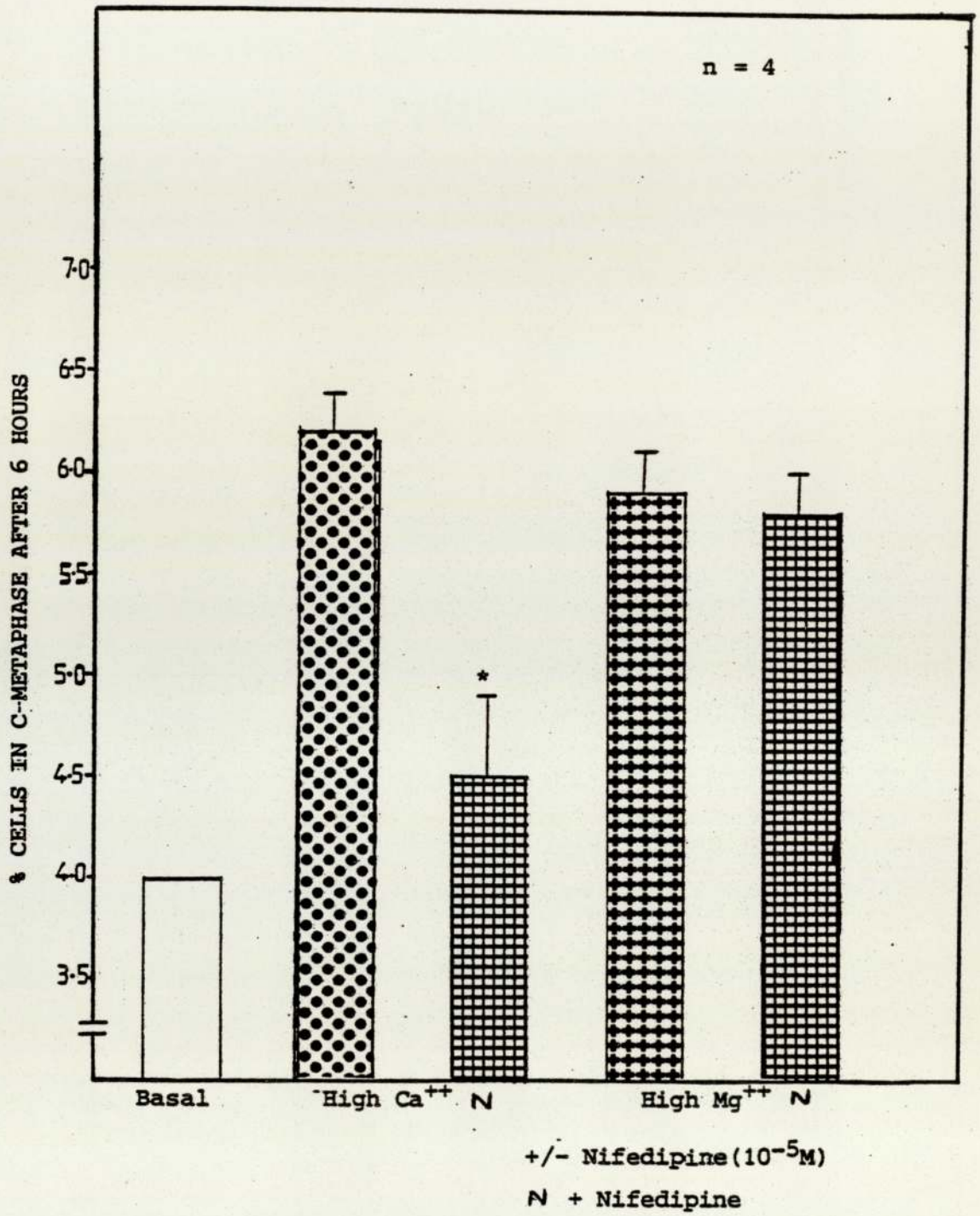


Figure 4 Effect of nifedipine on mitosis induced by high Ca^{2+} and high Mg^{2+}

Values significantly different from corresponding cultures without the drug are indicated by asterisk. Other details as in Figure 2.

not inhibit basal mitotic activity and did not significantly impair high Mg^{2+} induced mitosis (Figure 4). Hence, Mg^{2+} does not appear to promote mitosis via an increased Ca^{2+} influx.

Since it has been suggested that a rise in cytosolic Ca^{2+} or Mg^{2+} content may promote mobilisation of Ca^{2+} from intracellular reservoirs in sub-cellular organelles, we attempted to block possible effects of released Ca^{2+} by using the supposed Ca^{2+} mobilisation blockers, TMB-8 (Sigma Ltd) and M&B40,678 (May & Baker Ltd). The mitogenic response to both cations was certainly blocked by M&B40,678 at concentrations ranging from 10^{-10}M to 10^{-4}M (Figures 5 & 6) suggesting that Ca^{2+} mobilisation is a prominent feature of cell division initiated by elevated levels of both high Ca^{2+} and Mg^{2+}. The antagonist also blocked mitosis attributed to high magnesium in calcium-free medium (Figure 7). Moreover, M&B40,678 blocked the increase in cytosolic calcium concentrations induced by the mitogenic calcium concentration (Table 9). Such inhibition provides confirmative evidence of the significance of intracellular Ca^{2+} movement during stimulation of cell division. The basal mitotic activity was not influenced by M&B40,678. TMB-8, the other antagonist, (Malagodi & Chiou, 1974) however inhibited the proliferogenic response attributed to both cations only at a very high concentration (10^{-4}M) which appeared to be highly toxic. In fact, other workers have also indicated that TMB-8 at 10^{-4}M influenced ATP levels in rat thymocytes (Brand & Felber, 1984). The basal mitotic activity was not influenced by either of the antagonists. In view of the toxic effects of TMB-8, further studies only employed M&B40,678 as a putative mobilisation blocker. It seems likely therefore that intracellular calcium and magnesium changes can serve as triggers for mitosis. However imposed calcium and magnesium gradients of this magnitude seldom ever occur physiologically. Many natural mitogenic hormones however do exhibit calcium and/or magnesium dependency. Acetylcholine (ACh)

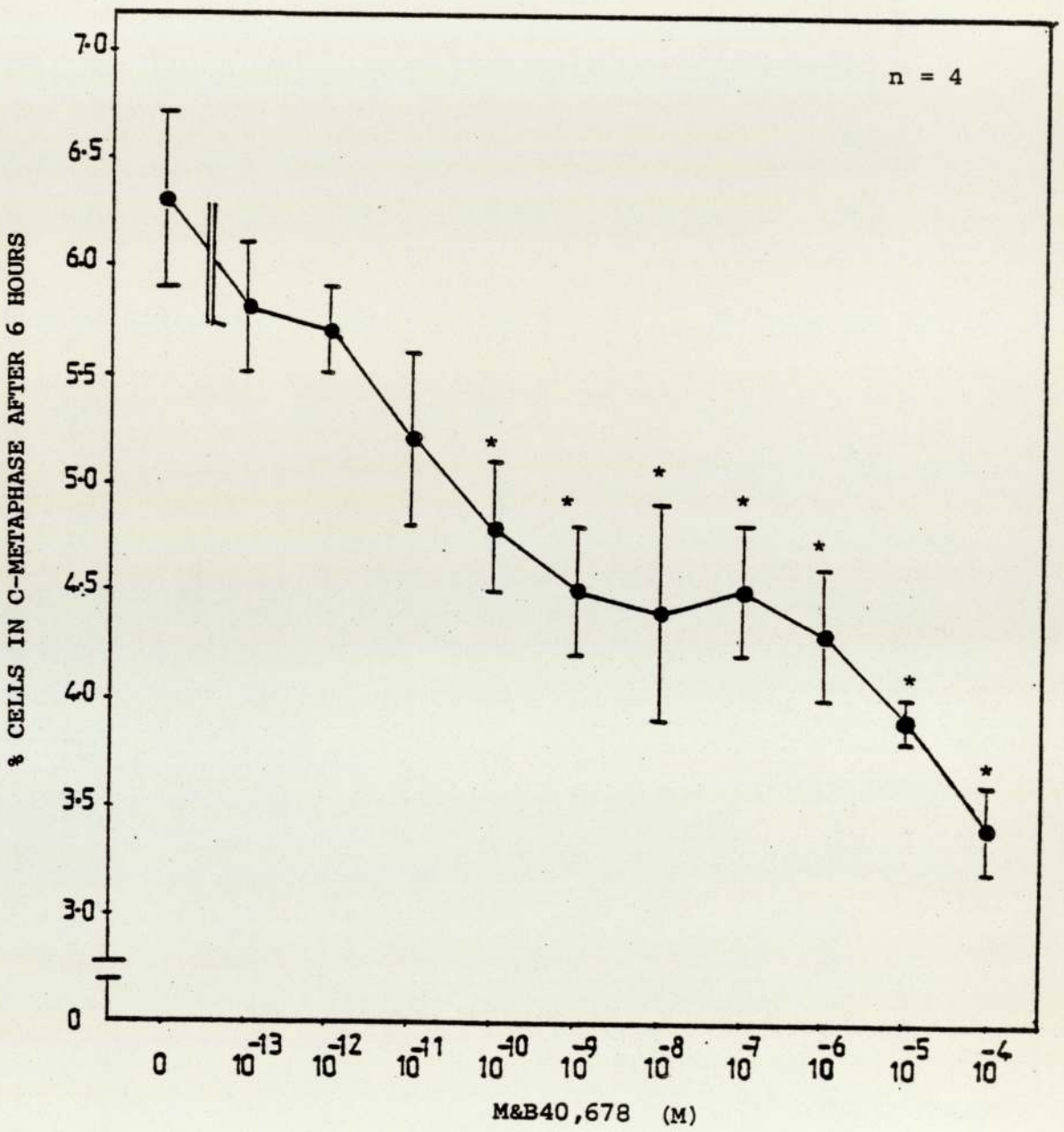


Figure 5 Effect of M&B40,678 on high Ca^{++}-induced mitotic activity.

High Ca^{++} represents a calcium concentration of 1.8mM in the culture medium. Values significantly different from those of cultures without M&B40,678 are indicated by asterisk.

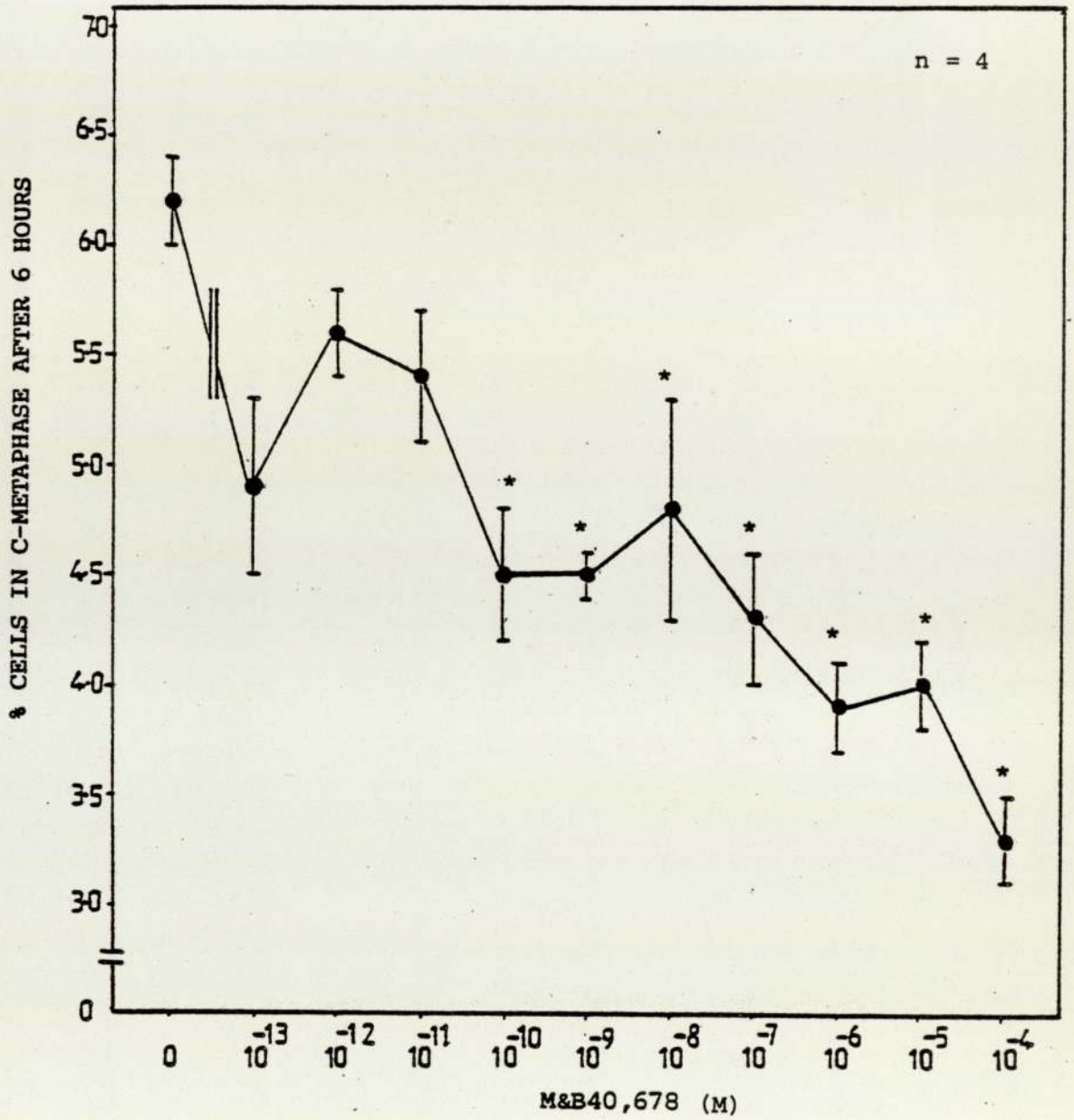


Figure 6 Effect of M&B40,678 on mitotic activity of thymic lymphocytes incubated in medium containing high Mg^{++}

High Mg^{++} represents a magnesium concentration of 2.5mM in the culture medium. Values significantly different from those of cultures without M&B40,678 are indicated by asterisk.

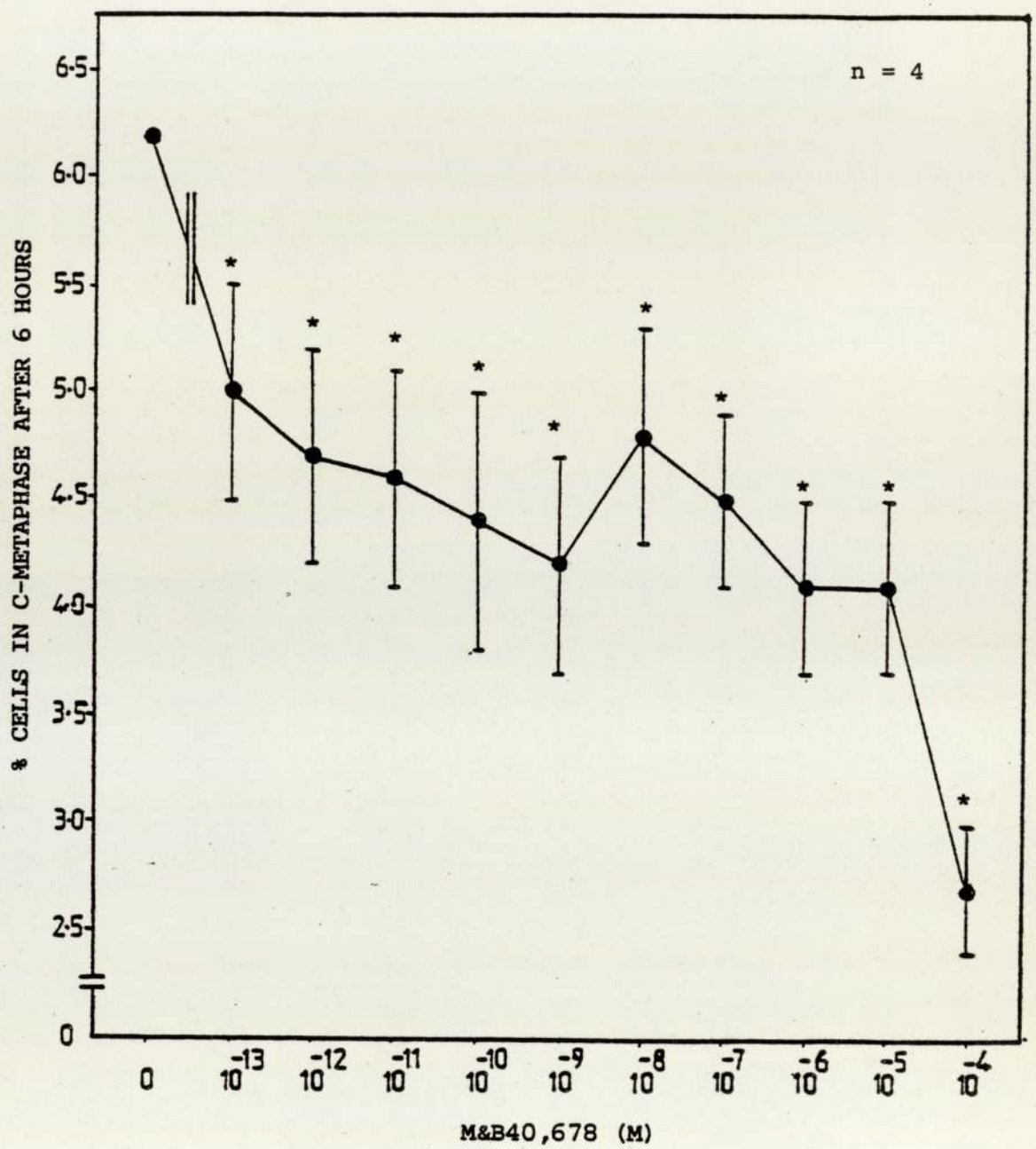


Figure 7 Effect of M&B40,678 on high Mg^{++}-induced mitotic activity in calcium-free media.

The culture medium contained 2.5mM Mg^{++} but no calcium. Values significantly different from those of corresponding cultures without the drug M&B40,678 are indicated by asterisk.

was selected for further study since it exhibits a biphasic effect with obligatory requirements for both ions. In the presence of eserine (10^{-9}M), an anticholinesterase agent which itself has no effect on basal mitotic activity, two peaks of mitogenic activity at concentrations of 10^{-5}M and 10^{-11}M were evident (Figure 8). These results confirmed the previous observations of a biphasic mitotic response to acetylcholine (Morgan et al, 1984).

Table 9 - The effect of calcium antagonists on intracellular calcium concentration of thymic lymphocytes in basal and high calcium media

Resting $[Ca^{2+}]_i$ levels (nm)	Medium cation concentration (mM) Ca^{2+}	Mg^{2+}	Treatment	Stimulated $[Ca^{2+}]_i$ levels (nM)
141	1.8	1.0	No addition	268
153	1.8	1.0	Verapamil	187
153	1.8	1.0	M&B40,678	153
187	0.6	1.0	Verapamil	187
161	0.6	1.0	M&B40,678	161

Verapamil and M&B40,678 were used at a concentration of 5×10^{-6} gml^{-1} and 10^{-5}M respectively

To investigate the biphasic mitogenic effect of acetylcholine further, the effect of atropine, a muscarinic receptor antagonist and a nicotinic receptor antagonist, hexamethonium was examined. Atropine (10^{-5}M) abolished the proliferative effect of the low concentration of acetylcholine (10^{-11}M) but not that of the high acetylcholine concentration (10^{-5}M) (Figure 8). The inhibitory effect of atropine was counteracted by a high concentration of acetylcholine (10^{-8}M/10^{-9}M) as in Figure 8, suggesting that ACh is exerting its effects via the

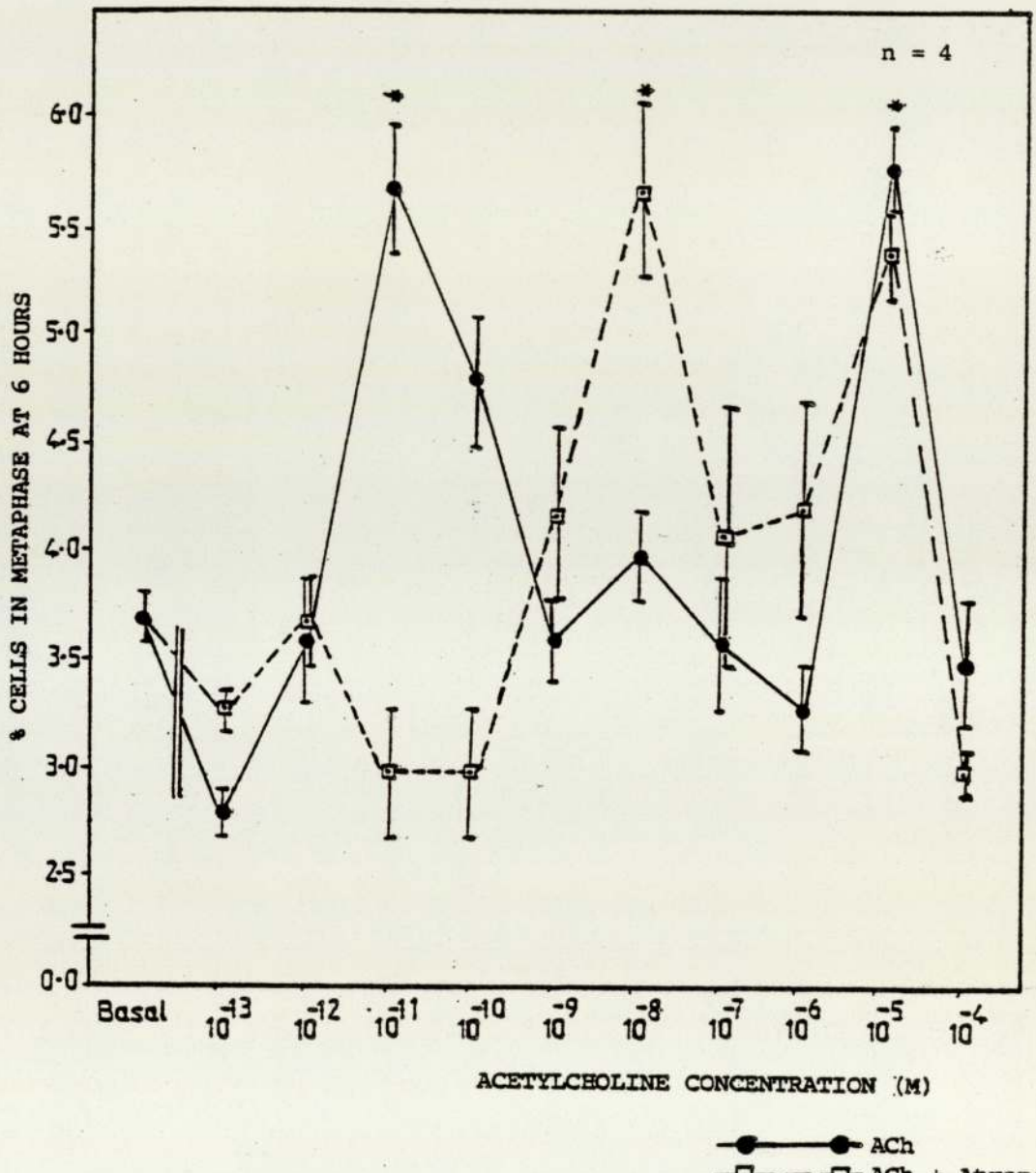


Figure 8 Effect of the muscarinic receptor antagonist, Atropine (10^{-5}M) on Acetylcholine-induced mitotic stimulation in basal media.

All cultures also contained the anticholinesterase agent eserine (10^{-9}M) which itself had no effect on mitotic activity. Values significantly higher than the basal cultures are indicated by asterisk.

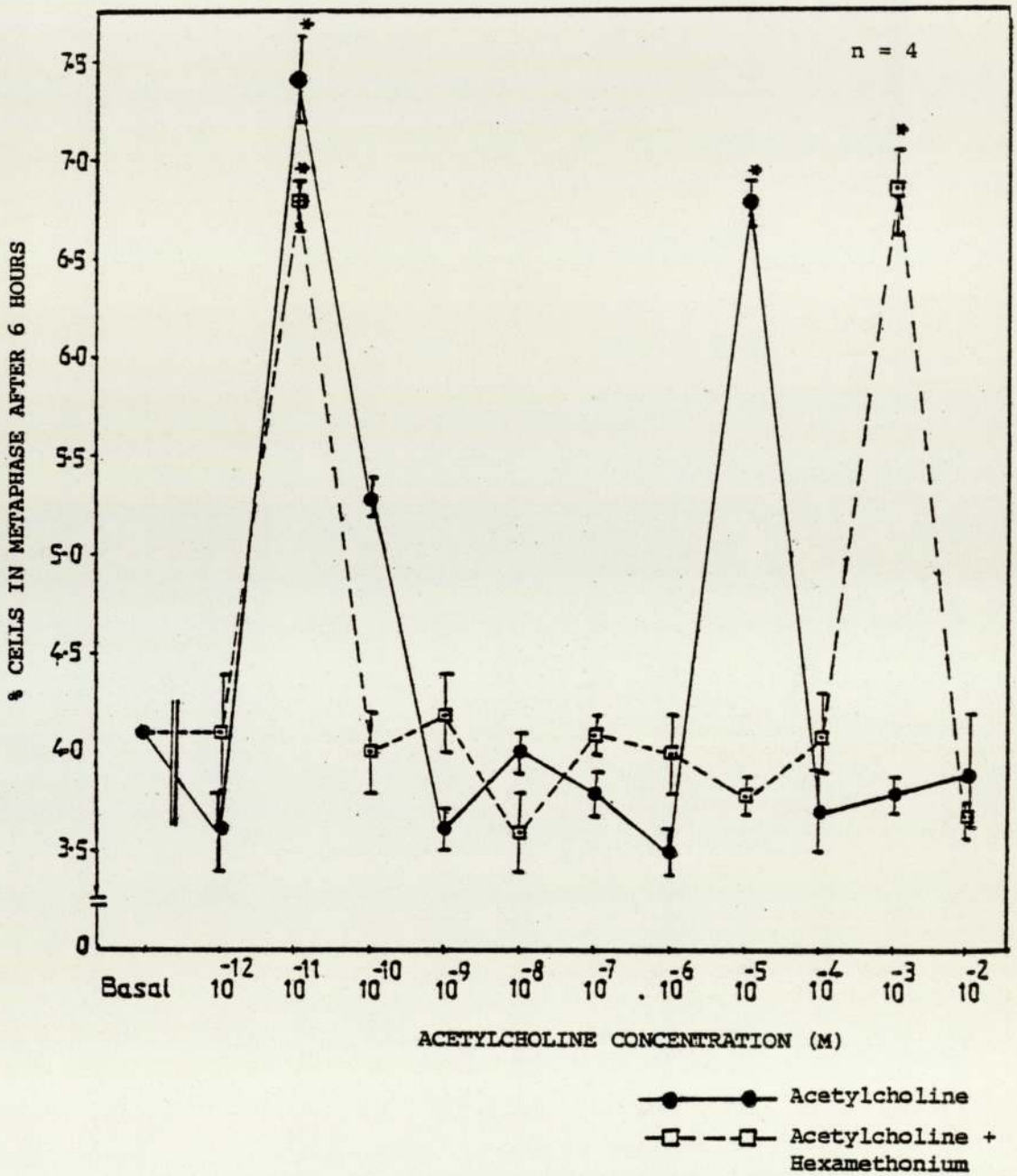


Figure 9 Effect of the nicotinic receptor antagonist, Hexamethonium (10^{-5}M) on mitosis induced by Acetylcholine in thymocytes incubated in basal media.

All cultures also contained the anticholinesterase agent, eserine (10^{-9}M) which itself did not influence mitotic activity. Values significantly higher than the basal culture are indicated by asterisks.

muscarinic receptor at low concentrations (10^{-11}M). In contrast, hexamethonium (10^{-5}M) only abolished the mitogenic effects of high concentrations of ACh (10^{-5}M) (Figure 9). Once again, the inhibition was overcome by application of supernormal concentrations of acetylcholine indicating that ACh can also activate nicotinic receptors for induction of mitosis.

As hormonal mitogens operate either via a calcium-dependent, oestradiol-blockable axis, or via a magnesium-dependent testosterone-blockabel route, it seemed relevant to examine the ionic dependence and the steroid sensitivity of ACh-induced mitogenesis. The low mitogenic concentration of ACh was found to be calcium-dependent while the high ACh concentration required magnesium to be effective (results not shown), thus confirming previous studies in this laboratory (Morgan, 1976). These same ionic dependencies were apparent when ACh exerted its proliferogenic effects despite the presence of atropine (Figures 10 and 11). As expected, the Ca^{2+} dependency of ACh (10^{-11}M) was inhibited by oestradiol (0.1 ugml^{-1}) whereas the mitosis due to the Mg^{2+} dependent high ACh concentration (10^{-5}M) was blocked by testosterone (Table 10). Whether a combination of oestradiol (0.1 ugml^{-1}) and ACh (10^{-11}M) increases Ca^{2+} influx as observed for an oestradiol and high calcium treatment (Figure 12) has not been determined.

Table 10 - The influence of sex-steroids on the mitosis induced by acetylcholine in rat thymic lymphocytes

	Percentage of cells in metaphase after 6 hours		
Treatment	Control	ACh 10^{-5}M	ACh 10^{-11}M
No addition	4.5 ± 0.1	6.2 ± 0.1	6.3 ± 0.2
Oestradiol	4.0 ± 0.2	6.1 ± 0.3	4.0 ± 0.2*
Testosterone	4.2 ± 0.1	4.3 ± 0.3*	6.4 ± 0.1

* Significantly different from untreated ACh cultures

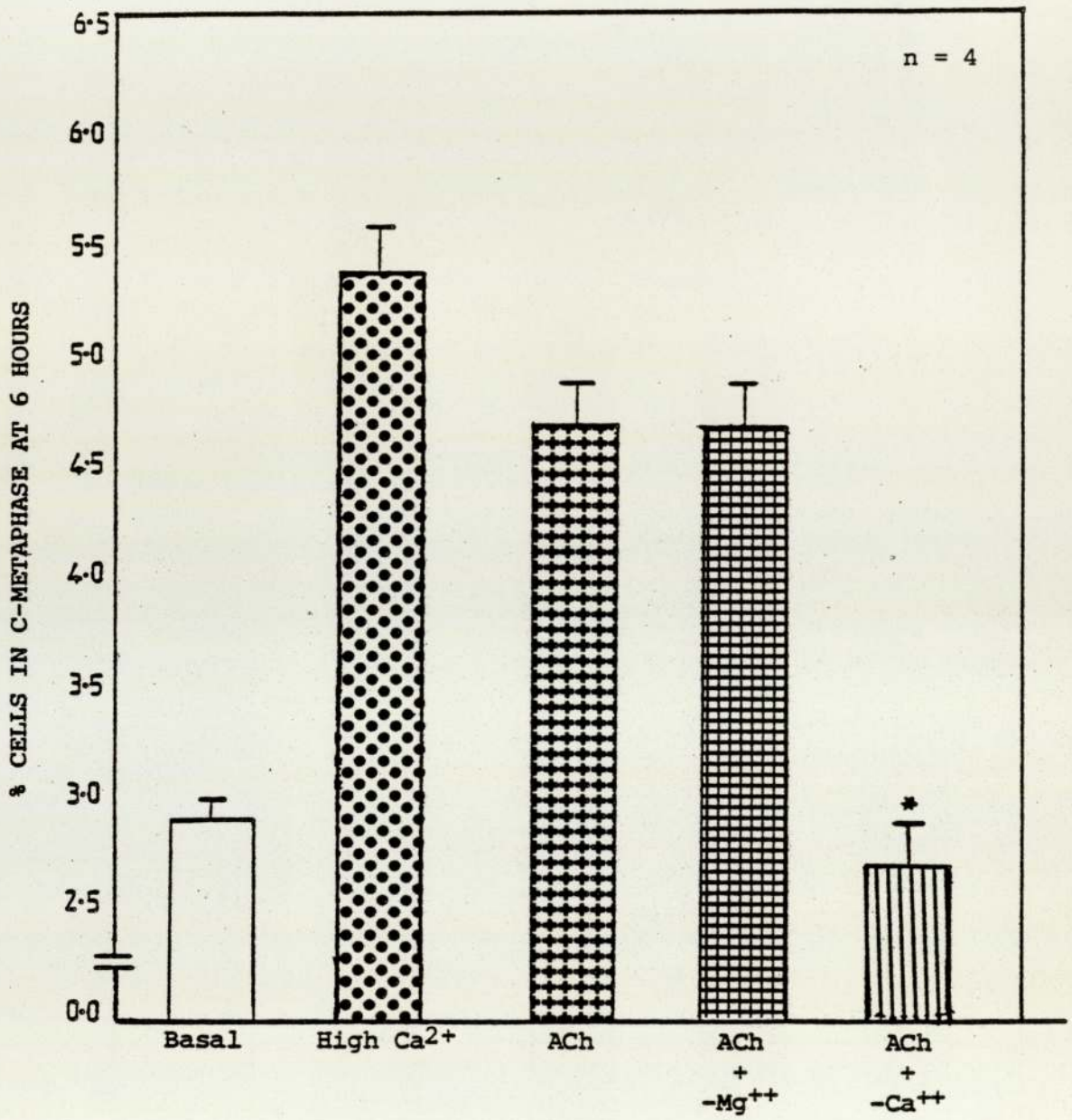


Figure 10 Effect of omission of calcium and magnesium ions upon the mitogenicity of low concentrations (10^{-8}M) of acetylcholine in the presence of atropine (10^{-5}M). All cultures containing acetylcholine contained eserine.

Values significantly different from the ACh in basal medium are indicated by asterisk

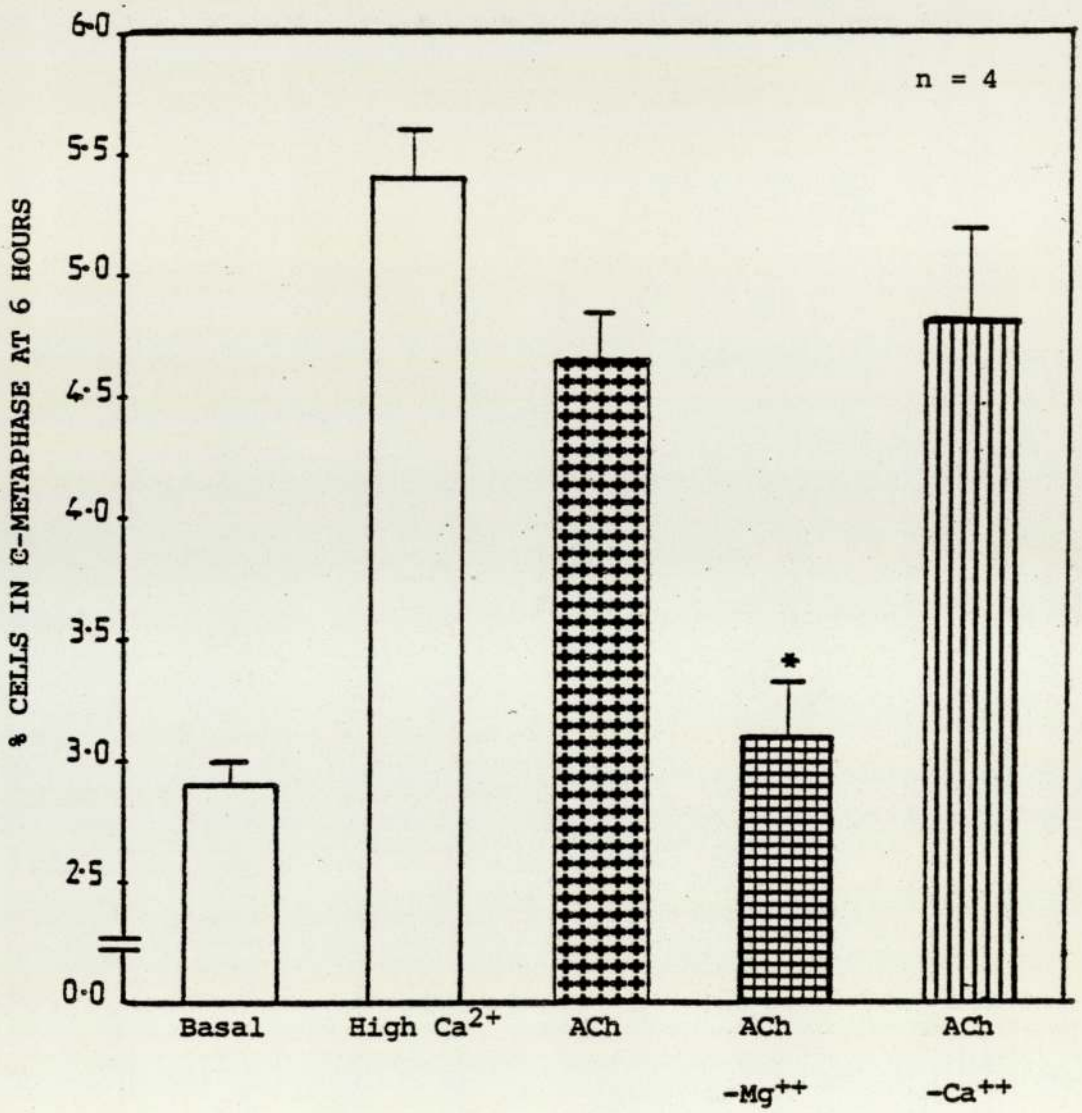


Figure 11 The effect of omission of calcium and magnesium ions from the medium upon the mitogenicity of high concentration (10^{-5}M) of acetylcholine in the presence of atropine (10^{-5}M).

Values significantly different from the ACh included in basal medium are indicated by asterisk. Other details as in Figure 10.

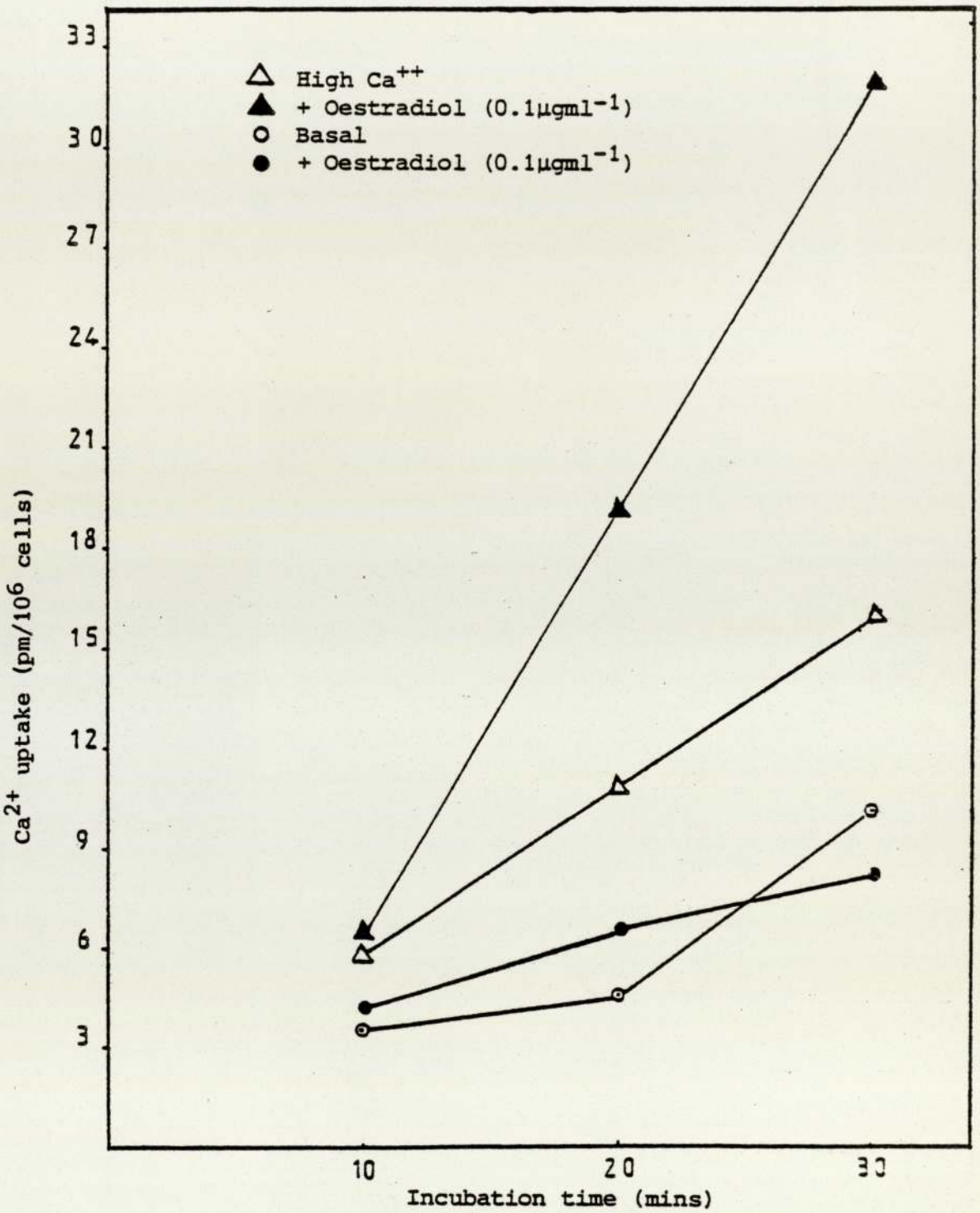


Figure 12 Effect of β-Oestradiol ($0.1\mu gml^{-1}$) on $^{45}Ca^{2+}$ uptake in rat thymocytes

Further studies employing the fluorescent indicator, Quin 2 clearly revealed a rise in cytosolic Ca^{2+} levels upon addition of the low concentration (10^{-11}M) of acetylcholine. No significant increase was evident in the absence of exogenous calcium (Table 11). The increase in cytosolic Ca^{2+} concentration is possibly due to enhanced influx (Table 12).

Table 11 - Effect of Acetylcholine on intracellular calcium concentrations of rat thymic lymphocytes

Resting $[Ca^{2+}]_i$ levels (nM)	Medium cation concentration (mM) Ca^{2+}	Mg^{2+}	Treatment (M)	Stimulated $[Ca^{2+}]_i$ levels (nM)
173	0.6	1.0	ACh (10^{-5})	204
115	0.6	1.0	ACh (10^{-11})	184
126	0	1.0	ACh (10^{-11})	150
150	0.6	0	ACh (10^{-11})	230

Table 12 - The effect of Acetylcholine on $^{45}Ca^{2+}$ influx in rat thymic lymphocytes in basal media

Treatment (M)	Ca^{2+} uptake pm/10^6 cells/20 mins
Basal	10.4
ACh (10^{-5})	9.0
ACh (10^{-10})	9.6
ACh (10^{-11})	17.0

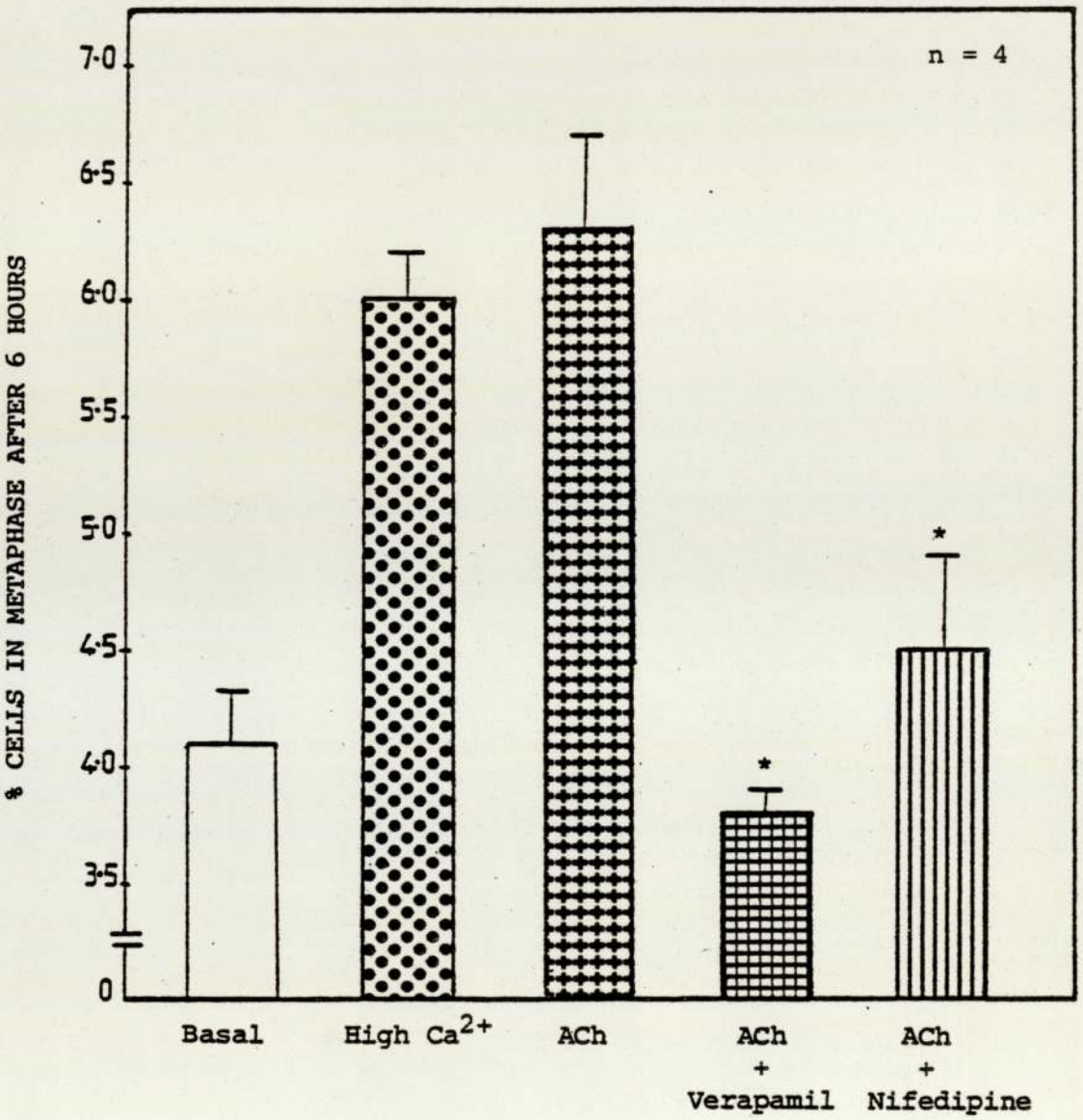


Figure 13 Effect of calcium-channel blockers upon the mitogenicity of low concentration (10^{-11}M) of acetylcholine. The concentrations of verapamil and nifedipine employed were 5×10^{-6} gml^{-1} and 10^{-5} M respectively.

Values significantly different from the corresponding cultures without the calcium antagonists are indicated by asterisk.

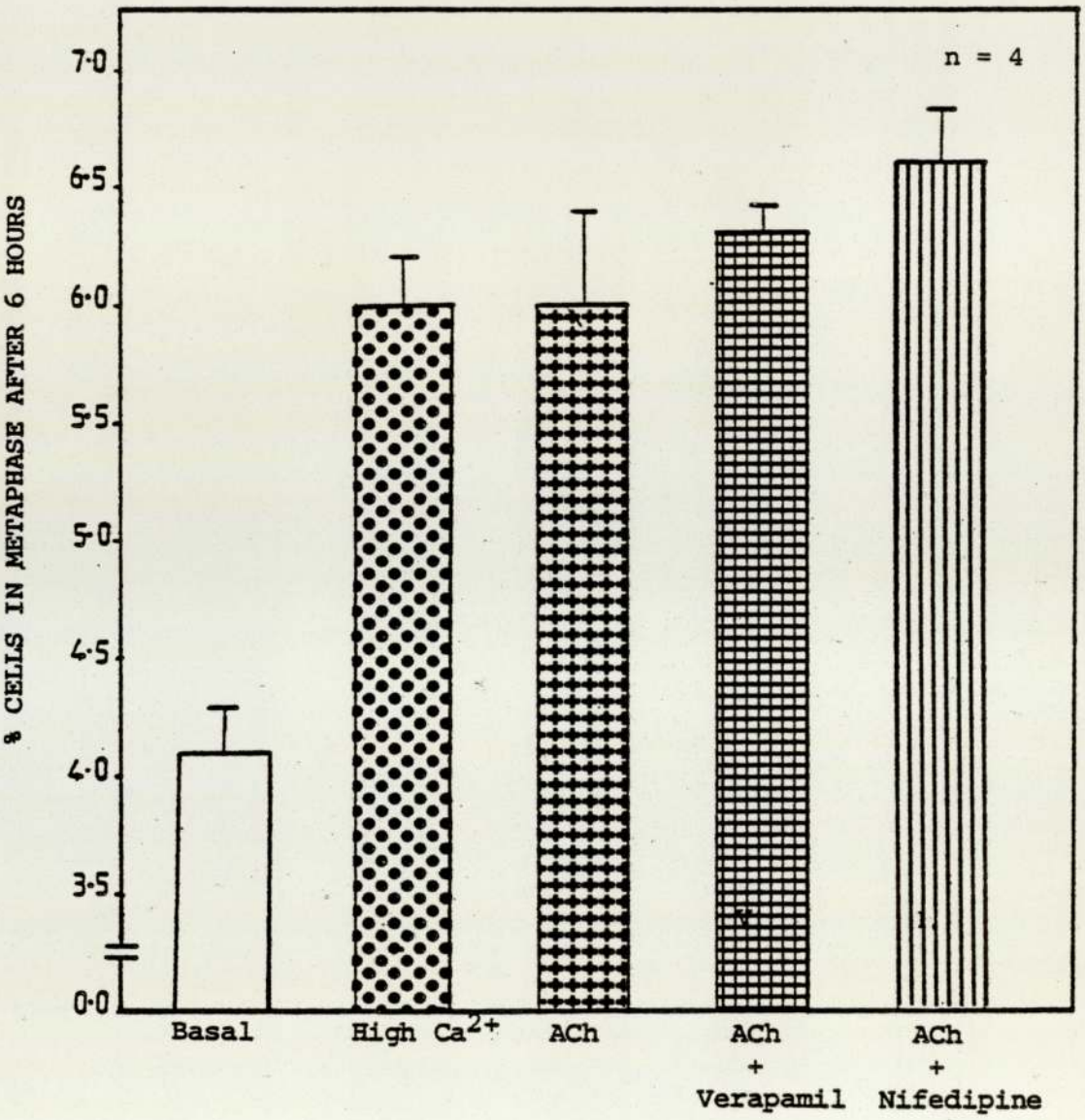


Figure 14 Effect of calcium channel-blockers on the mitogenic activity of high concentration (10^{-5}M) of acetylcholine.

Verapamil and Nifedipine were employed at concentrations of 5×10^{-6} g ml^{-1} and 10^{-5}M respectively.

The calcium dependency of the low ACh concentration was further confirmed by addition of calcium channel blockers, verapamil and nifedipine which compromised the mitogenic response to low ACh concentrations without any affect on the Mg^{2+}-induced mitogenic response (Figures 13 and 14). M&B40,678 also had an inhibitory effect on Ca^{2+} dependent mitosis of low ACh concentrations but surprisingly did not inhibit the stimulatory effect of the high Mg^{2+} dependent ACh concentration (Figure 15). Thus, M&B40,678 inhibits the potentiated mitogenic response to high Mg^{2+} but not Mg^{2+} dependent mitogen. The Ca^{2+} released from intracellular storage sites may be involved in the Ca^{2+} dependent mitotic effects of low ACh concentrations but not those of the Mg^{2+} dependent high ACh concentrations. Thus, it seems feasible to presume that ACh (10^{-11}M) promotes the movement of Ca^{2+} down its concentrration gradient and also intracellularly and may consequently raise cytosolic Ca^{2+} levels which may be responsible for initiation of DNA synthesis and finally mitosis. A rise in $[Ca^{2+}]_i$ may probably activate the calcium regulatory protein, CaM. A phenothiazine antipsychotic agent, trifluoperazine (TFP) selectively binds and inactivates the calcium calmodulin complex probably by altering the conformation of the Ca^{2+} binding sites (Roufogalis, 1983). If calmodulin is activated, the mitogen-induced mitosis should be affected by calmodulin antagonists. As expected, TFP abolished the cell division triggered by the low ACh concentration but did not effect the proliferative ability of the high concentration of the neurotransmitter (Figure 16). Thus, it appears that the activation of either muscarinic or nicotinic receptors on the thymocytes initiates events culminating in mitosis in a different cationic mechanism.

A physiological agent which can also exert biological actions in target tissues via the Ca^{2+} messenger system is the parathyroid hormone (PTH). In

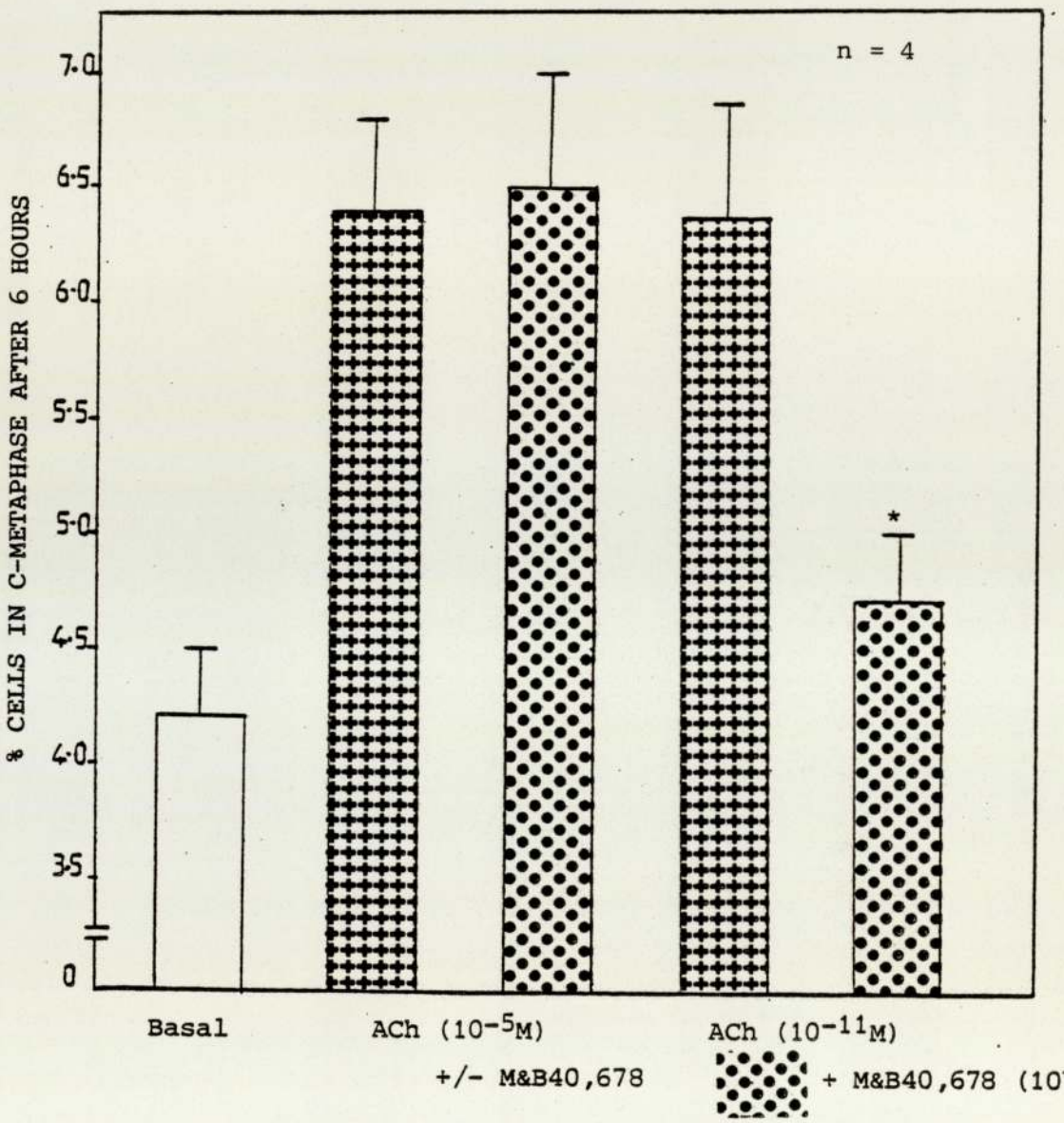


Figure 15 Effect of the drug, M&B40,678 on the mitotic activity of thymic lymphocytes in the presence of both high and low concentrations of acetylcholine.

Values significantly different from those of acetylcholine without the drug are indicated by asterisk.

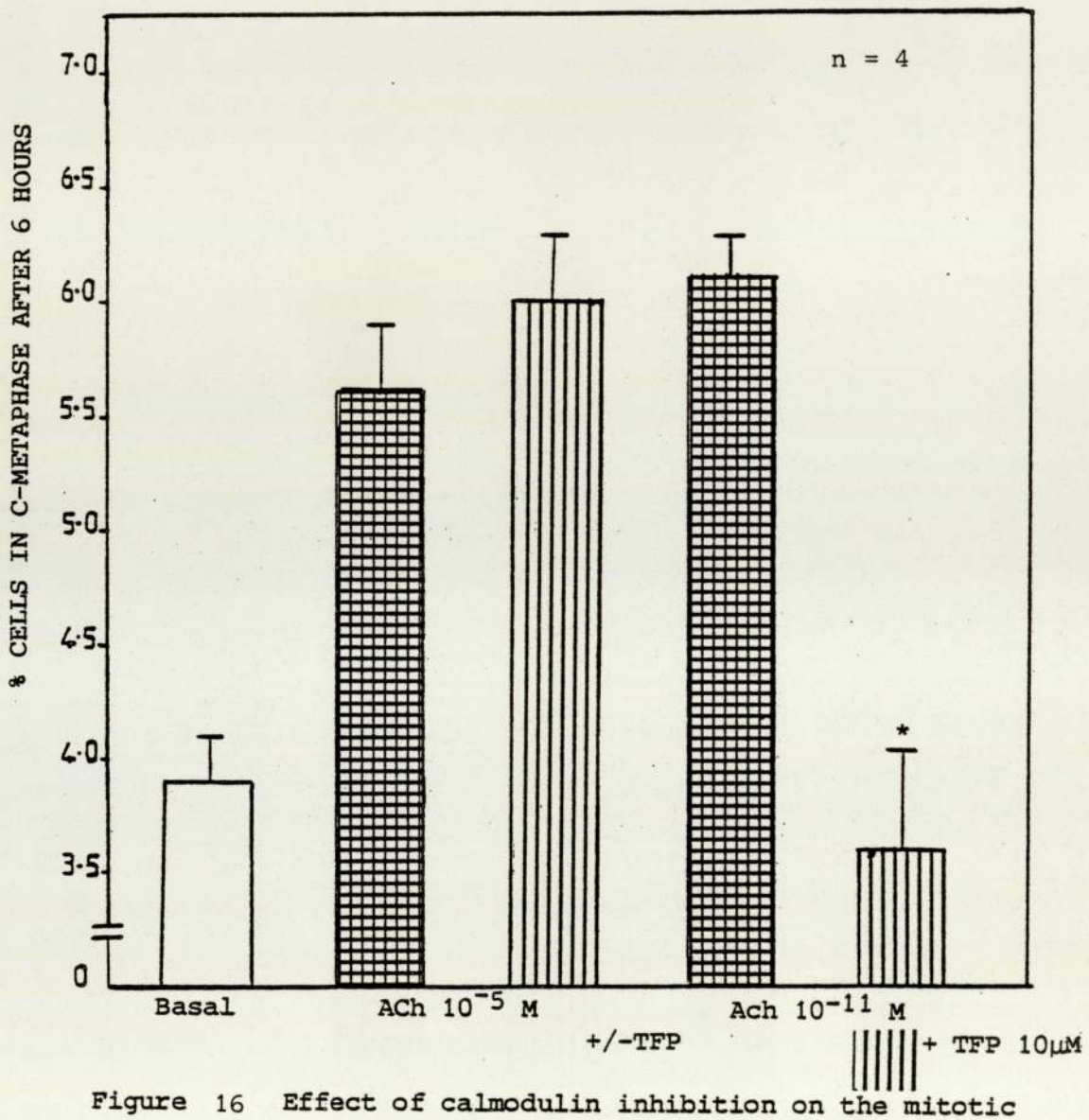


Figure 16 Effect of calmodulin inhibition on the mitotic activity of thymocytes in the presence of high and low concentrations of acetycholine.

Values significantly different from those of ACh without TFP are indicated by asterisk.

fact, PTH is principally involved in the regulation of mammalian calcium homeostasis and hence serves to raise plasma calcium levels in response to hypocalcemia or even antigenic stimulation. In the present study, a biologically active PTH fragment (1-34) and a super agonist hPTH 8,18 Nleu (1-34) have been employed. Both the synthetic human fragments clearly exhibited a mitogenic effect at a concentration of $10^{-8}gml^{-1}$ (Figures 17 & 18). These fragments apparently stimulated proliferation via an increase in cytosolic Ca^{2+} concentrations as Quin 2 studies revealed a rise in cytosolic Ca^{2+} concentration (Table 13).

Table 13 - Effect of PTH fragments on intracellular Ca^{++}concentration of thymic lymphocytes

Resting $[Ca^{2+}]_i$ levels (nm)	Treatment	Stimulated $[Ca^{2+}]_i$ levels (nm)
106 ± 7	PTH (1-34) 10 $ngml^{-1}$	205 ± 27
87 ± 8	PTH 8,18 Nleu (1-34) 10 $ngml^{-1}$	169 ± 13
98 ± 4	PTH 8,18 Nleu 34 Tyr (3-34) 100 $ngml^{-1}$	171 ± 6

The increase in cytosolic calcium content may be due to an enhanced Ca^{2+} influx (Table 14) and possibly due to a liberation of Ca^{2+} from sequestration sites within the organelle pools.

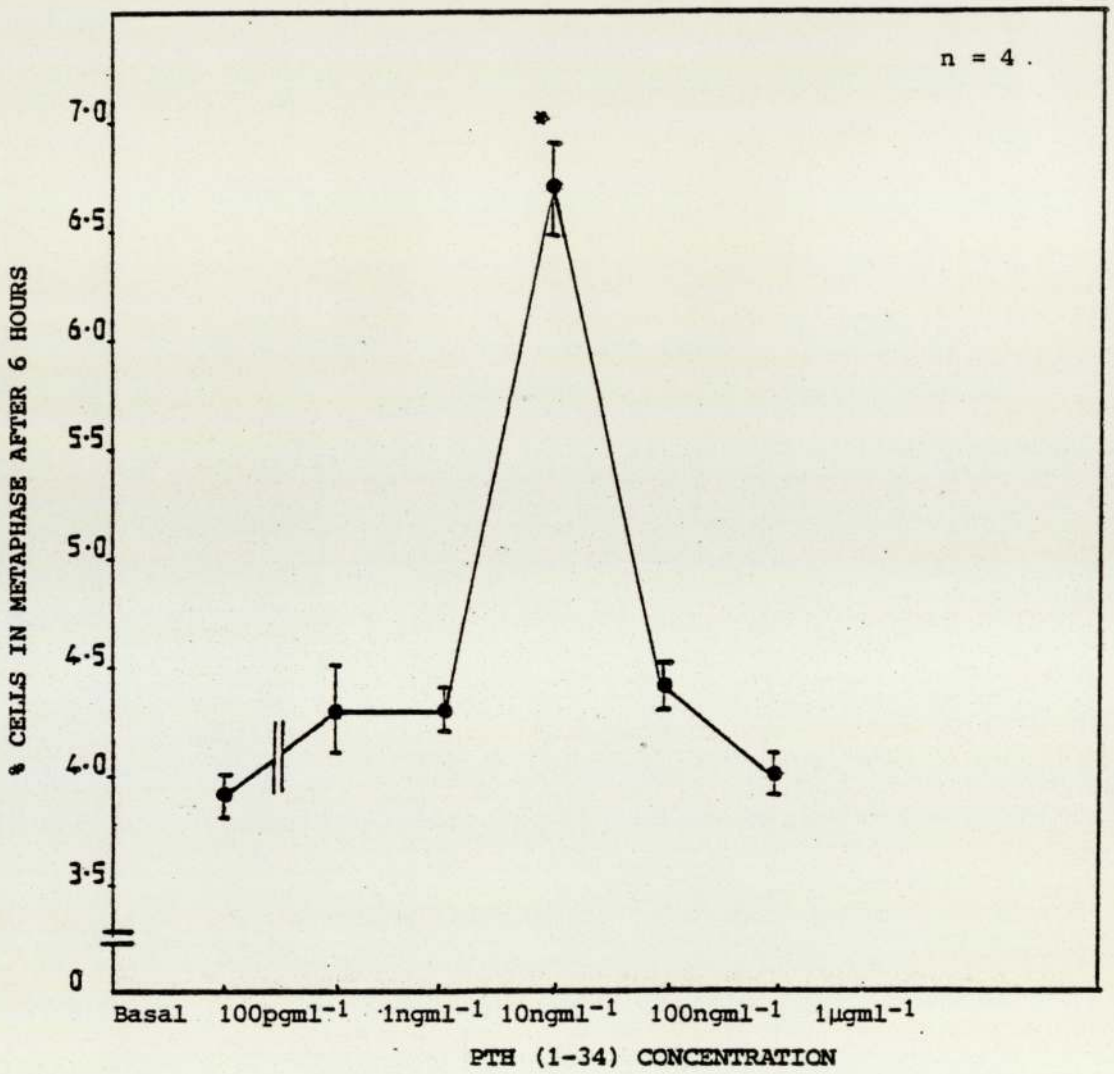


Figure 17 Effect of different concentrations of PTH (1-34) on mitotic activity of thymic lymphocytes in basal media.

Basal media contains 0.6mM Ca^{2+} and 1.0mM Mg^{2+}. Values significantly different from those of basal mitotic activity in the absence of any PTH (1-34) are indicated by asterisk.

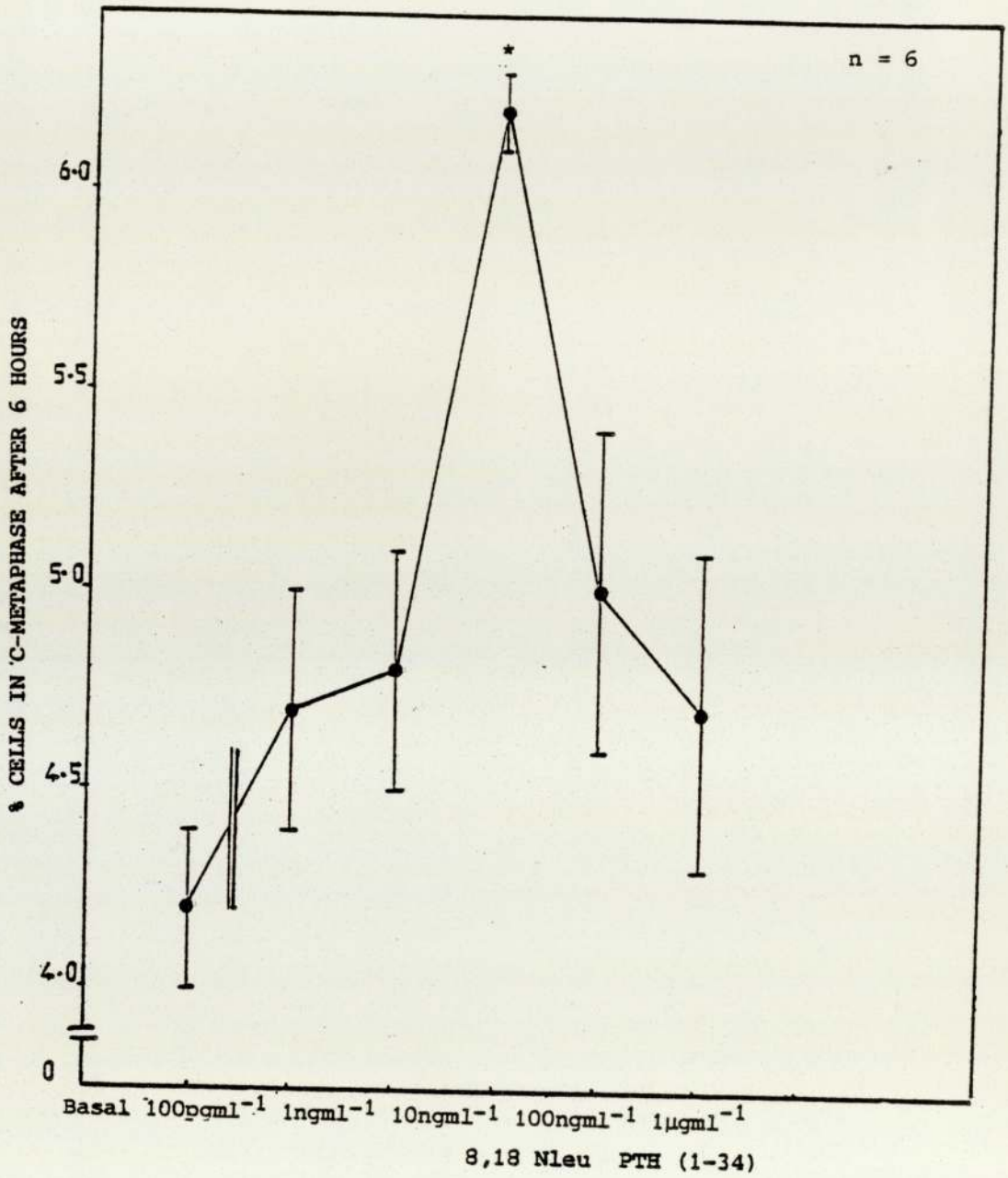


Figure 18 Effect of different concentrations of PTH 8,18 Nleu PTH (1-34) on mitosis of thymocytes in basal media.

Values significantly different from those of basal mitotic activity in the absence of any PTH are indicated by asterisk

Table 14 - The effect of PTH (1-34) on $^{45}Ca^{2+}$ influx in rat thymic lymphocytes in basal media

Treatment gml^{-1}	Ca^{2+} uptake $pm/10^6$ cells/20 mins
Basal	6.3
PTH (1-34) 10^{-7}	5.6
PTH (1-34) 10^{-8}	11.6
PTH (1-34) 10^{-9}	6.8

The PTH fragments indeed required extracellular Ca^{2+} to manifest their mitogenic potential but were not influenced by depletion of Mg^{2+} ions from the culture medium (Figures 19 and 20). This calcium dependency was further demonstrated by the dimunition of mitosis following application of the calcium antagonists, verapamil (5×10^{-6} gml^{-1}), nifedipine (10^{-5}M) and M&B40,678 (10^{-5}M) (Table 15). An inhibitory effect of mitosis was also observed on addition of the sex steroid, oestradiol (0.1 μgml^{-1}). Testosterone (0.1 μgml^{-1}) had no effect (Table 16). As the mitogenic effecs of PTH were further abrogated in the presence of the calmodulin inhibitors, TFP and M&B13 753 (Table 17), Ca^{2+} appears to be an important mediator of PTH actions via an activation of the regulatory protein, calmodulin.

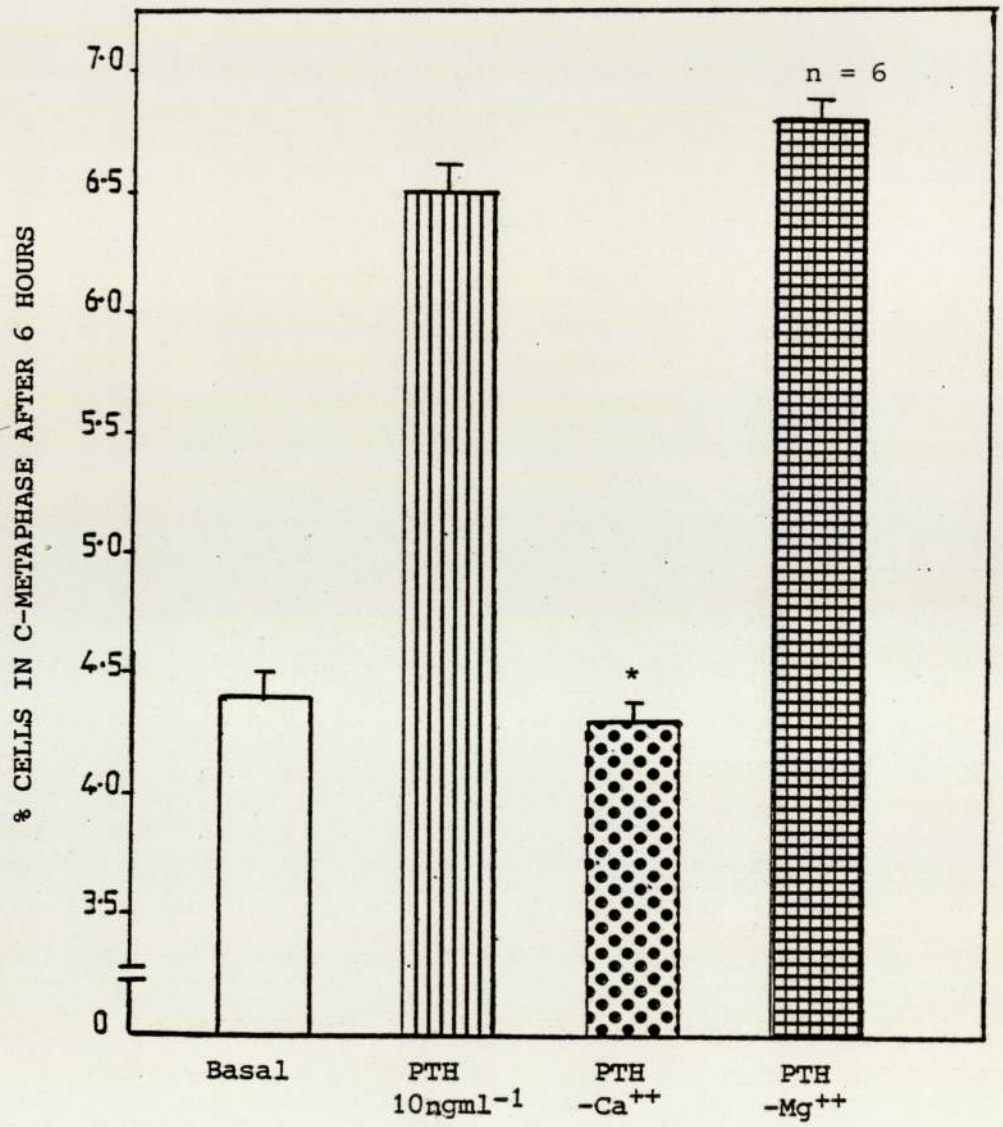


Figure 19 Ionic dependency of PTH (1-34)-induced mitosis.

PTH (1-34) at a concentration of $10_n gml^{-1}$ was included in 0.6 mM Ca^{++}, 1.0mM Mg^{2+} or in media where either of the divalent cations had been omitted. Values significantly different from those of basal cultures incubated with PTH are indicated by asterisk.

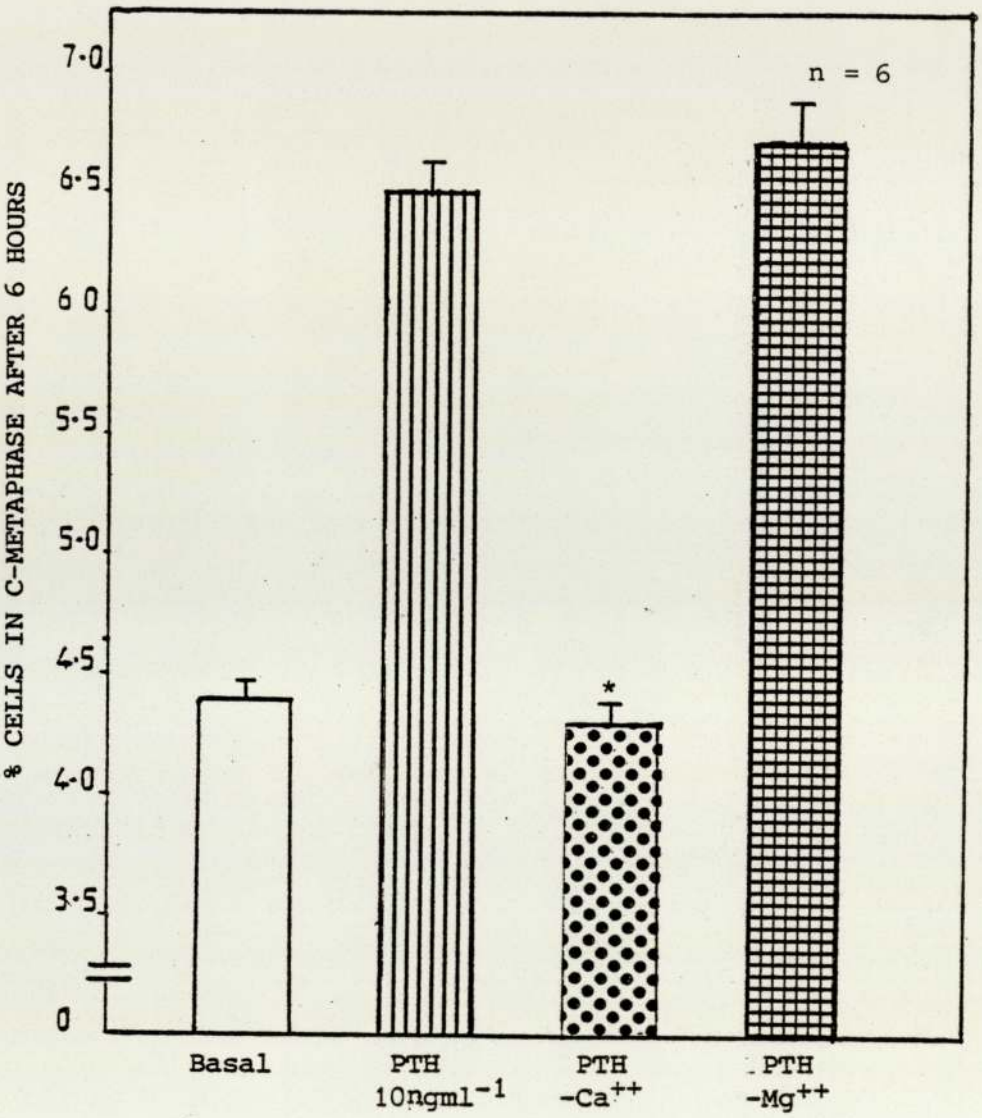


Figure 20 Ionic dependency of 8,18 Nleu PTH (1-34) induced mitosis.

PTH (10 $ngml^{-1}$) was included in 0.6mM Ca^{++}, 1.0 mM Mg^{2+} or in media where either calcium or magnesium had been omitted. Values significantly different from those of basal cultures incubated with PTH are indicated by asterisk.

Table 15 - The influence of the calcium antagonist on the PTH-induced mitotic response

Treatment	Percentage of cells in metaphase at 6 hrs ± sem		
	Control	PTH (1-34) 10^{-8} gml^{-1}	PTH 8,18 Nleu (1-34) 10^{-8} gml^{-1}
No addition	4.4 ± 0.2	6.5 ± 0.2	6.5 ± 0.2
Verapamil (5×10^{-6} gml^{-1})	4.0 ± 0.2	4.6 ± 0.3*	4.2 ± 0.2*
Nifedipine (10^{-5}M)	4.2 ± 0.3	4.6 ± 0.4*	4.6 ± 0.3*
M&B40,678 (10^{-5}M)	4.3 ± 0.2	4.5 ± 0.5*	4.5 ± 0.4*

* Significantly different from untreated PTH cultures

Table 16 - The influence of sex steroids on the PTH-induced mitotic response

Treatment	Percentage of cells in metaphase at 6 hrs ± sem		
	Control	PTH (1-34) 10^{-8} gml^{-1}	PTH 8,18 Nleu (1-34) 10^{-8} gml^{-1}
No addition	4.4 ± 0.2	6.5 ± 0.2	6.5 ± 0.2
Oestradiol 0.1 µg ml^{-1}	4.2 ± 0.1	4.6 ± 0.3*	4.4 ± 0.2*
Testosterone 0.1 µg ml^{-1}	4.1 ± 0.2	6.8 ± 0.2	6.6 ± 0.3

* Significantly different from untreated PTH cultures

Table 17 - The effect of calmodulin inhibition on PTH-induced mitosis

Treatment	Percentage of cells in metaphase at 6 hrs ± sem		
	Control	PTH (1-34) 10^{-8} gml^{-1}	PTH 8,18 Nleu (1-34) 10^{-8} gml^{-1}
No addition	4.4 ± 0.2	6.5 ± 0.2	6.5 ± 0.2
TFP 10^{-6}M	4.0 ± 0.1	4.7 ± 0.4*	4.5 ± 0.3*
M&B13753 10^{-5}M	4.1 ± 0.2	4.3 ± 0.3*	4.6 ± 0.2*

* Significantly different from untreated PTH cultures

A PTH analogue 8, 18 Nleu 34 Tyr b PTH (3-34) amide, an antagonist of cyclic AMP-mediated PTH action was further employed to verify the above results. Preliminary investigations indicated that it had agonistic properties, as it promoted proliferation at a concentration of 10^{-7}M (Figure 21). This agonistic effect again appeared to be Ca^{2+} dependent as a rise in cytosolic $[Ca^{2+}]_i$ levels was evident at this concentration (Table 18). These results suggest that the PTH action in thymocytes may be coupled to the calcium messenger system rather than the adenylate cyclase system. This was indeed substantiated by determination of adenylate cyclase activity in crude thymic lymphocyte membrane preparations. The basal levels were 155.2 pmoles cAMP/min/mg protein. Following addition of either isoproterenol (10 μm) or prostaglandin E2 (5 μm) the enzymatic activity was significantly increased. In marked contrast to the effect of the PTH fragments on mitosis and cytosolic calcium, none of the fragments had any effect on adenylate cyclase activity (Table 18).

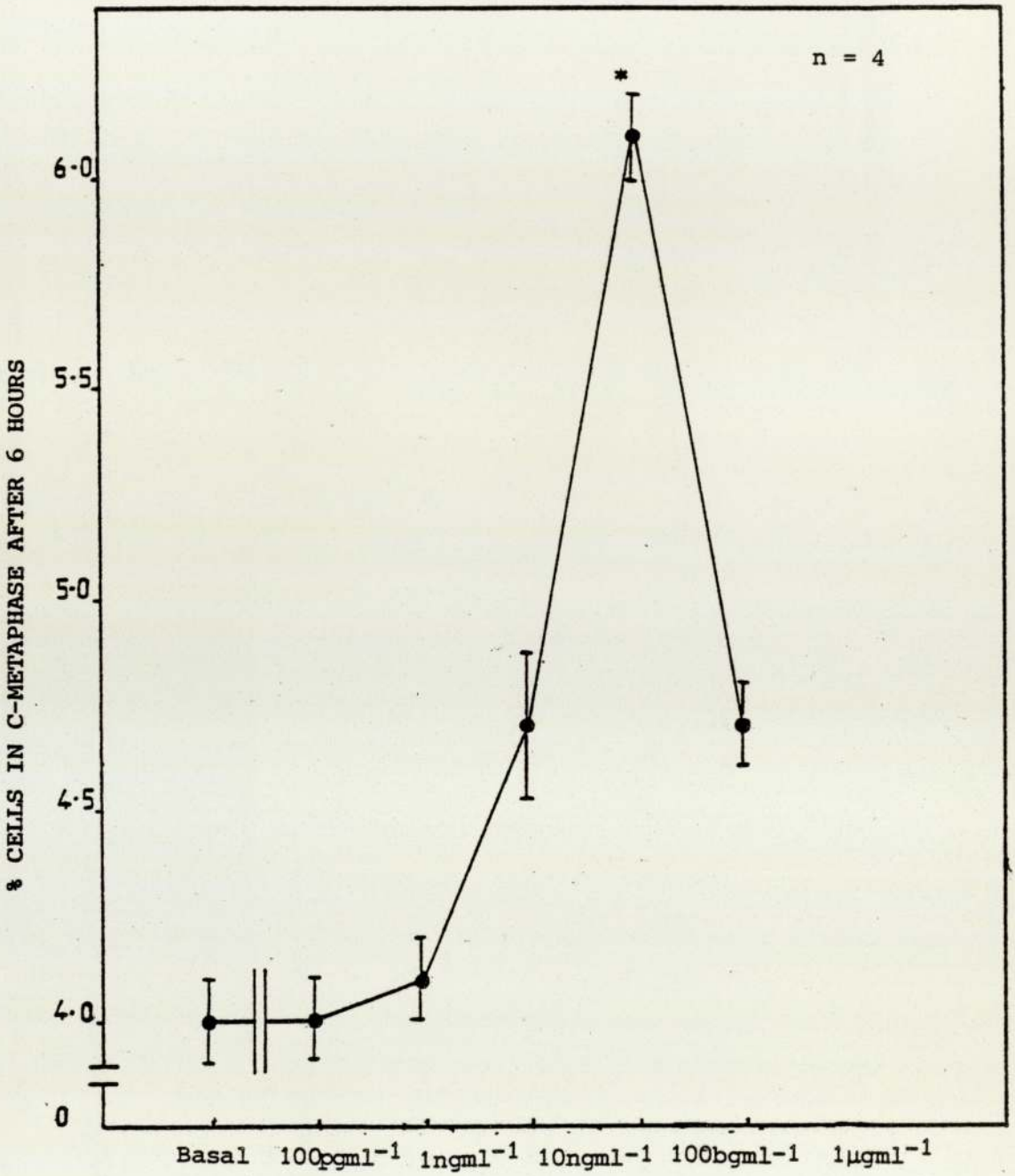


Figure 21 Effect of 8,18 Nleu 34 Tyr bPTH (3-34) on mitosis of thymic lymphocytes in basal media

Value significantly different from those of basal mitotic activity in the absence of any PTH are denoted by asterisk.

Table 18 - Adenylate cyclase activity of thymocyte membrane preparations

Treatment	pMol cAMP/min/mg protein
Control	155.2 ± 9.3
Isoproterenol (10 μm)	239.7 ± 22.7*
Prostaglandin E2 (5 μm)	328 ± 39.2*
PTH (1-34) 10^{-8} gml^{-1}	165.7 ± 10.5
8,18 Nleu PTH (1-34) 10^{-8} gml^{-1}	150.0 ± 10.9
8,18 Nleu 34 Tyr PTH (3-34) 10^{-7} gml^{-1}	173.0 ± 15.6

Furthermore, while cyclic AMP levels increased within five minutes of the addition of isoproteronol or PGE_2 in the a bsence of PDE inhibitors in whole basal cell preparations, PTH fragments at their respective stimulatory concentrations provoked a decline in intracellular cyclic AMP (results not shown). The ability of PTH to reduce cellular cAMP was abolished in the presence of trifluoperazine (10 μm) or 1-isobutyl 3-methylxanthine (100 μM). This response to PTH may be a consequence of calcium-mediated activation of calmodulin dependent cAMP phosphodiesterase. It is therefore evident that the mitogenic effects of PTH are attributed to the Ca^{2+} CaM messenger system and not to the adenylate cyclase system.

Although it appears that a relatively pure population of thymic lymphocytes is being recruited into the cell cycle, we cannot exclude the possibility that the mitogens discussed herewith rather than acting directly on these cells may be mediating their ultimate mitogenic effects via the release of IL2 by some activated T cells. IL2, thus released may cause the progression of other IL2-receptor bearing cells into the cell cycle. As Ca^{2+} ion flux appears to mediate the effects of

many hormone-receptor interactions including those discussed above, the role of Ca^{2+} ions in the stimulation of T-cell proliferation by IL2 was investigated.

A partially purified rat IL2 preparation essentially free of lectin and culture additives (NIBSC) markedly enhanced mitosis in rat thymocytes in basal medium. The maximal response was evident at 1:10 dilution of the stock preparation. In Ca^{2+}-free medium, the proliferative response was eliminated (Table 19). The same concentration of rat IL2 also stimulated cell division in Con A blasts and the IL2-dependent cytotoxic lymphocyte cell line, CTLD. In these cell types, chelationof Ca^{2+} to below 5×10^{-6}M by addition of 1 mM EGTA abolished the mitogenic activity (Table 19). When normal Ca^{2+} concentrations were re-established by addition of $CaCl_2$ (final concentration 0.9 mM), the proliferative response to IL2 was restored in both cell types, the minimum concentration required for normal cell proliferation being 0.7 mM $CaCl_2$. Exposure to calcium-channel blockers, verapamil and nifedipine also abolished the mitotic response to IL2 (Table 20). It is hence reasonable to suggest that IL2 on interaction with its receptor leads to an influx of extracellular calcium which is essential for mitosis.

Experiments with the Ca^{2+}-sensitive fluorescent probe, Quin 2 showed that IL2 increased cytosolic calcium in both CTLD cells and thymocytes (Figure 22). In the absence of calcium no such elevation was observed after IL2 treatment. Various calmodulin antagonists also inhibited IL2 induced mitogenesis (Table 21) suggesting that the Ca^{2+} signal is further transduced by the calmodulin molecule.

Table 19 - The effect of calcium depletion on the proliferative response to IL2

Treatment	Proliferation [^{3}H] thymidine uptake (cpm) ± sem		% thymocytes in C-metaphase after 6 hours ± sem
	Blasts	CTLD	
Basal	380 ± 143*	307 ± 11*	3.1 ± 0.2*
IL2	7842 ± 890	11361 ± 265	5.9 ± 0.3*
IL2 low Ca^{2+a}	207 ± 38*	334 ± 61*	3.0 ± 0.2*

a - calcium concentration was below 5 x 10^{-6}M; * significantly different from the proliferative activity induced by IL2 treatment in normal media. For further details, refer to Appendices (A) and (B).

Table 20 - The effect of calcium channel blockers on the proliferative response to IL2

Treatment	Proliferation [^{3}H] thymidine uptake (cpm) ± sem		% thymocytes in C-metaphase after 6 hours ± sem
	Blasts	CTLD	
Basal	1216 ± 111	408 ± 21	4.0 ± 0.3
IL2	44355 ± 1209	23562 ± 532	5.8 ± 0.2
Verapamil[a]	1130 ± 197	388 ± 31	3.9 ± 0.2
Nifedipine[a]	1686 ± 88	512 ± 54	ND
Verapamil[a] + IL2	1509 ± 92*	4150 ± 1336*	4.1 ± 0*
Nifedipine[a] + IL2	18754 ± 691*	3594 ± 691*	ND

ND = not determined; a = 10^{-5} gml^{-1}; * values significantly different from untreated IL2 cultures

Table 21 - The effects of calcium antagonists on the proliferative response to IL2

Treatment	Proliferation [^{3}H] thymidine uptake (cpm) ± sem		% thymocytes in C-metaphase after 6 hours ± sem
	Blasts	CTLD	
Basal	1351 ± 87	491 ± 12	2.3 ± 0.2
IL2	38045 ± 4278	17133 ± 1419	4.8 ± 0.3
TFP[a]	1120 ± 131[b]	320 ± 59	2.1 ± 0.2
M&B 13753[b]	ND	360 ± 101	2.4 ± 0.2
TFP + IL2	1267 ± 29*	484 ± 47*	2.3 ± 0.2*
M&B13753 ± IL2	ND	398 ± 20*	2.5 ± 0.2*

ND = not determined; a = 10^{-6}M; b = 10^{-5}M; * values significantly different from untreated IL2 cultures

Table 22 - The effect of recombinant Interleukin-2 on intracellular Ca^{2+} concentration in thymocytes

Resting $[Ca^{2+}]_i$ levels (nM)	Medium cation concentration (mM)		Treatment (M)	Stimulated $[Ca^{2+}]_i$ levels (nM)
	Ca^{2+}	Mg^{2+}		
160 ± 14	0.6	1.0	IL2 (3×10^{-9})	348 ± 37
119 ± 3	0	1.0	IL2 (3×10^{-9})	138 ± 18
135 ± 5	0.6	0	IL2 (3×10^{-9})	252 ± 25
150 ± 21	0.6	1.0	IL2 (3×10^{-11})	205 ± 51

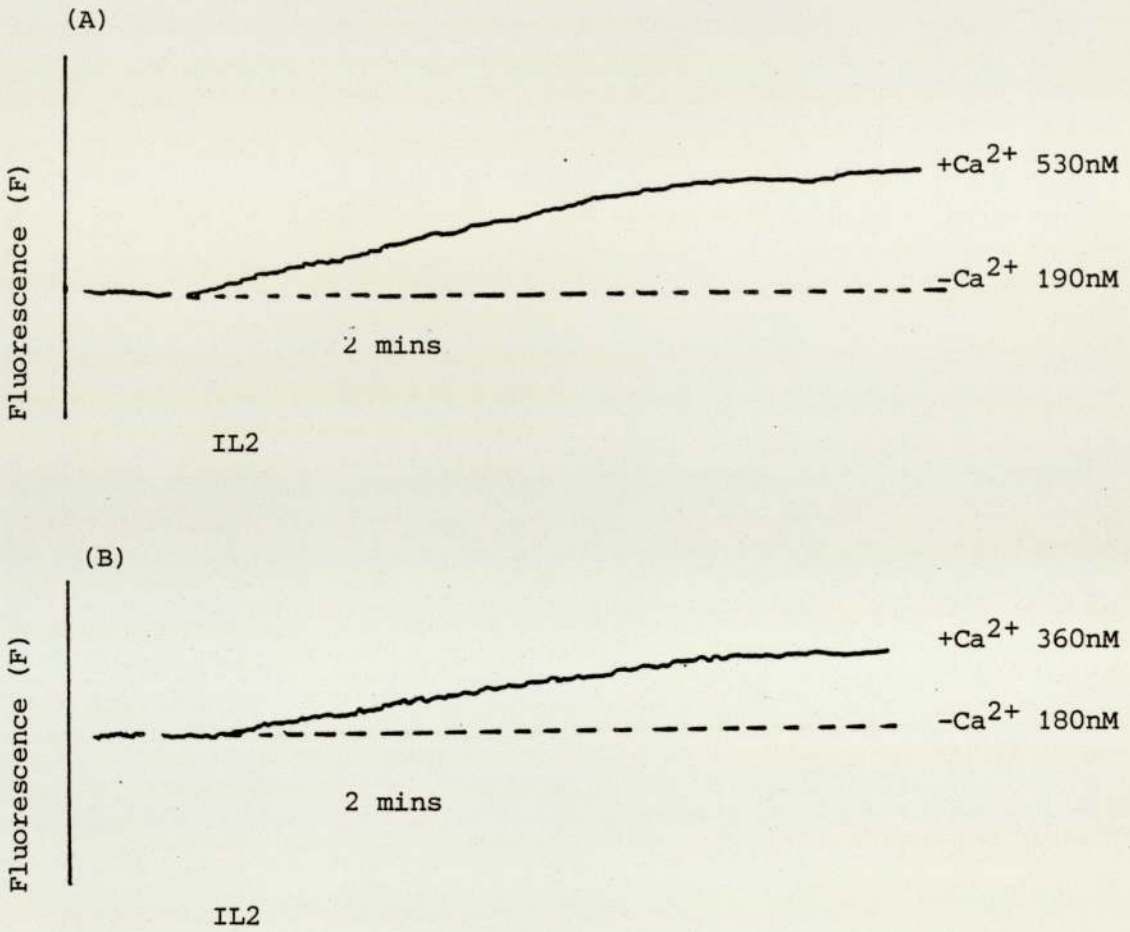


Figure 22 The effect of IL2 addition on the fluorescence at 492nM of Quin 2 loaded CTLD cells (A), and thymocytes (B), excited at 339nM

The fluorescence values correspond to resting calcium levels of 190 nM (A), and 180 nM (B), and stimulated levels of 530 nM (A), and 360nM (B). No increase was seen in calcium-free medium ($-Ca^{2+}$).

Table 23 - The effect of calcium antagonists on intracellular calcium concentration in IL2-stimulated thymocytes in basal media

Resting $[Ca^{2+}]_i$ levels (nM)	Treatment	Stimulated $[Ca^{2+}]_i$ levels (nM)
166 ± 14	r IL2	323 ± 31
171 ± 15	r IL2 + Verapamil[a]	199 ± 23
196 ± 15	r IL2 + M&B40,678[b]	254 ± 23

The concentration of r IL2 employed was 3×10^{-9}M; a = 5×10^{-6} gml^{-1}, b = 10^{-5}M

To combat the possibility that mitosis in the above experiments may be due to unknown contaminating mitogenic factors in the partially purified rat IL2 preparation, these proliferation experiments were repeated when human recombinant IL2 became available.

The recombinant IL2 (NIBSC) certainly stimulated cell division. A sigmoid-dose response curve was evident with maximal proliferation at 6×10^{-10}M IL2. This curve correlated well with the dose-response obtained for the IL2 dependent CTLL cell line (Figure 23). The responses to such low doses of IL2 suggest that both cell types bear high affinity receptors for this growth factor.

Further experiments were performed to determine if the proliferative effects of IL2 were indeed attributable to the calcium-messenger system. Once again, the enhanced division proved to be calcium dependent (Figure 24) inhibitable by calcium antagonists (Figures 25 & 26), prevented by calmodulin antagonists (Figure 27) and oestradiol (Figure 28). The Ca^{2+} dependent nature was also substantiated by the rise in intracellular free calcium concentration subsequent to IL2 treatment and the negligible increase in the absence of extracellular Ca^{2+} or in the presence of the antagonists (Tables 22 & 23). Thus, it is clear that IL2 elicits

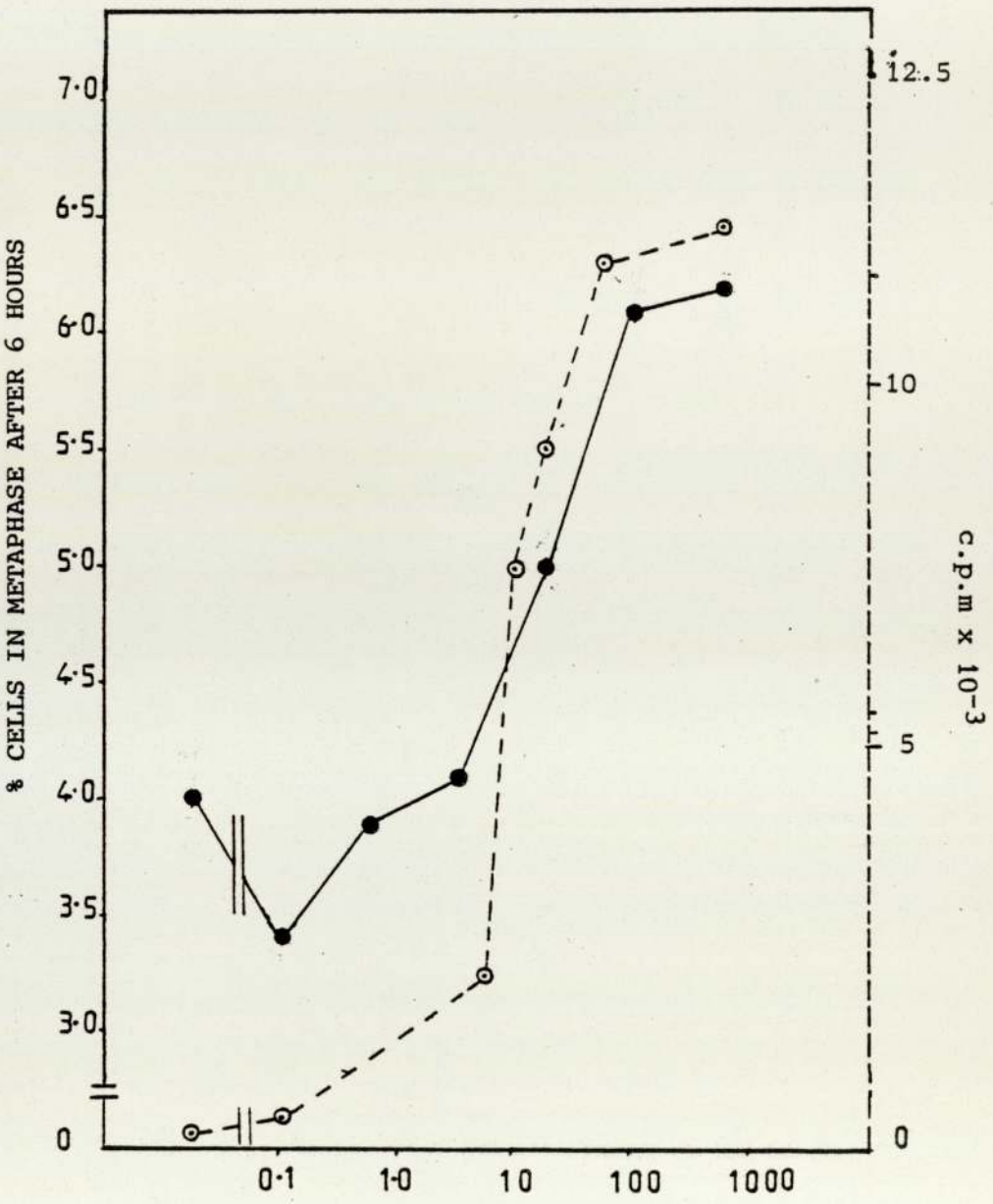


Figure 23 A comparison of the effects of rIL2 on the proliferation of rat thymocytes <u>in vitro</u>, expressed as a percentage of colchicine metaphases accumulated over 6h in culture (●) and on the IL2-dependent mouse CTLL line, expressed as cpm x 10^{-3} of [^{3}H] thymidine incorporated over 24h in culture (⊙). For further details, refer to Appendix (G).

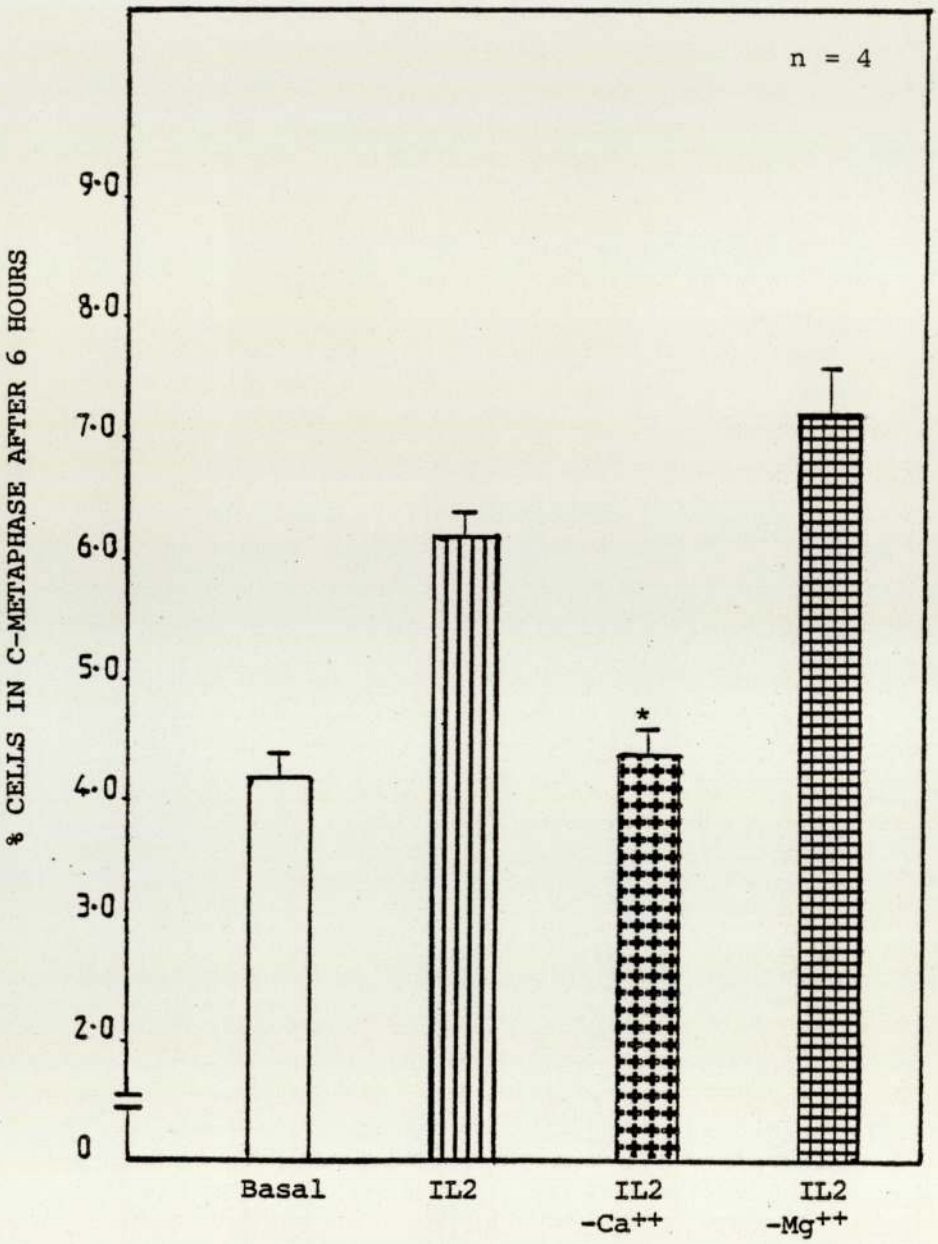


Figure 24 Ionic dependency of recombinant IL2-induced mitosis.

IL2 (3×10^{-9}M) was included in basal, calcium free ($-Ca^{++}$) and magnesium-free ($-Mg^{++}$) media. Values significantly different from those of corresponding cultures incubated in basal conditions are indicated by asterisk.

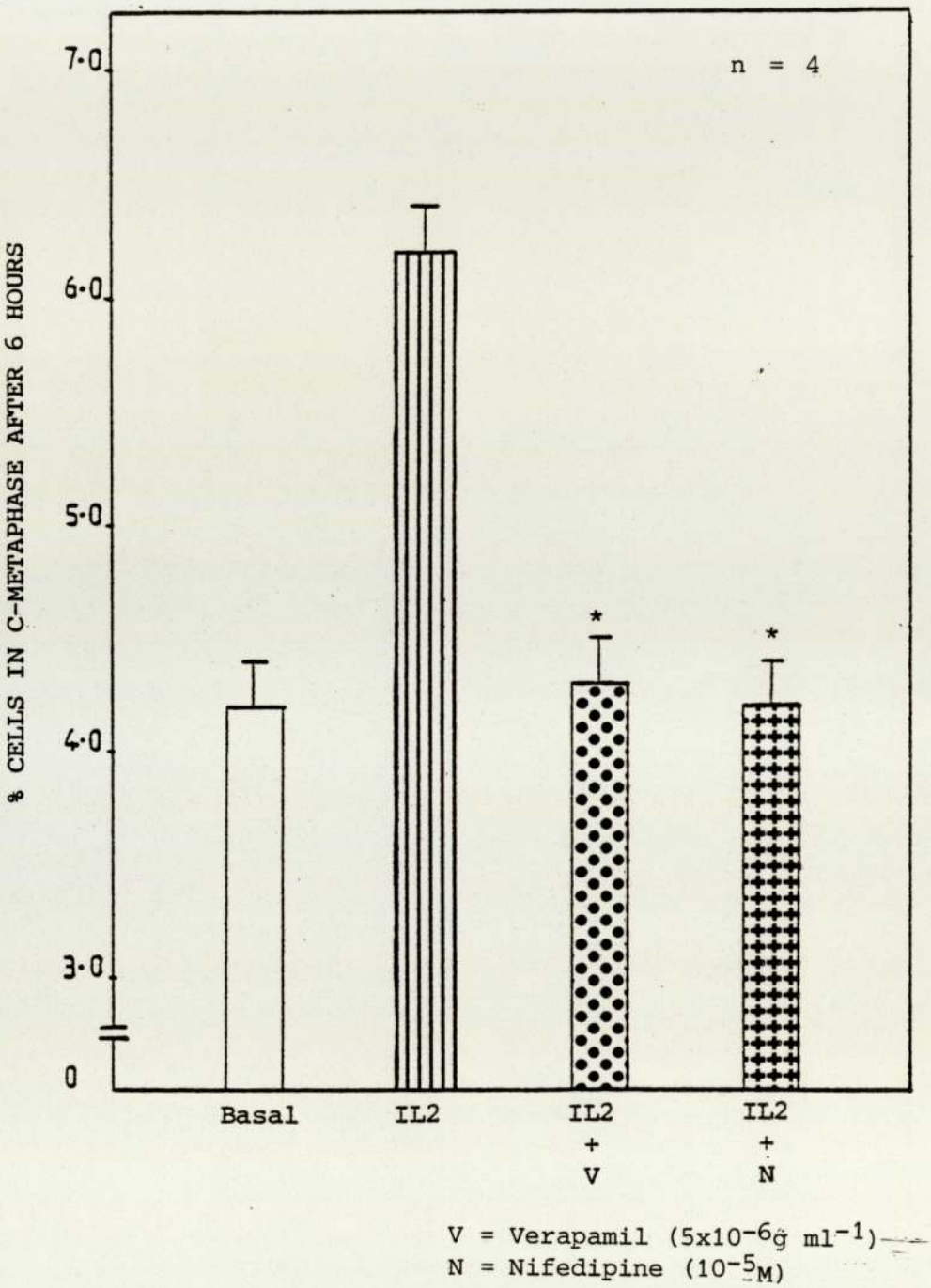


Figure 25 Effect of calcium-channel blockade on interleukin-2 induced mitogenesis

The concentration of rIL2 was 3 x 10^{-9}M. Values significantly different from corresponding cultures without any antagonists are indicated by asterisk.

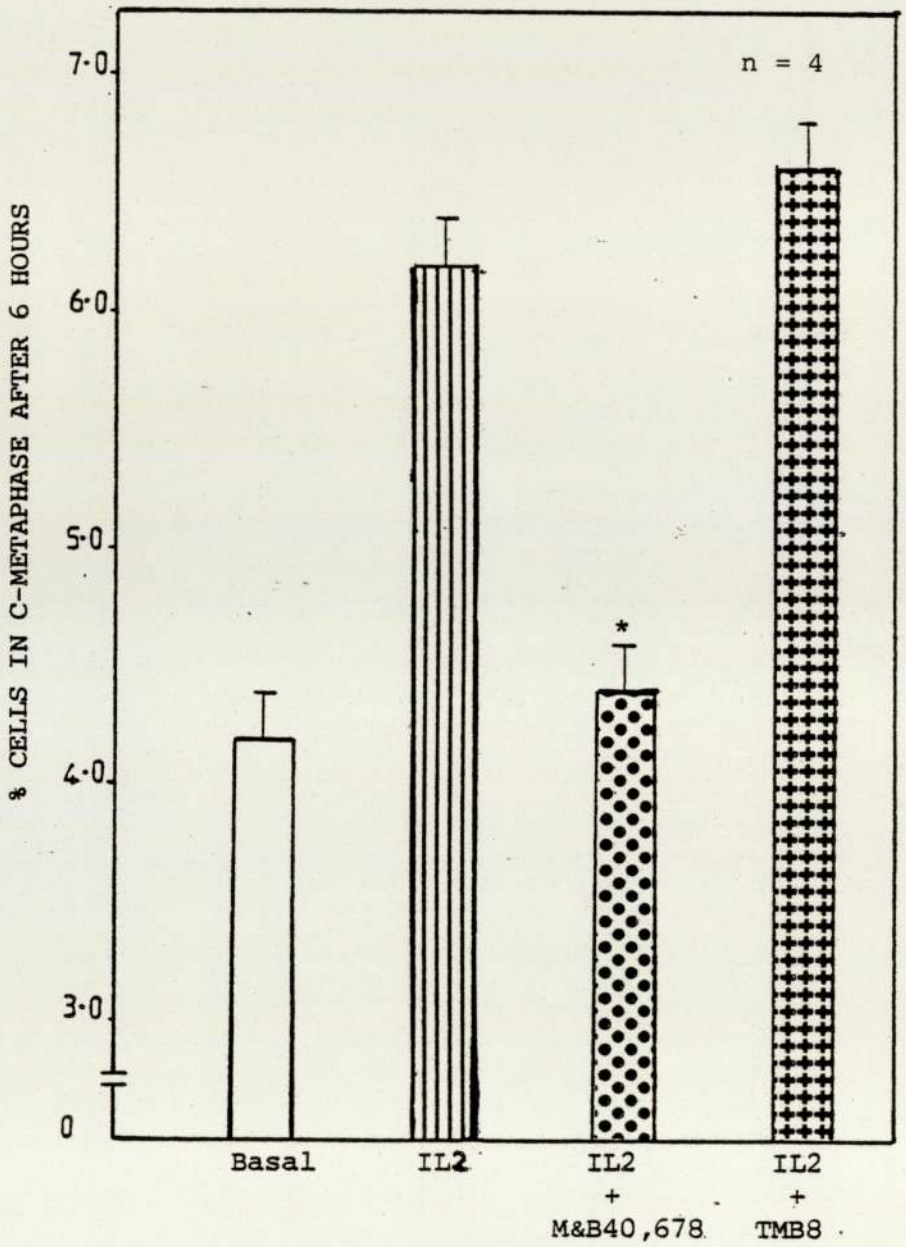


Figure 26 Effect of the putative mobilisation blockers on Interleukin-2-induced mitogenesis.

The mobilisation blockers were used at a concentration of 10^{-5}M. Other details as in Figure 25

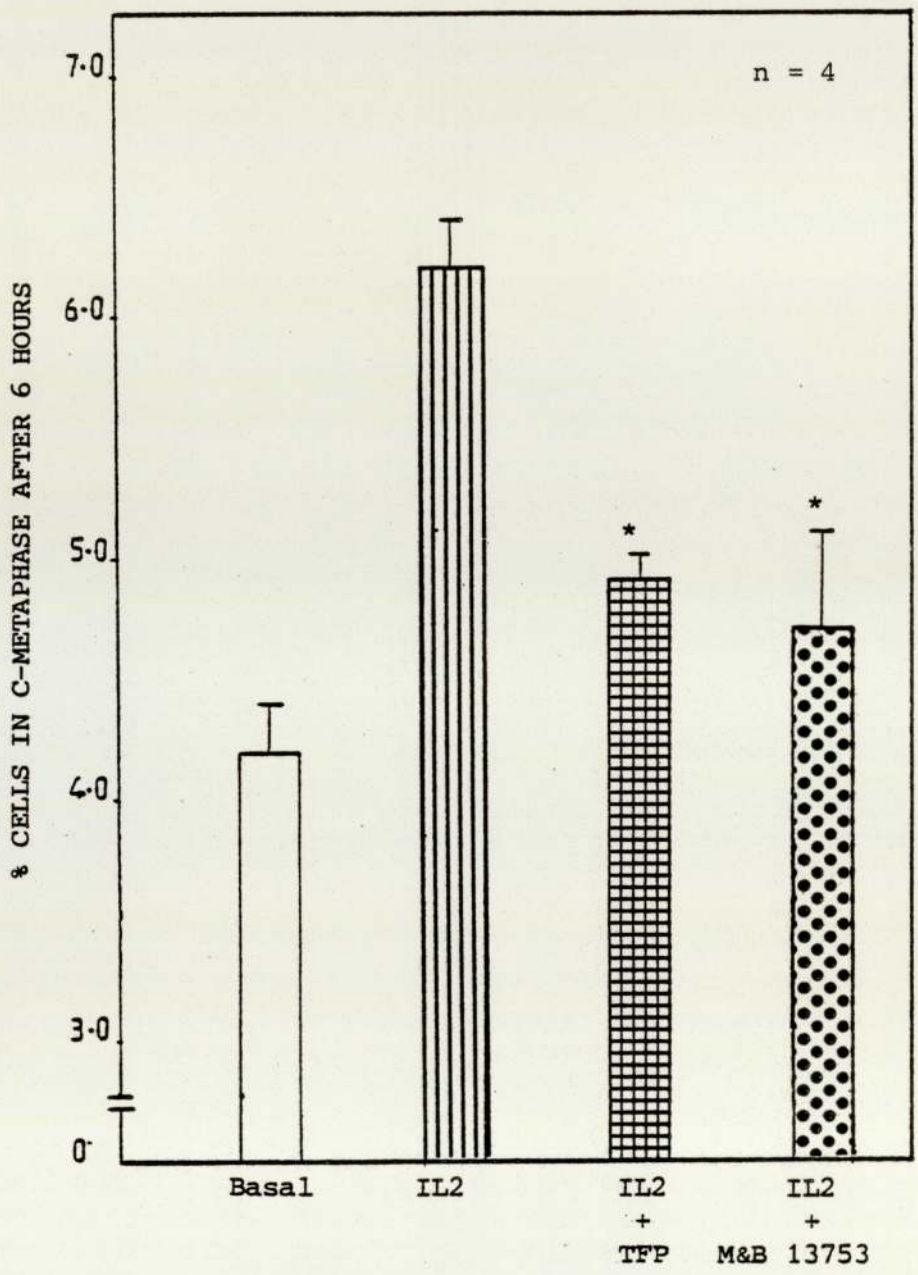


Figure 27 Effect of calmodulin inhibition on interleukin-2 induced mitotic activity

The concentration of the calmodulin inhibitors was 10^{-5} M. Statistical details as in Figure 26

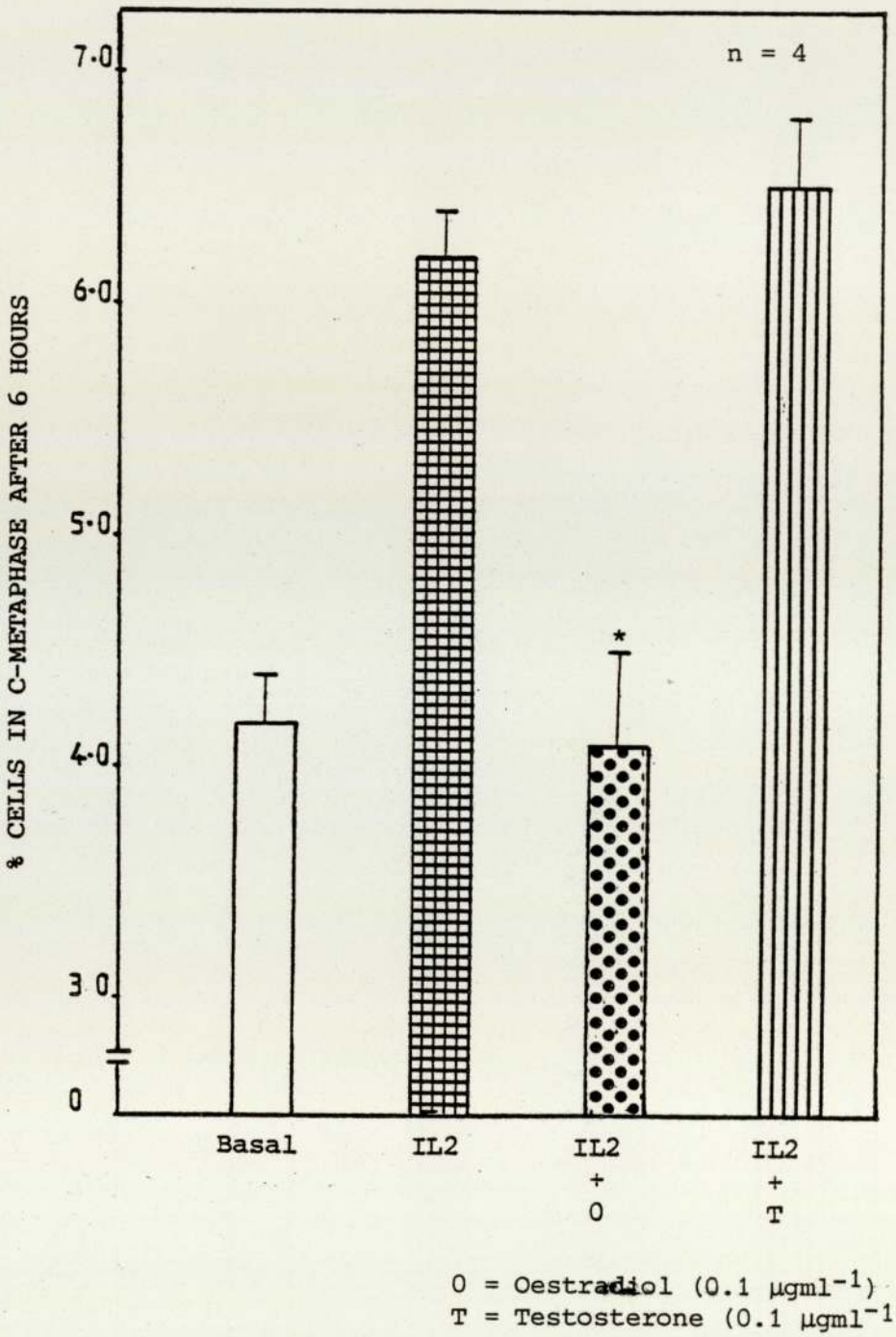


Figure 28 Sex-steroid blockade of Interleukin-2-induced mitogenesis

The concentration of rIL2 employed was 3×10^{-9}M. Values significantly different from those of corresponding cultures without sex steroids are indicated by asterisk.

responses via the various Ca^{2+} signalling mechanisms.

As thymocytes usually undergo DNA synthesis in less than one hour after mitogenic stimulation (Whitfield et al, 1974), it was crucial to determine the critical period of time which elapses between addition of IL2 and commitment to the division cycle. After an initial incubation period of 5, 10, 20 and 30 minutes, the IL2 treated cells were exposed to antimitotic agents such as verapamil and TFP. The cells were incubated further for 6 hours and the mitotic activity assessed in a usual manner. Since the mitogenic response of IL2 treated cultures was not masked by addition of verapamil after the initial five minutes and of TFP after twenty minutes (Figure 29), it seems logical to presume that the triggering mechanism for mitotic events is initiated within the initial ten minutes of IL2 application. Such rapid responses to IL2 suggest that in the rat thymus, IL2-receptors bearing cells are already present.

As IL2-receptor expression has been reported to be higher in fetal and newborn animals in comparison to adult animals, the response of 20-day foetal, newborn and aged thymocytes to IL2 was therefore examined. In Lou rats, there appears to be a progressive decline in the basal mitotic activity of thymic lymphocytes with age as illustrated in Figure 30. Nevertheless, exposure to concentrations of calcium or IL2 which were previously found to be mitogenic in cultured thymocytes of young adult animals stimulated cell division in isolated thymocytes at each of the ages tested; the response was greatest in new-born animals.

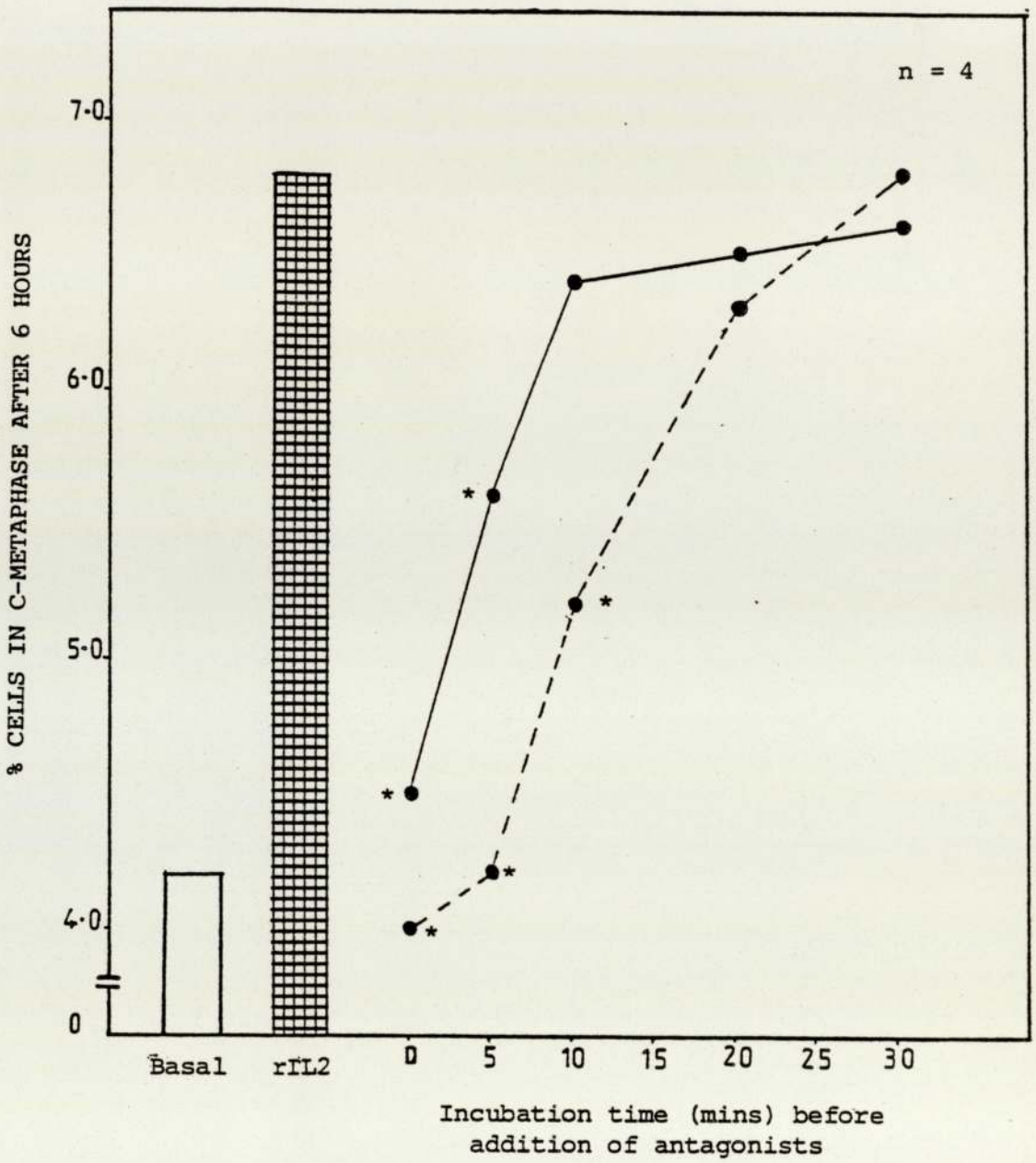


Figure 29 Determination of the activation period for rIL2 induced mitosis thymocytes.

The antimitotic agents verapamil ●—● (5×10^{-6}g ml^{-1}) and trifluoperazine ●---● (10^{-5}M) were added to cultures containing rIL2 (3×10^{-9}) at different times during the initial thirty minutes of the 6 hour incubation period.

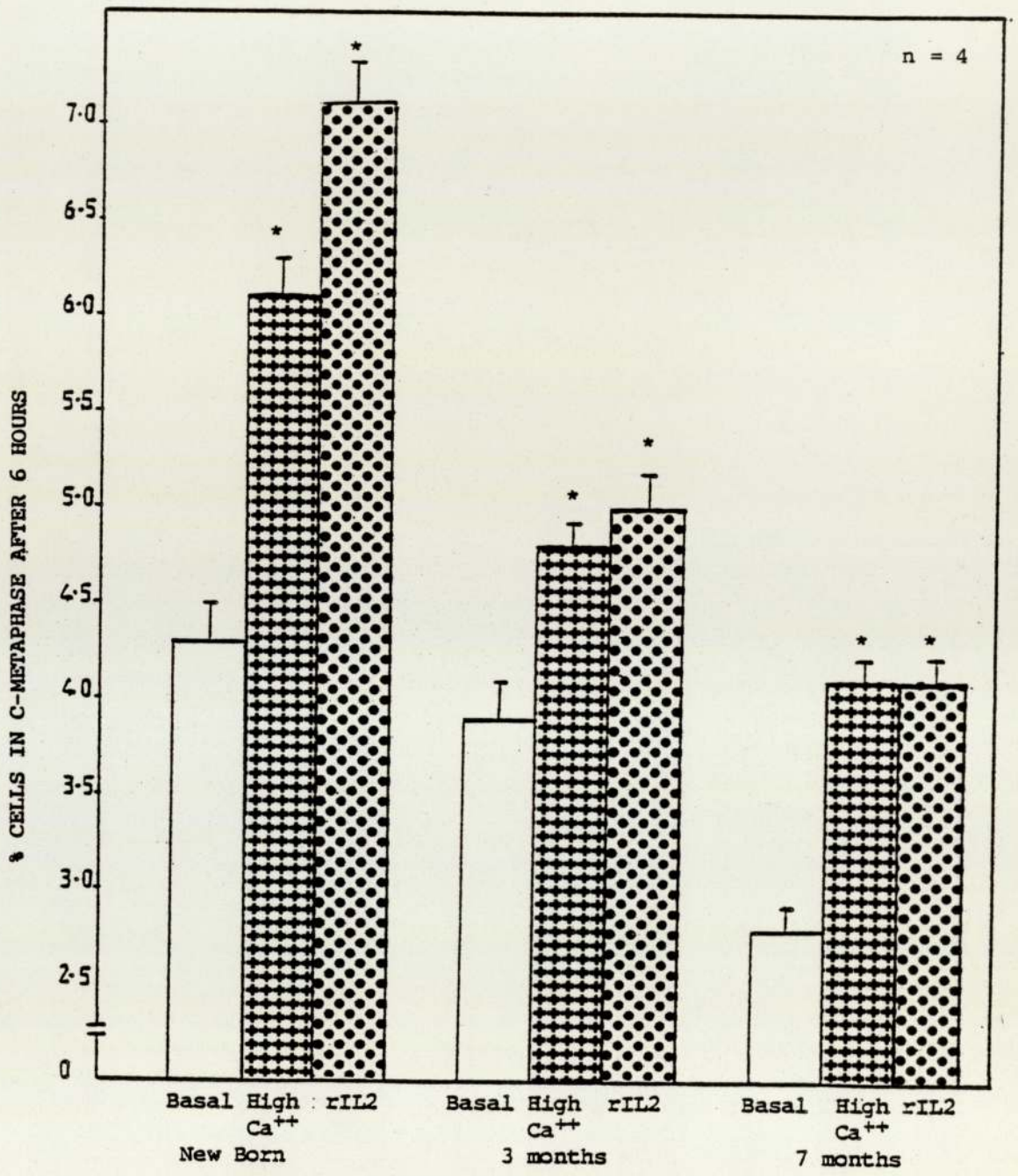


Figure 30 A comparison of the effects of rIL2 (3×10^{-9}m) on the <u>in vitro</u> proliferation of thymocytes from 3-day (new born), 3 month, and 7 month-old Lou rats in basal media.

Basal media contains 0.6 mM Ca^{++}; 1.0 mM Mg^{++}. High Ca^{++} represents media containing 1.8 mM Ca^{++}. Values significantly different from their respective basals are indicated by asterisk.

Table 24 - The effect of r IL2 on the mitotic activity of thymocytes isolated from foetal and adult animals

Treatment	% thymocytes in C-metaphase after 6 hours	
	Foetal (20-d)	Adult (6 weeks old)
Basal	10.6 ± 0.8	4.3 ± 0.2
IL2 300 nM	14.8 + 1.0*	ND
IL2 3 nM	11.7 ± 1.2	6.3 ± 0.3*
IL2 30 pM	8.8 ± 1.4	4.3 ± 0.2

* Significantly different from basal cultures

For proliferative studies in foetal thymocytes, thymus glands were obtained from animals of Wistar strain. After 20 days of gestation, foetuses were removed from the uterus and thymus glands excised after cervical dislocation. The thymuses were rinsed and the cell suspension incubated for six hours in the usual manner. The basal mitotic activity in the foetal cells was apparently twice that in the adult. Recombinant IL2 at 3×10^{-7}M and 3×10^{-9}M once again proved to be mitogenic, albeit, the magnitude of the mitotic response seemed to be similar in both foetal and adult thymocytes (Table 24). Clearly, rat thymic lymphocytes isolated from animals of different ages will respond to IL2.

Since IL2 is the natural or physiological growth factor to lymphoid cells, the mitogenic properties of the various agents described above may possibly be ascribed to the release of this lymphokine. In an attempt to test this premise, an immunosuppressive drug, cyclosporin A, CsA (Sandoz Ltd) was employed. CsA is considered to impair IL2 production and IL2 receptor expression (Larsson, 1980; Bunjes et al, 1981; Dos Reis & Shevach, 1982) and should therefore abrogate the mitogenic effects of hormones and other agents if these actions are

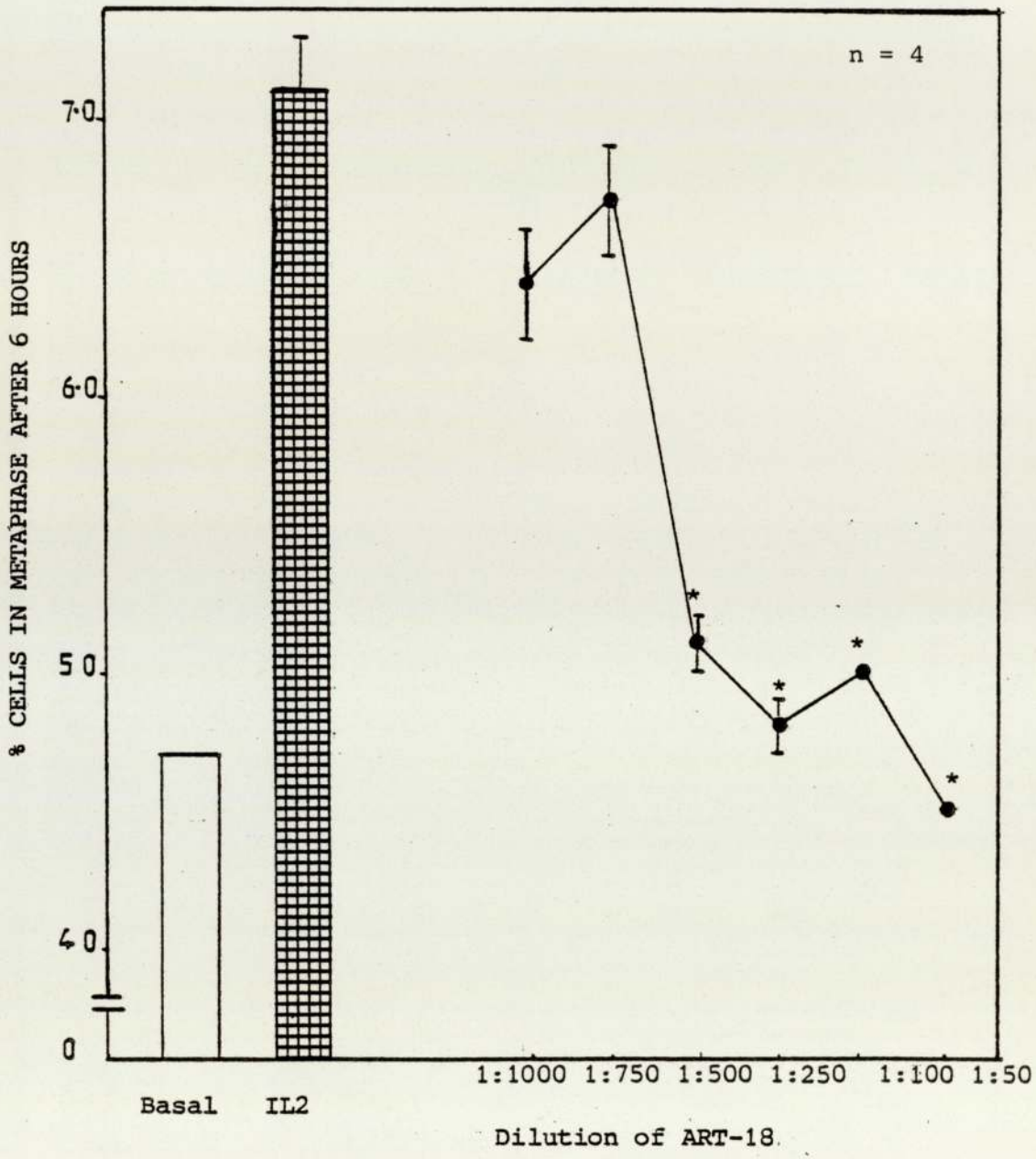


Figure 31 Effect of the different concentrations of the rat anti-IL2 receptor antibody, ART-18 on r IL2 induced mitosis of rat thymocytes in basal media.

Values significantly different from the corresponding cultures without antibody are denoted by asterisk.

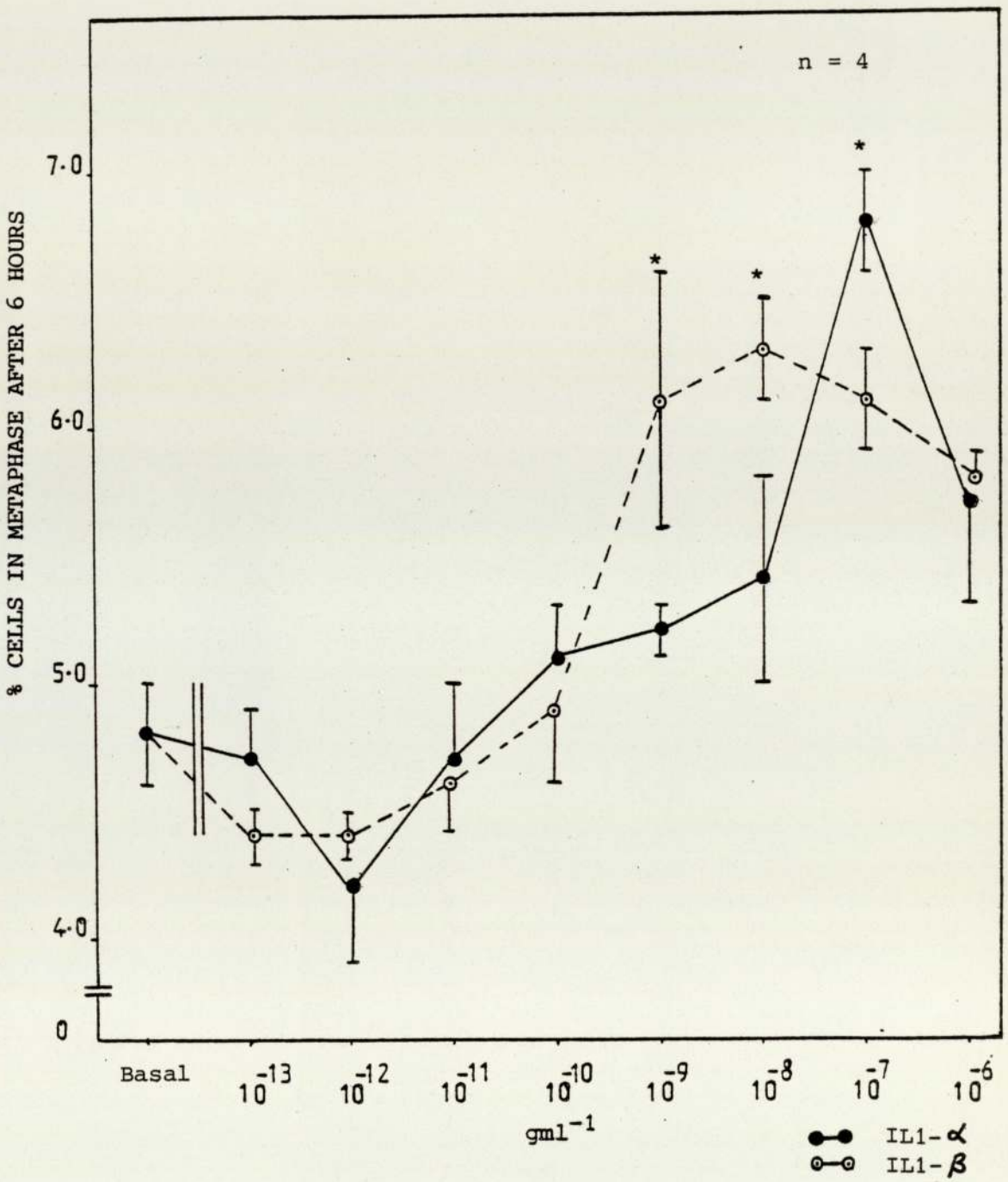


Figure 32 Effect of the forms of Interleukin-1, IL1- α and IL1-β on mitotic activity of thymocytes in basal media.

Values significantly different from cultures free of Interleukin-1 are denoted by asterisk.

mediated by IL2 release. Initial experiments revealed that CsA at a non-toxic concentration of 50 ng ml^{-1} did indeed prevent the mitogenic propensity of all agents tested (results not shown). Recently, however other workers have indicated that CsA has adverse effects on nuclear morphology and metabolism and compromises calmodulin action (Colombani et al, 1985; Simons et al, 1986). Thus, it cannot be concluded with certainty that the range of mitogens tested exerted their effects via IL2.

A highly specific monoclonal anti-IL2 receptor antibody ART-18 (Diamantstein, T; Berlin) was therefore employed to further investigate the issue of an IL2 mediated effect. As expected ART-18 clearly blocked the proliferogenic response of IL2. Such inhibition occurred when thymocytes were treated with 1:50 to 1:500 dilution of the stock antibody thirty minutes prior to the 6-hr incubation with IL2 (Figure 31). ART-18 which itself had no affect on basal proliferation did not influence the stimulatory response of other mitogens (Table 25). This suggests that the mitogenic factors tested do not induce cell division via an IL2 release.

Many workers believe that the mitogenic effects of IL1 are indirectly related to IL2 production and receptor expression. If this is so, ART-18 should block the mitogenic propensity of IL1. Both forms of recombinant IL1, IL1α and IL1ß (NIBSC) enhanced cell division; IL1ß was effective at slightly lower concentrations than IL1α (Figure 32). Surprisingly ART-18 did not abolish the proliferation induced by either of the IL1 forms (Table 25).

Table 25 - Effect of the anti IL2 receptor antibody, ART-18 on the proliferative activity of various mitogens in thymic lymphocytes

Treatment	% Cells in metaphase after 6 hrs	
	No addition	ART-18 1:500
Control	5.0 ± 0.2	5.6 ± 0.4
High Ca	7.3 ± 0.2	7.1 ± 0.3
High Mg	7.2 ± 0.2	7.3 ± 0.1
r IL2 3×10^{-9}M	7.3 ± 0.2	5.5 ± 0.2*
PTH (1-34) 10^{-8} gml^{-1}	7.1 ± 0.3	7.4 ± 0.2
Isoprenaline 10^{-6}M	7.3 ± 0.3	7.3 ± 0.2
r IL1 α 10^{-7} gml^{-1}	7.8 ± 0.2	7.5 ± 0.1
r IL1 ß 10^{-8} gml^{-1}	7.8 ± 0.2	7.4 ± 0.2

* Significantly different from the culture devoid of the antibody

We must consider that either IL1 is directly mitogenic in these cells or that IL1 induced IL2 production is sufficient to displace the antibody from the receptor thus allowing all activation to proceed.

As all the mitogens discussed thus far seem to stimulate the same small percentage of cells to divide, it seems likely that they are acting upon the same sub-population of thymocytes. Indeed, when IL2-treated thymic lymphocytes were incubated for 6 hours along with mitogenic concentrations of calcium or magnesium no additive or synergistic effect was observed (Table 26). Similarly, when thymocytes were exposed to a combination of a mitogenic (3×10^{-9}M) or sub-mitogenic concentration of IL2 (3×10^{-11}M) and two different mitogenic concentrations of ACh, no synergy was observed. In fact, when a high concentration (10^{-5}M) of ACh was used, the combination of neurotransmitter and interleukin appeared inhibitory (Table 27).

Table 26 - Effect of r IL2 on high Ca^{++} and high Mg^{++} induced mitosis

Treatment	% cells in C-metaphase after 6 hrs	
	Control	r IL2 (3×10^{-9}M)
Control	4.2 ± 0.2	6.6 ± 0.2
High Ca^{++}	6.4 ± 0.3	6.6 ± 0.3
High Mg^{++}	6.6 ± 0.4	6.7 ± 0.3

High Ca^{++} and high Mg^{++} denote 1.8 mM Ca^{++} and 2.5 mM Mg^{++} respectively

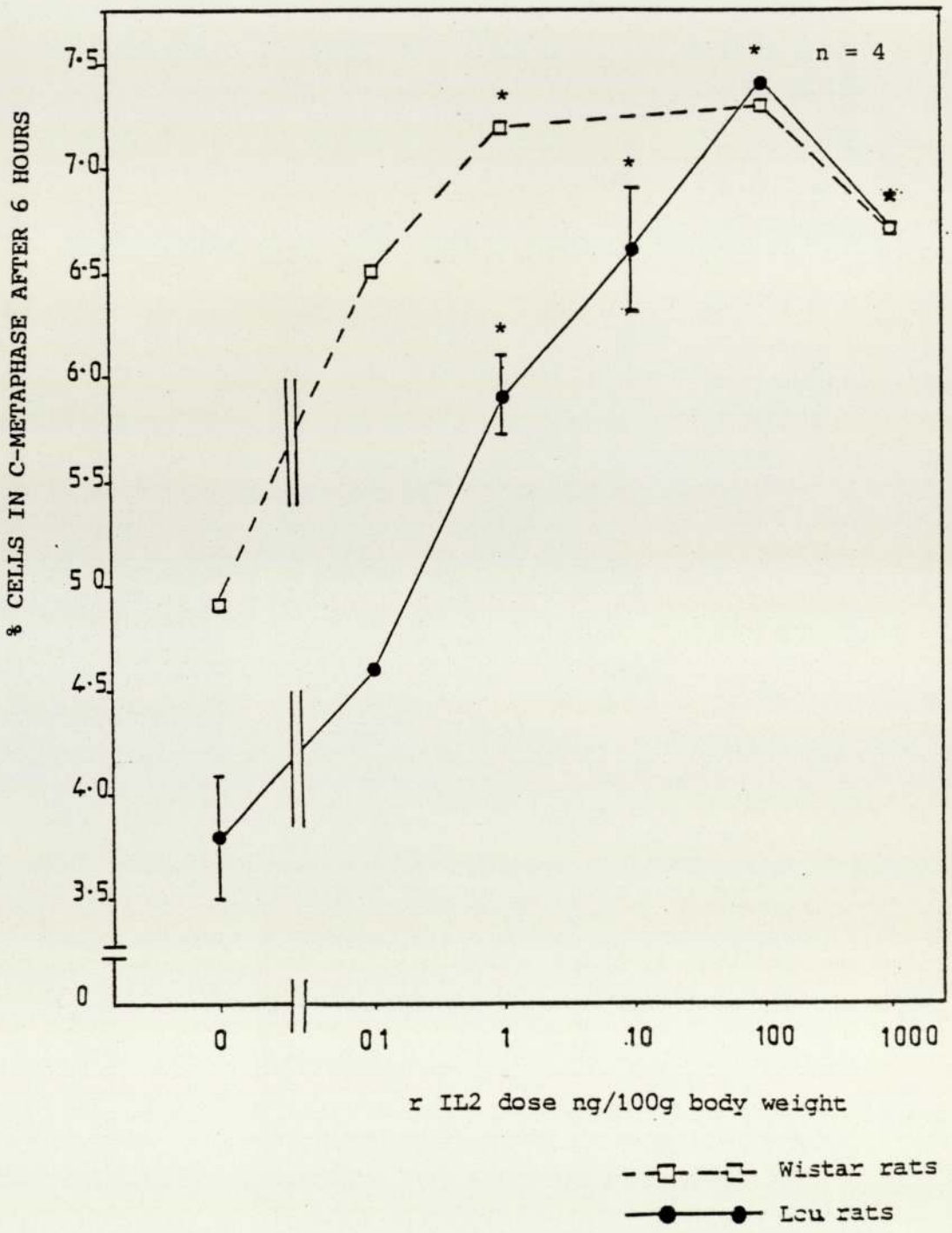


Figure 33 The effect of a single intraperitoneal injection of different doses of r IL2 on proliferation of rat thymocytes.

Values significantly different from mitotic activity of thymocytes derived from saline-injected animals are indicated by asterisk.

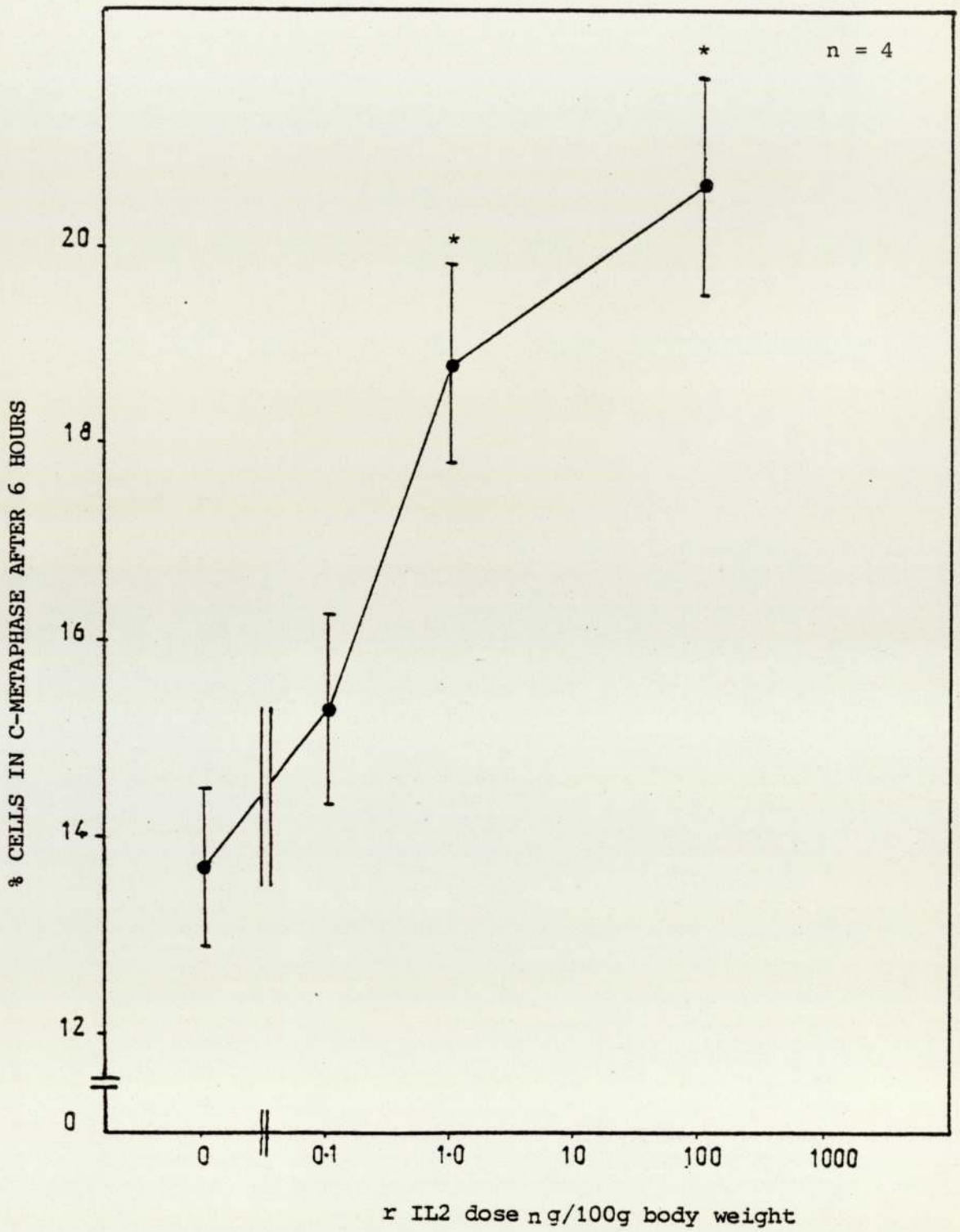


Figure 34 The effect of a single intraperitoneal injection of different doses of r IL2 on the proliferation of bone-marrow cells of Wistar rats.

Values significantly different from mitotic activity of thymocytes derived from saline-injected control animals are indicated by asterisk

Table 27 - Effect of r IL2 on mitosis induced by Acetylcholine in basal media

Treatment	Control	r IL2 (3×10^{-9}M)	r IL2 (3×10^{-11}M)
Basal	4.3 ± 0.2	6.3 ± 0.3	4.3 ± 0.2
ACh 10^{-5}M	6.5 ± 0.3	4.8 ± 0.5*	5.0 ± 0.2*
ACh 10^{-11}M	6.5 ± 0.4	5.4 ± 0.3	5.8 ± 0.1

* Values significantly different from cultures devoid of r IL2

Having shown that IL2 can recruit thymocytes into mitosis over a 6 hour period in vitro, it seemed relevant to examine whether IL2 could exert the same effect in vivo. Therefore, Lou or Wistar rats were injected intraperitoneally with r IL2. A dose of 100 ng IL2/100g body weight induced maximal division although as little as 1 ng IL2/100g also significantly enhanced cell division (Figure 33). The bone marrow also showed a similar increase in proliferation in response to the exogen ous human recombinant IL2 (Figure 34).

In an attempt to demonstrate that endogenous rat IL2 could likewise stimulate mitosis in these tissues, syngeneic spleen cells were activated in vitro by 5 $\mu g ml^{-1}$ Con A. After 4 hours in culture, the cells were washed with 0.05M methyl-α-D-man noside to remove Con A and injected intraperitoneally into young Wistar rats at a cell density of 10^7 cells ml^{-1}. When cultured for 24 hours, these cells produce sufficient amounts of IL2 to stimulate a proliferative response in the IL2-dependent cell line. On injection of these activated spleen cells, an enhanced cell division was observed in both thymus and bone-marrow compared to both saline-injected control animals and those receiving spleen cells not activated by Con A (Figure 35). Hence, it is clear that IL2 produced during the cellular immune response may effectively elevate mitotic activity in thymus and

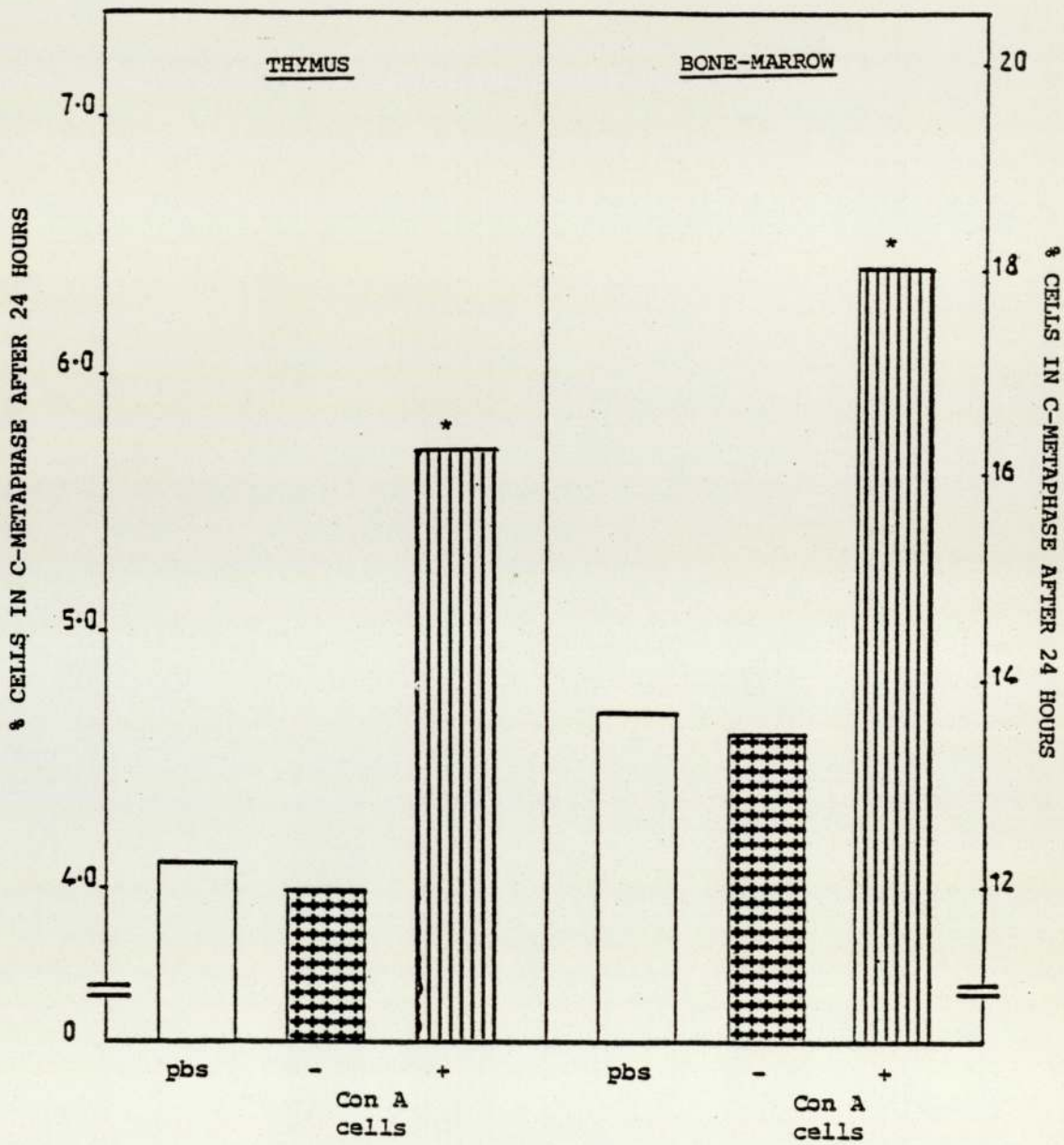


Figure 35 The effect of a single intraperitoneal injection of saline (pbs), spleen cells (-) or spleen cells stimulated <u>in vitro</u> with Con A (+) on proliferation in thymus or bone-marrow of Wistar rats.

Values significantly different from control are denoted by asterisk

For further details refer to Appendix (C)

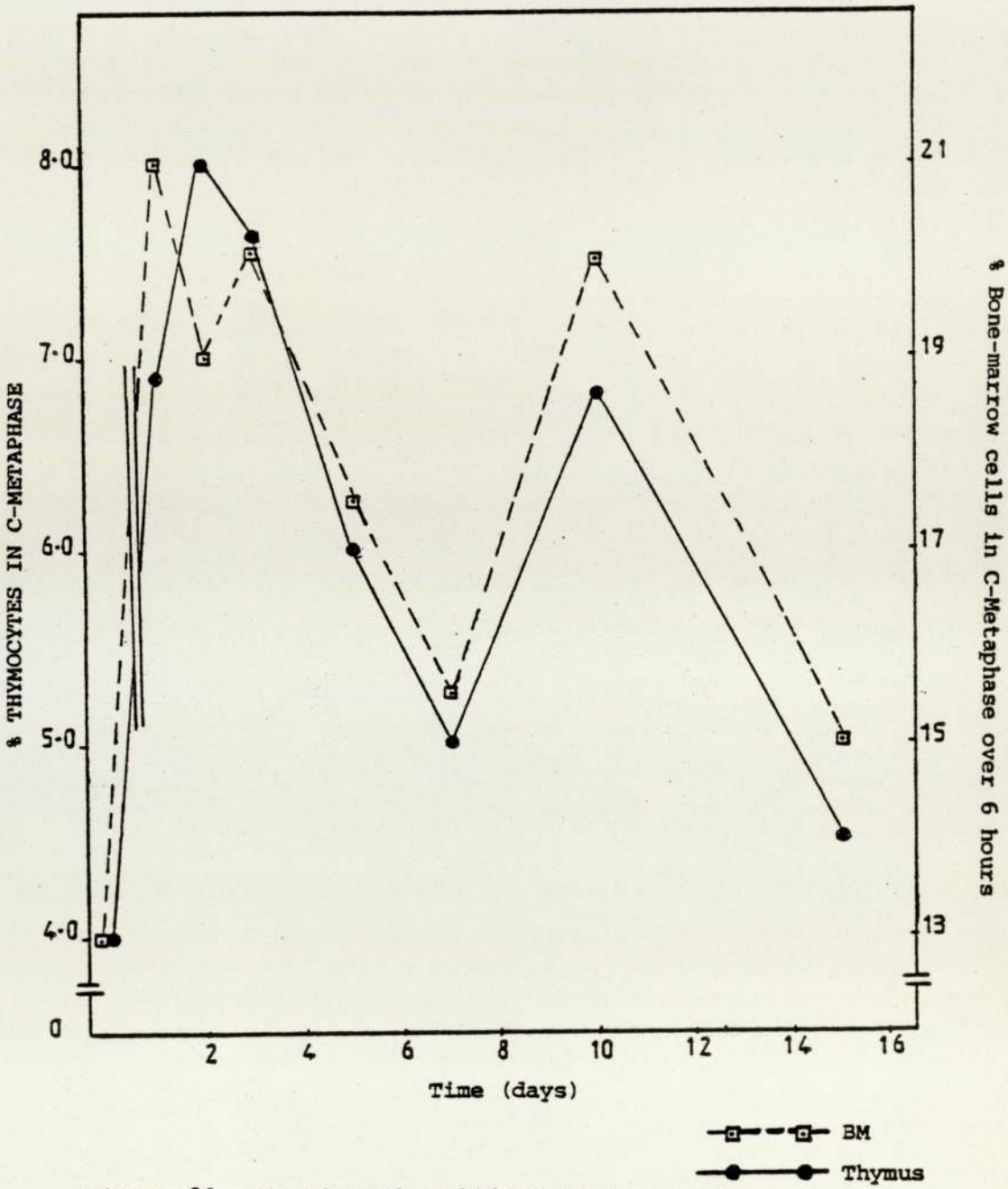


Figure 36 Kinetics of proliferation in rat thymus (o) and bone-marrow following footpad injection of WCPV.

For details, refer to Appendix (D)

bone-marrow tissues.

The above results were further substantiated in experiments in which rats were immunized in the hind foot pads with whole cell B.pertussis vaccine (WCPV). A characteristic biphasic mitotic response indicative of some IL2 production and consequently T-cell activation was certainly evident in both the thymus and bone-marrow (Figure 36). Thus, it seemsd feasible to suggest that IL2 produced during antigenic challenge is capable of inducing proliferation at least in the primary lymphoid tissues.

CHAPTER FIVE

DISCUSSION

In addition to the proliferation induced by various systemic and local hormones, thymic lymphocytes in culture can likewise respond to certain neurotransmitters (Morgan et al, 1975, 1984). Although at first sight, the sensitivity to such an array of agonists would suggest a lack of specificity, this is certainly not the case. This is because the mitogenic action of many of these agents can be specifically blocked by pharmacological antagonists indicating that these cells must bear specific receptors for these stimulatory agents. Occupancy of receptor sites by these agonists would perhaps modify the state of opening of closely-linked ion channels and cause ionic fluxes responsible for cell proliferation. In the present study, examination of the mitotic response of thymic lymphocytes on exposure to acetylcholine (ACh) clearly reveals a biphasic action (Figures 8 & 9). Such mitogenic potential of ACh at two different concentrations is achieved via two different mechanisms linked to the divalent cations; Ca^{2+} and Mg^{2+} (Figures 10 & 11). Such an association is not altogether surprising as these divalent cations by themselves can induce cell division on elevation of their concentration in the culture medium (Figure 2). Furthermore many other mitogenic hormones seem to exert their effects via a calcium or a magnesium axis (Table 3). While the cell division at the higher acetylcholine concentration (10^{-5}M) is coupled to nicotinic receptor activation and Mg^{2+} ions; the lower acetylcholine concentration (10^{-11}M) is associated with muscarinic receptor stimulation and a Ca^{2+} dependent response. As a high acetylcholine concentration is required for activation of nicotinic receptors, these receptors must be of low affinity. Muscarinic receptors on the other hand, are of high affinity as a lower concentration of acetylcholine is sufficient to produce an effective cellular response. Hence, acetylcholine can be considered as both a type I

and type II mitogen depending upon the concentrations employed for promoting division in thymocytes.

The biphasic mitogenic action of acetylcholine has also been observed by other workers with optima at approximately 5×10^{-5}M and 5×10^{-8}M (Macmanus et al, 1975). The somewhat different mitogenic concentrations apparent in our study are probably a result of the presence of eserine, an anticholinesterase agent, thus preventing acetylcholine degradation in the course of a 6 hour incubation. The work of Macmanus and co-workers (1975) however indicated that the low stimulatory acetylcholine concentration was Ca^{2+} independent. The different strains of rats from which the thymocytes were isolated or alternatively the higher cell density employed by them many account for such controversial results. The present work after exhaustive repetition clearly illustrated the calcium requirement of the low acetylcholine concentration. Calcium antagonists, eg. nifedipine and verapamil (Fleckenstein, 1977; Spedding & Cavero, 1984) which block the mitogenic potential of high calcium concentrations also inhibited the mitotic capacity of the calcium-dependent low concentration of acetylcholine whilst leaving the magnesium coupled mitogenesis unimpaired (Figures 13 & 14). Furthermore, the sex steroid ß-oestradiol ($0.1\ gml^{-1}$) which inhibits the mitogenic action of high calcium (Figure 2) and other Ca^{2+}-dependent mitogens (Morgan et al, 1975) also abolished the proliferative response (Table 10). It is possible that oestradiol following interaction with its receptors on thymocytes increases Ca^{2+} influx so that the intracellular Ca^{2+} concentration rises to supernormal and toxic levels ultimately causing inhibition of mitogenic stimulation (Hesketh et al, 1982; Gulino et al, 1985). An increased Ca^{2+} uptake was certainly evident when thymocytes were exposed to oestradiol in high Ca^{2+} medium (Figure 12) confirming previous results obtained by Cade (1983). Whether an enhanced Ca^{2+} entry occurs when thymocytes are exposed to a combination of oestradiol ($0.1\ \mu gml^{-1}$) and

acetylcholine (10^{-11}M) has not been determined.

The events which follow the binding of acetylcholine to its receptor are not clear. It has been suggested that in addition to the intracellular divalent cationic climate, fluctuations in concentrations of cyclic nucleotides might initiate events culminating in mitosis (Rasmussen & Goodman, 1977; Rebhun, 1977; Whitfield et al, 1979). Thus, the Ca^{2+} dependent increase in mitosis on treatment with low acetylcholine concentrations could be due to an elevation of cGMP levels. Acetylcholine can certainly elevate cGMP levels in a wide variety of tissues such as heart (George et al, 1970), brain (Lee et al, 1972) and ductus deferens (Schultz et al, 1973). In fat cells, exposure to low concentrations of acetylcholine (10^{-10}M and 10^{-11}M) comparable to those employed here resulted in an accumulation of cGMP (Iliano et al, 1973). Such enhancement in cGMP production may be due to an increase in cytosolic Ca^{2+} concentration as a consequence of increased Ca^{2+} influx or alternatively due to mobilisation from intracellular storage sites (Schultz et al, 1973; Ferrendeli et al, 1970). Moreover, since the exogenous addition or endogenous genesis of the nucleotide is capable of inducing cell division, an association between cGMP and proliferation seems plausible (Rebhun, 1977; Morgan et al, 1977). However, other evidence suggests this is probably not the case. Although acetylcholine did increase cGMP levels in thymocytes, it did so only at non-mitogenic concentrations (Macmanus et al, 1975). Furthermore in our laboratory even high concentrations of acetylcholine failed to increase cGMP (unpublished results) although such high concentrations can produce elevations in cGMP levels in unstimulated human lymphocytes (Haddock et al, 1975). As cGMP is probably not involved, it is possible that mitotic activity may be induced directly by a rise in cytosolic $[Ca^{2+}]_i$.

An increase in intracellular calcium concentration was certainly evident from Quin 2 studies; (Table 11) this may be a consequence of a calcium redistribution, an

enhanced Ca^{2+} influx or even a reduced Ca^{2+} efflux. As receptor activation by cholinergic agents often evokes an increased hydrolysis of phospholipids (Michell, 1982; Salmon & Honeyman, 1980), the increased Ca^{2+} content may be attributed to the release of Ca^{2+} from intracellular organelles by the formation of yet another second messenger, IP_3 (Berridge, 1984; Vicentini et al, 1985, 1986; Pozzan et al, 1986). This indeed seems feasible as the addition of the putative calcium mobilisation blocker, M&B40, 678 impaired the proliferative response of the low acetylcholine concentrations (Figure 15). It should be noted that the precise mechanism of action of this agent is not known. Calcium redistribution alone however, is not exclusively responsible for proliferogenesis (Pozzan et al, 1986). Extracellular calcium must also be considered as cell division does not occur in Ca^{2+} free medium. Indeed, preliminary $^{45}Ca^{2+}$ uptake studies indicated an increased Ca^{2+} influx in thymocytes on exposure to low acetylcholine concentrations (Table 12). This influx was pertinent for cellular response and probably occurred through specific channels as the calcium-channel blockers, verapamil and nifedipine compromised the mitotic response (Figure 13). Although these blockers specifically block voltage-opreated channels, it is possible that acetylcholine by exerting its effects on the plasma membrane may cause activation of receptor-operated channels which may further indirectly lead to a partial cellular depolarisation automatically triggering the opening of voltage-operated channels (Cavero and Spedding, 1983). Thus acetylcholine at low concentrations required both calcium influx and mobilisation for stimulation of mitosis in rat thymocytes.

As acetylcholine at low concentrations appear to be operating via the Ca^{2+} dependent axis, the next step following the rise in $[Ca^{2+}]_i$ is the association of Ca^{2+} with its regulatory protein, calmodulin. Following Ca^{2+} binding, calmodulin undergoes activation and ultimately induces the physiological response. Chafouleas and Means (1982) have suggested that an elevation of cytosolic levels of calmodulin

at the G1/S boundary of the cycle is essential for progression of cells into the S phase. This is because calmodulin regulates specific functions such as cytoskeletal formation and phosphorylation of proteins, particularly the histones which may be important at the G1/S transit. Thus, if the mitogenic capacity of acetylcholine involves regulation by calmodulin, the addition of specific calmodulin inhibitors may negate the increase in calmodulin concentration or prevent the actions of calmodulin essential for progression through the S phase thereby inhibiting mitosis. The proliferative ability of acetylcholine was certainly blocked by the anticalmodulin drug trifluoperazine (Figure 16). However, it has been suggested that TFP can also inhibit protein-kinase C; high concentrations (4×10^{-5}M) were needed to produce such inhibition (Wise et al, 1982). The antimitotic concentration employed in the present study was 10^{-6}M so it seems more likely that calmodulin is implicated here. Hence, calcium via the formation of calci-calmodulin complexes seems to trigger the cascade of mitotic events linked to the low acetylcholine concentration.

In contrast, the magnesium-dependent proliferative response of high concentrations of acetylcholine links it with other Mg^{2+} dependent mitogens such as isoprenaline, adrenaline, dopamine and glucagon (Table 3, Figure 11). As these agonists elevate cAMP levels in thymic lymphocytes and other tissues (Macmanus et al, 1971; Kebabian & Cote, 1981), the Mg^{2+} dependency may in some way be coupled to cAMP production. Regretably cAMP concentrations after acetylcholine application were not measured in the present study. The exact role of Mg^{2+} is not clear. In addition to serving as a cofactor for adenylate cyclase activation (Maguire, 1984), it may perhaps be involved in a second reaction triggered by an increase in cAMP levels (Morgan et al, 1977). Indeed, evidence from adipocytes in which there is increased Mg^{2+} uptake following exposure to adenylate cyclase stimulants, adrenaline and ACTH (Elliott & Rizack, 1974) indicates that cAMP may regulate Mg^{2+} ion transfer into the cells. Such increases in Mg^{2+} may consequently initiate

DNA synthesis (Perris & Morgan, 1978). A positive correlation between the level of intracellular magnesium and DNA synthesis is apparent in some cells (Hosseini & Elin, 1985). Some workers have suggested that even if a slight rise in intracellular free Mg^{2+} concentration occurs, it should be adequate to exert enough metabolic control to initiate proliferative events (Sanui & Rubin, 1982).

Clearly, acetylcholine is capable of stimulating proliferation in thymic lymphocytes at both low and high concentrations. Heightened lymphopoiesis on exposure to acetylcholine has also been noted by Singh (1979) using thymic organ cultures. Remarkably, the concentrations employed were not specified. Our own observations clearly pont to dual acetylcholine receptors on thymocytes. It is of considerable interest that both muscarinic and nicotinic receptors have also been described on human and murine lymphocytes (Strom et al, 1974; Maslinski et al, 1980; Adam et al, 1986). Furthermore, stimulation of muscarinic receptor augments mitogen-stimulated DNA synthesis and cytolytic effect of certain T lymphocytes (Strom et al, 1974, 1981).

As acetylcholine clearly promotes mitosis in vitro, it is possible that acetylcholine may be of some significance in thymic lymphopoiesis in vivo. If it does, it would not be surprising as the thymus has extensive autonomic neuronal input in the cortex and the cortico-medullary boundary (Bullock & Moore, 1981; Bullock & Pomerantz, 1984). Such innervation of the thymus by fibres derived from the vagus is evident by days 10-11 of gestation prior to vascularisation and differentiation into a lympho-reticular organ suggesting that the neurotransmitter may be essential for the integrity of the thymic microenvironment. In the nude (nu/nu) mouse, the rudimentary thymic epithelium fails to receive appropriate parasympathetic innervation which may possibly contribute to the failure of proper development of the gland. In addition to a parasympathetic input, the normal thymic tissues also receives sympathetic fibres. These nerves are however detectable later

in ontogeny, around 17/18 days of embryonic life (Singh, 1984). As these fibres are usually adjacent to the blood vessels; it is possible that they control blood flow and influence lymphocyte traffic. Non-vascular associated fibres have also been detected in both the cortex and the medulla adjacent to the lymphocytes (Felten et al, 1985). It has been reported that sympathetic nerve fibres exert an inhibitory or restraining effect on lymphocyte division in the developing thymus. Conversely, sympathectomy heightens lymphopoiesis in foetal thymic rudiments from nu/nu mice; the lymphoid cells thus generated have characteristics of thymocytes, such as the binding to peanut agglutinin and expression of Thy-1 antigen (Singh, 1985). It is clear that thymic innervation is important for thymocyte maturation and when innervation dimishes with age, there is a concomitant involution of the tissue (Ghali et al, 1980).

In addition to the classical sympathetic and parasympathetic innervation, the thymic tissue may also contain neuropeptides such as vasoactive intestinal peptide (VIP), oxytocin and neurophysin (Geenen et al, 1985). The significance of these peptides in the thymus is speculative. As vasopressin and oxytocin are capable of replacing IL2 as a stimulus for γ- IFN production by Lyt 2^+ mouse lymphocytes, thymocytes could indeed be the targets for oxytocin (Johnson & Torres, 1985). VIP may play a potential role in the modulation of T-cell migratory or recognition capabilities for homing to the secondary lymphoid tissue. A role for these neuroendocrine peptides in the control of cell division has also been suggested (Hunt et al, 1977; Johnson et al, 1982; Hanley, 1985). Thus, some comitogenic, inductive or repressive actions of these peptides during thymocyte differentiation seems plausible.

Although it is clear that the central nervous system provides signals via neuroendocrine circuits and the autonomic nerve supplies to the thymus; the mechanism by which these signals co-ordinate and control intrathymic differentiation

is not known. It is quite likely that the neuroendocrine hormones and neurotransmitters available within the thymic environment may directly affect the thymocytes which have receptors or may indirectly affect their maturation by affecting other cellular components of the stroma such as the macrophages and epithelial cells; which in turn have been shown to be the source of chemical factors such as IL-1 and thymic hormones respectively. Consistent with this hypothesis, ACh receptors have been detected on thymic epithelial cells (Kao & Drachman, 1977; Engel et al, 1977). Such cells may secrete the peptides, eg. facteur thymique serique, thymosins and thymopoietin which promote thymocyte differentiation. In fact, such a function for thymosins after autonomic stimulation has been indicated previously as chemical sympathectomy can alter circulating levels of Tα1 (Hall et al, 1982). Intriguingly thymopoietin, TPO, at physiological concentrations appears to bind to muscular nicotinic receptors impairing their activation. Hypersecretion of TPO is found in myasthenia gravis and widespread nicotinic blockage and receptor loss may be the consequence and account for some of the symptoms of this disease (Verkatasubramanian et al, 1986). It is not certain whether interaction ot TPO with the nicotinic receptors of thymic lymphocytes could in part at least account for maturation in the thymic microenvironment.

Another hormone which appears to modulate the growth and development of the thymus gland is the PTH. As mentioned previously, PTH influences intrathymic cell production. Surgical removal of the parathyroid glands induces a hypocalcemia and subsequently hypoplasia of the thymus which can be reversed by supplementation with PTH or calcium (Perris et al, 1970. In addition, PTH also stimulates mitotic activity in vitro (Whitfield et al, 1969). The mechanism of PTH-stimulated mitosis however is not clear as it employs either cAMP and/or Ca^{2+} as second messengers in its hormonal actions on the classical target tissues; the bone and kidney. Thus, the calcium ionophore A23187 when applied to radiolabelled

cultured foetal long bones mimics the action of PTH and causes ^{45}Ca release (Dziak & Stern, 1975). Furthermore, PTH-induced bone resorption can be inhibited by the calcium-channel blocker verapamil (Herrmann Erlee et al, 1977). Certainly PTH has been shown to increase calcium influx into isolated bone cells in culture (Dziak & Stern, 1975). This effect on calcium transport can be clearly dissociated from the effect of the hormone on cAMP formation in foetal mouse bone cells (Herrmann Erlee et al,1983).

An effect of PTH calcium transport in the kidney is well established and leads to increases in cytosolic calcium in proximal convoluted tubule cells (Hruska et al, 1986). In renal cells, however, the enhanced transport may be a consequence of increases in cAMP. PTH also heightens Ca^{++} influx into several other diverse cell types such as intestinal epithelium (Nemere & Szego, 1981), liver (Chausmer et al,1972) and cultured HeLa cells (Borle, 1968). Whether the ion movement is linked to cyclic nucleotide metabolism in these instances has not been examined.

The present study was undertaken to dissect the second messenger involvement in PTH-induced mitogenesis in cultured thymic lymphocytes. The evidence certainly suggests that cAMP plays no role but that a hormone-induced alteration of cellular calcium homeostasis is responsible for PTH-stimulated thymocyte proliferation. The thymocytes did not exhibit PTH-sensitive adenylate cyclase activity (Table 18), rather the intracellular cAMP levels decreased as a result of PTH addition. This effect was blocked by both TFP and isobutyl methylxanthine, indicating a PTH-mediated activation of calmodulin sensitive cAMP phosphodiesterase. An earlier report also demonstrates that a crude preparation of bovine PTH does not stimulate thymocyte adenylate cyclase activity (Whitfield et al, 1971).

In the present study, the NH_2-terminal fragment PTH (1-34) in which the full biological activity of the native hormone resides (Herrmann Erlee et al, 1976;

Habener et al, 1984) induced proliferative activity in a dose-dependent manner with a maximum response at $10^{-8}gml^{-1}$(Figure 17). Other PTH analogues, the super agonist 8,18 Nleu PTH (1-34) also stimulated mitosis (Figure 18). Previous evidence indicated that PTH (1-34) stimulated mitosis in thymocytes in a Ca^{2+} dependent manner (Morgan et al, 1975; Perris et al, 1983). It was not surprising, therefore, to find that the PTH fragments also had an obligatory requirement for this ion (Figures 19 and 20). Preliminary experiments certainly showed an enhanced $^{45}Ca^{2+}$ influx within twenty minutes of PTH (1-34) addition (Table 14). Moreover, experiments performed using Quin 2 confirmed that this fragment caused an increase in the free intracellular Ca^{2+} concentration. Indeed in the presence of extracellular Ca^{2+}, all three PTH fragments at their respective mitogenic concentrations provoked an approximately two-fold increase in $[Ca^{2+}]_i$ within twenty-five minutes as compared to the levels of $[Ca^{2+}]_i$ in unstimulated thymocytes (Table 13).

The effects of PTH (3-34) mitosis and calcium metabolism were unexpected (Figure 21, Table 13). In the kidney, this fragment inhibits PTH-induced stimulation of adenylate cyclase and in contrast to the other fragments, it fails to increase cytosolic calcium concentrations in proximal tubule cells and in an osteoblast like cell line (Rosenblatt et al, 1977; Lowik et al, 1985; Hruska et al, 1986). Presumably in thymic lymphocytes, PTH (3-34) both binds to and activates the PTH receptor rather than acting as an antagonist as it does in other tissues.

Although the PTH fragments require a supply of extracellular calcium in order to raise cytosolic concentrations and stimulate mitosis, some of the calcium increment may be derived from intracellular stores. Certainly the drug M&B40,678 thought to impair calcium mobilisation, inhibited PTH-induced mitogenesis (Table 15). Another supposed mobilisation blocker, TMB 8, reduced the rise in cytosolic calcium caused by PTH in proximal tubule cells (Hruska et al, 1986). Although the

specificity of these materials is far from clear, PTH may well release Ca from sequestered stores by enhancing phospholipid turnover which certainly occurs in dog kidney cortical tubule cells and fragments (Meltzer et al, 1982; Khalifa et al, 1983) and also in red blood cells (Brautbar et al, 1985). Whether IP_3 is generated in these cells and indeed the other targets for PTH action including thymocytes has not yet been established.

The mitogenic action of PTH certainly involves some aspect of calcium metabolism (influx, mobilisation or both) since its action, like that of all other calcium-dependent mitogens, was abolished by the calcium-channel blockers, calmodulin antagonist and oestradiol (Tables 15, 16 & 17). Thus the dependency of the mitogenic action of PTH upon extracellular calcium, the enhanced calcium entry, the sensitivity to calmodulin antagonist and the decline in cyclic AMP during PTH stimulation suggest that calcium, and not cAMP is the second messenger of PTH action in these cells. It therefore appears that thymocytes, unlike osteoblasts, possess only one kind of PTH receptors coupled to the second messenger, calcium.

It is obvious that the proliferative activity of thymic lymphocytes is closely linked to calcium and PTH levels in vivo and in vitro (Perris et al, 1970; Morgan et al, 1975; Perris et al, 1983). A similar response is also apparent in the bone marrow. In fact, parathyroidectomy causes a hypocalcemia and a dramatic bone marrow hypoplasia. Moreover, aparathyroid animals exhibit an impaired ability to restore normal erythrocyte numbers after haemorrhage as the parathyroid-dependent increments in plasma calcium concentration and bone-marrow mitosis which normally follow bleeding fail to occur (Perris et al, 1971).

Considering the importance of PTH in the promotion of mitosis in thymus and bone-marrow, it seems logical to assume that parathyroidectomy would adversely affect the immune response. In fact in aparathyroid Sprague Dawley rats, the enhanced splenic DNA synthesis and the increased immune response normally

elicited by sheep red blood cell injections were significantly reduced (Swierenga et al, 1976). In a parallel study in this laboratory using Wistar rats, secondary splenic lymphoid tissue was not susceptible to ablation of the parathyroid gland. Such withdrawal of PTH however could not induce an increment in either plasma Ca^{2+} concentration or in bone marrow proliferation normally apparent on antigenic challenge (Edwards et al, 1981). Recent experiments have revealed that PTH influences cell mediated immunity more than humoral immunity (Perris et al, 1984). Although antigenic encounter often induces hypercalcemia, the events uynderlying this episode are not clear. It was suggested that lymphokines released by lymphocytes during antigenic stimulation may cause bone resorption and along with normal circulating concentrations of PTH induce hypercalcemia and heightened bone marrow mitosis (Perris et al, 1984). One lymphokine with bone-resorbing properties is interleukin 1 (Gowen et al, 1983; Oppenheim et al, 1986). Whether this lymphokine in conjunction with PTH is in fact responsible for the increment in plasma Ca^{2+} concentration after antigenic challenge is not known. An interaction of IL1 and/or the IL2 which would subsequently be released with PTH in the control of lymphoid cell proliferation remains a distinct possibility.

Exposure to thymocytes to a partially purified rat IL2 preparation certainly stimulated cell division (Table 19). Such proliferation also evident for Concanavalin A (Con A) blasts and the IL2 dependent cell line, CTLD occurred only in the presence of normal concentrations of calcium in the extracellular medium. Absence of the ion, its chelation with EGTA or a blockade of its influx by drugs such as nifedipine, and verapamil, prevented mitosis (Table 20). Birx et al (1984) have also observed a suppression of IL2-induced proliferation in T lymphocytes on addition of these calcium antagonists. In contrast, Weiss et al (1984) showed that the IL2 response was unaffected by the addition of 0.5mM EGTA to the culture medium and have claimed that IL2 does not act via a Ca^{2+} flux. However, a higher

concentration (0.75mM) of EGTA reduced the IL2 response by at least twenty percent suggesting calcium does have some role. We found that if EGTA is increased to 1mM, the IL2 response of Con A blasts and CTLD cells is totally blocked, and that the addition of at least 0.7mM $CaCl^2$ is required to restore the response.

These results suggest that IL2 acts by promoting Ca^{2+} influx into the cells. However, in a single experiment, we were unable to detect any enhanced $^{45}Ca^{2+}$ uptake into thymocytes within twenty minutes of IL2 addition. In activated peripheral blood T-lymphocytes, however, IL2 certainly enhanced $^{45}Ca^{2+}$influx within one minute of addition (Larsen et al, 1986). In the IL2-dependent cell line also, IL2 induced an early influx of Ca^{2+} followed by a shutdown of the calcium channel (Johnson et al, 1985). An increased Ca^{2+} entry normally increases the free cytosolic Ca^{2+} concentration. Indeed, in IL2-stimulated thymocytes and Con A blasts, a marked rise in intracellular Ca^{2+} concentration was apparent (Figure 22). Such an elevation however failed to occur in thymocytes in Ca^{2+}-free medium. Presumably therefore IL2 does induce a Ca^{2+} influx in thymocytes but the tracer technique was probably insensitive enough to detect it.

Despite the doubts expressed by some workers (Mills et al, 1985; Larsen et al, 1986), others are convinced that cytosolic Ca^{2+} does increase after IL2 application (Rossio et al, 1985) and suggest this is a consequence of phophatidylinositol phosphate breakdown. The IP_3 and diacylglycerol thus generated would mobilise calcium and activate protein kinase-C respectively (Farrar & Anderson, 1985; Farrar et al, 1986). In thymocyte preparations, the supposed mobilisation blocker, M&B40,678 impaired the calcium elevating and mitogenic properties of IL2 (Figure 26, Table 23) suggesting that calcium influx and mobilisation may both contribute to the mechanism of IL2 action. No direct studies of phosphatidyl inositol metabolism in the thymus have yet been undertaken.

These studies suggest that IL2 is a Ca^{2+}-dependent or type II mitogen for thymocytes. This Ca^{2+} dependency was further substantiated by the addition of calmodulin antagonists. These drugs certainly prevented cell division in all the cell types tested (Table 21). A calcium-calmodulin mediated protein phosphorylation may thus further trigger the events leading to mitosis.

Although our studies demonstrated that partially purified rat IL2-provoked mitosis via a Ca^{2+} influx, the possibility that contaminants in the IL2 preparation were responsible could not be discounted. Other workers using recombinant IL2 have indicated that proliferation in peripheral blood lymphocytes, murine thymus and spleen cell lines is independent of increases in cytosolic calcium (Mills et al, 1985). Furthermore, although Larsen et al (1986) indicated IL2 caused increased ^{45}Ca uptake, they failed to observe any simultaneous elevation in cytosolic Ca^{2+}. In our hands, highly purified recombinant IL2 gave identical results to the partially purified rat preparation suggesting that in the thymus, at least calcium has a critical role in IL2 action.

In peripheral T lymphocytes, IL2 addition does not directly stimulate cell division. An antigenic or mitogenic stimulus is first required for cellular activation and expression of IL2 receptors. A second signal provided by IL2 subsequently drives the progression of these activated cells from G1 to the replicative S phase (Stadler et al, 1981). In thymocytes, proliferation in response to IL2 however occurred in the absence of a primary exogenous antigenic or mitogenic stimulant. Other workers using preparations of conditioned medium enriched in IL2 from lectin-stimulated thymocytes have also reported increases in proliferation of thymocytes (Bodeker et al, 1980; Nishimura et al, 1984). Such stimulation of normal, non-activated thymic lymphocytes suggests that antigenic stimulation due to the contact of the animals with their environment may have led to the expression of IL2 receptors. In addition, it has been shown that IL2 alone can induce the

expression of receptors on human T-cells and thymocytes that have not been activated previously by lectins or other inducing agents (Reem et al, 1985). Further more, studies on the proliferogenic response of IL2 by variation of incubation time before application of antimitotic agents revealed that the inhibitor must be present at the time the mitotic stimulus is applied inorder to exert its inhibitory effect (Figure 29). This confirms that IL2 is acting rapidly by binding to its receptors. Il2-receptor (IL2-R) expression has now been detected by immunohistochemistry with monoclonal antibodies on thymocytes from adult humans, mice and rats (Ceredig et al, 1985; Takacs et al, 1985). In the mouse, a minor subpopulation of phenotypically immature thymocytes mostly located in the subcapsular cortex bear these receptors (Ceredig et al, 1985; Raulet, 1985). In the rat and human thymus, however, the cells reacting with the respective anti IL2 receptor antibodies were located exclusively in the medulla (Takacs et al, 1985). Whatever the location, the role of the IL2 receptor-positive cells is not yet established.

In the adult thymus, approximately 1-2% of the lymphocytes bear IL2-R. This level is higher in new born or foetal thymus (Takacs et al, 1984; Habu et al, 1985; Raulet, 1985). Evidence that these receptors are functional in early foetal development is contradictory. While Van Bohmer et al (1985) showed no effect of affinity-purified or recombinant DNA-derived IL2 on foetal thymocyte proliferation in vitro, Habu (1986) certainly detected DNA synthesis in both embryonic and adult thymocytes. Low doses of IL2 stimulated proliferation whereas higher doses inhibited division whilst promoting differentiation into natural killer cells (Habu, 1986). Other workers have also shown an increased division in murine and human foetal thymocytes (Teh & Ho, 1985; De La Hera et al, 1985; Hardt et al, 1985). In fact, a role for IL2 in the growth and maturation of cells committed to the T-cell lineage during intrathymic differentiation has been suggested (De La Hera et al, 1985).

In the present study, IL2 recruited thymocytes of the foetal, new-born young and aged animals into division (Figure 30). The precentage of cells undergoing proliferation corresponds closely to IL2 receptor-positive cells reported by several workers for animals of these ages (Ceredig et al, 1985; Raulet, 1985). In the 3-month-old thymus, dose response curves were similar to those established for CTLL cells; maximal response occurring at a concentration of ~ 100 pM (Figure 23). This suggests that in thymocytes of this age, the receptors for IL2 are of high affinity. In foetal (19/20 day old) thymocytes, enhanced division was evident at these low concentrations but increasing the IL2 concentration to 30 nM caused additional recruitment (Table 24). This suggests that during foetal and neonatal development, there is a transition from low to high affinity receptors.

Most parties have thus agreed that both foetal and adult thymocytes bear functional IL2 receptors. If is of some interest that the response to IL2 can be heightened by the simultaneous presence of IL1 (Hardt et al, 1985; Mannel et al, 1985). Conceivably this may represent direct mitogenic effect of IL1, enhanced IL2 receptor expression or enhanced production of IL2 by the thymocytes themselves. This latter possibility has been extensively investigated with conflicting results. Habu (1986) and Lugo et al (1986) suggest that 16 and 18 day old embryonic thymocytes cannot produce IL2 whereas Ceredig (1986) claims that even 14 day old foetal thymocytes will produce this lymphokine when treated with phorbolmyristateacetate (PMA) and Ionomycin. It is not clear whether natural stimuli can likewise stimulate IL2 production in foetal thymocytes (Habu, 1986).

The ability of foetal thymocytes to manufacture and respond to IL2 may account for the extensive proliferation and expansion of the lymphocyte pool within the embryonic thymus. In adult thymocytes, IL1 will stimulate the release of IL2 by medullary PNA^- cells; these same cells will then divide in response to IL2 in an autocrine manner (Chen et al, 1982; Conlon et al, 1982).

In our study, both forms of IL1, IL1α and IL1ß stimulated mitosis (Figure 32). Such proliferative action is consistent with the presence of IL-1 receptors on thymocytes (Dower & Urdal, 1987). The proliferative capacity of IL1 is probably not attributable to IL2 release and subsequent utilisation, since the anti IL2-receptor antibody, ART-18 failed to block IL1-induced division (Table 25). It thus appears that IL1 is directly mitogenic. It is just possible that sufficient IL2 could be produced so as to dislodge the antibody from the receptor. The latter possibility is unlikely however, since Takacs et al (1985) have shown that excess IL2 is unable to displace the ART-18 antibody and stimulate proliferation. ART-18 also failed to inhibit the action of other thymocyte mitogens such as PTH, isoprenaline and stimulatory concentrations of calcium and magnesium (Table 25). The anti-IL2 receptor antibody completely abolished the mitogenic effect of IL2 (Table 25, Figure 31). Since all the mitogens including IL1 and IL2 stimulate division to the same extent, they are probably activating the same sub-population of thymocytes in each case. Simultaneous applications of two mitogens never produced an additive effect; indeed the combinations were sometimes inhibitory (Tables 26 & 27).

It was of considerable interest that IL2 could also stimulate division in vivo. Intraperitoneally administered IL2 or endogeneously produced rat IL2 by syngeneic spleen cells enhanced mitosis in both thymus and bone marrow (Figures 33, 34 & 35). In contrast, Ettinghausen et al (1985) could not detect any increase in ^{125}I-labelled deoxyuridine incorporation in thymus or hind limbs following high dose IL2 injection. No explanation for these contrasting results is readily apparent. In a further study, peripheral immunisation of rats with B.pertussis had profound systemic effects on thymus and bone marrow mitosis (Figure 36). Although the soluble components themselves may mainly be causing some of this activity directly, the proliferation observed may mainly be a consequence of IL2 produced in lymph nodes draining the injection site. This IL2 could circulate to thymus and bone

marrow and stimulate division of cells despite the presence of circulating inhibitors (Hardt et al, 1981; Honda et al, 1985). In support of this, it has been shown that popliteal lymph-node cells 3 days after immunisation with sheep red blood cells (SRBC) can produce adequate amounts of IL2 into the culture medium over 24 hours (Spitz et al, 1985). The amounts of IL2 secreted could provoke cell division. Previous studies in this laboratory have indeed revealed that bone marrow proliferation is elevated following injection of SRBC (Edwards et al, 1981). The significance of this effect is unknown; however, it is tempting to speculate that it represents a feedback mechanism whereby peripheral antigenic challenge normally associated with hypercalcemia causes IL2 production, which, in turn causes recruitment of T cells from the thymus to help meet the challenge. The T cells expressing IL2 receptor in bone marrow are of cytotoxic/suppressor phenotype, and may be involved in the regulation of antibody production in bone marrow, or may be a source of cytotoxic T cells which can immigrate to peripheral sites to combat the antigenic challenge (Mills et al, 1985; Dosch et al, 1985).

From the results presented here, it is obvious that proliferation of thymic lymphocytes is influenced to a considerable extent by agents such as acetylcholine, parathyroid hormone and interleukins. Hence, in addition to a physiologial link between the endocrine and immune systems, a functional link between the nervous and immune systems is also apparent. Such interrelationships of lymphocytes with hormones and neurotransmitters are likely to be quite subtle but an understanding of these intricate mechanisms which may perhaps involve some additional mediators derived from the thymic epithelium is fundamental to our appreciation of thymocyte development/proliferation as an integrated physiological phenomenon.

APPENDIX

(A) The Concanavalin A blast cell assay

Mouse spleen cells were washed 3 times in RPMI 1640 containing 3% foetal calf serum (FCS). To induce blast formation and expression of IL-2 receptors, the cells were resuspended in RPMI 1640 medium containing 5% FCS and 5 x 10^{-5}M 2-mercaptoethanol (subsequently referred to as culture medium) at a density of 2 x 10^6 cells/ml and cultured for 72h with 2.5 µl/ml of concanavalin A. The blasts were washed twice in saline and resuspended in culture medium containing 25mM methyl-mannoside, which inhibits the mitogenic effect of any residual concanavalin A.

The cells were distributed into 96 well microtitration plates (Flow) at 5 x 10^4 cells/well. The appropriate additions of test solutions were made and the volume made up to 100 µl with culture medium. After 18 h of culture, 50 µl (0.5µCi) of [^{3}H] thymidine (specific activity 25 Ci/mmol) was added to each well and the cells harvested 4 h later onto glass fibre filters. The incorporated radioactivity was determined by scintillation counting. All samples were assayed in triplicate and the results expressed as a mean cpm ± SD.

(B) The IL2 Dependent Cell Line Assay

The CTLD cells were grown in culture medium containing IL2, for which they have an absolute dependence. Two days after their last feeding, the cells were washed extensively with RPMI 1640 containing 3% foetal calf serum (FCS). The cells were cultured with the appropriate supplements in 100µl aliquots at a density of 5 x 10^3 cells/well. After 24 hours at 37°C, the cells were pulsed with 0.5 µCi/well of [^{3}H] thymidine (Amersham Ltd) for 4 hours and subsequently harvested using a cell harvester. After drying, the filters were counted in a liquid scintillation counter.

The other cell line, CTLL has been used in some experiments. These cells were cultured in RPMI 1640 containing 5% FCS. The cell density and the proliferation assay employed was the same as above.

(C) Activation of spleen cells by Concanavalin A (Con A)

Spleen cells were prepared and suspended at a concentration of 10^7 leukocytes/ml in RPMI 1640 medium containing 5% fetal calf serum. Where desired, the medium was supplemented with 5 $\mu g\ ml^{-1}$ Con A. After 4 hours, the cells were washed thrice in RPM1 1640 containing 0.05M methyl-α-D-mannoside to remove Con A. Subsequently, 2 x 10^7 cells/ml^{-1} were resuspended in RPMI 1640 and cultured in 0.5 ml aliquots for 24 hours. IL2 activity in the culture supernatants was measuring using the CTLL assay.

Injection of spleen cells

In some experiments, Wistar rats were injected intraperitoneally with 10^7 syngeneic Con A-activated or unstimulated control spleen cells or phosphate-buffered saline. After 18 and 21 hours, colchicine was injected intraperitoneally at a concentration of 0.2 mg/100 g rat. At 24 hours, the thymus and bone-marrow were removed and C-metaphases counted as described previously in Section 3.2.1b.

(D) Determination of Proliferation in rat thymus and bone-marrow following immunisation with B Pertussis vaccine

A British reference preparation for Pertussis vaccine (66/84) reconstituted in phosphate buffered saline, PBS, was used as the whole cell B Pertussis vaccine (WCPV). The material was washed once in PBS to remove soluble antigens, prior to its use for immunisation.

Wistar rats were immunized with 5×10^8 bacteria in 50 µl saline in each hind footpad. On different days after immunization, rats were injected intraperitoneally with colchicine, 0.2mg/100g rat in 0.5 ml pyrogen-free saline. After 3 hours, a second injection of colchicine was given and 3 hours later the animals were sacrificed. The thymus and bone-marrow were removed and C-metaphases subsequently counted as described previously in Section 3.2.1b.

(E) ANOVAR - The One-way Analysis of Variance

Worked Example:

The effect of varying concentrations of PTH (3-34) on mitosis in isolated rat thymic lymphocytes:

Treatment	Basal	High Ca^{2+}	PTH (3-34) ——>				
			$10^{-6}gml^{-1}$	$10^{-7}gml^{-1}$	$10^{-8}gml^{-1}$	$10^{-9}gml^{-1}$	$10^{-10}gml^{-1}$
1	45	6.8	3.5	7.3	4.6	5.0	4.3
2	4.6	6.5	4.0	7.1	3.7	4.6	4.3
3	4.4	6.6	4.3	6.9	4.4	3.6	4.2
4	4.9	7.1	3.9	6.7	3.5	3.7	4.6
Treatment Total	18.4	27.0	15.7	28.0	16.2	16.9	17.4
Mean	4.6	6.8	3.9	7.0	4.1	4.2	4.4

Procedure

The sum of squares (total deviance) is calculated for the experiment

$$\text{Sum of squares (SS)} = \varepsilon (x - \bar{x})^2 = \varepsilon x^2 - \frac{(\varepsilon x)^2}{n}$$

$\frac{(\varepsilon x)^2}{n}$ is known as the correction factor and x is each observation in the experiment. Thus,

$$\varepsilon (x - \bar{x})^2 = 740.44 - \frac{19488}{28} = 44.44 \text{ with } 27 \text{ df } (n-1)$$

The sum of squares and degrees of freedom are divided into two component parts:

1 The between treatments sum of squares:
(treatment deviance)

$$\varepsilon (x - x)^2 = \varepsilon \frac{(TT)^2}{N} - \frac{(\varepsilon x)^2}{n} \quad \text{where}$$

TT is the treatment total and N, the number of observations which make up the treatment total.

$$\frac{(\varepsilon x)^2}{n} = \text{the correction factor} = 696$$

$$\varepsilon (x - x)^2 = \frac{2948.86}{4} - 696 = 41.22$$

2 The within treatments sum of squares (error):

This is obtained by subtraction of the treatment deviance from the total deviance,

Therefore, 44.44 - 41.22 = 3.22

An Anovar summary table is then constructed. The Mean square (or Variance) is calculated by dividing the sum of squares by the degrees of freedom (df). The Variance ratio (F) is obtained by dividing the between treatments mean square by the within treatments mean square.

Variation	df (n-1)	Sum of squares	Mean squares	F
Between treatments	6	41.22	6.87	44.90***
Within treatments	21	3.22	0.153	
Total	27	44.44		

In order to find the values of 'p', the F tables are consulted. In this case F (6) (21) = 44.90 $p < 0.001$ (1-tail). Thus, the differences between the means of the seven treatments are highly significant and are not due to chance sampling variation.

The Least Significant Difference (LSD):

$$LSD = t \times \sqrt{\frac{2S^2}{n}}$$

where S is the error mean square, and n the number of observations which make up each mean. A value for 't' is obtained from the tables at a given probability level and df for error is substituted in this formula.

Thus for $p = 0.05$, $t = 2.080$ (21 d.f. error)

$$LSD_{(0.05)} = 2.080 \times \sqrt{\frac{2 \times .153}{4}} \sim 6$$

Therefore between treatment differences greater than 0.6 are significant at the p - 0.005 level.

Thus, in this particular experiment there is a significant difference at the $p = 0.05$ level between the Basal and the PTH (3-34) at a concentration of 10^{-7} gml^{-1} mean.

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