MOUSE SPLENOCYTE PROLIFERATION AND ITS MODULATION BY SEX STEROIDS

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SUMMARY

Concanavalin A, a T cell mitogen enhanced DNA synthesis in murine splenocytes. Amongst the early signals prior to this event was an increase in cytosolic calcium derived from both intra- and extracellular sources. The requirements for extracellular calcium persisted for four hours after the lectin administration which itself was needed for six hours. Putative calcium channel antagonists and calmodulin inhibitors blocked the increase in DNA synthesis. The calcium signal was mimicked by application of the ionophore, A23187, although no increase in DNA synthesis occurred. An activator of protein kinase C, 12-0-tetradecanoylphorbol 13-acetate, had little effect in isolation but the combined application of these two agents greatly enhanced DNA synthesis. The natural mediators of these events are presumed to be inositol trisphosphate and diacylglycerol derived from phosphatidylinositol bisphosphate hydrolysis. Lectin application and protein kinase C activation both increased intracellular pH possibly as a result of Na^+/H^+ exchange since amiloride an inhibitor of this antiporter inhibited lectin induced DNA synthesis. The calcium and hydrogen ionic changes occur within minutes of lectin application; the protracted requirement for this mitogen suggests further signalling mechanisms occur to elicit maximum DNA synthesis in these cells.

Gonadectomy caused an increase in thymic and splenic weight. Splenocytes derived from castrated mice showed no change in mitogen response whereas those from ovariectomised mice demonstrated a reduced lectin sensitivity. Testosterone, 5α dihydrotestosterone, α and ß oestradiol all inhibited lectin induced DNA synthesis but only at pharmacological concentrations. Testosterone glucuronide and cholesterol were without effect. Studies with mouse serum fractions of differing steroidal status were unable to confirm the presence or absence of serum factors which might mediate the effects of steroid on lymphoid cells, all fractions tested inhibited lymphocyte transformation.

Both interleukin-2 and lipopolysaccharide induced splenocyte mitogenesis was also impaired by high steroid concentrations in vitro, suggesting that steroids mediate their effect by a non-specific, non-receptor-mediated event.

Keywords: Splenocytes
Sex steroids
Proliferation
Signal transduction
Calcium

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CHAPTER ONE

INTRODUCTION

1.1 Preface

Ideally cell growth occurs only when required either for development or for tissue maintenance. Although the growth in size of individual cells is a small component in the growth of tissues and organs, an increase in the number of cells is of much greater significance in both normal and neoplastic growth. Since the increase in cell number is achieved by mitotic division of cells, an understanding of normal growth and its aberrations must be based on a study of the mechanisms that regulate cell division.

The control of normal growth in eukaryotic cells is achieved by a complex signalling system beginning with the action of a variety of extracellular effectors which, having bound to specific receptors in or on the cell, are able to initiate changes in intracellular processes which ultimately lead to DNA synthesis and mitosis (Rozengurt & Collins 1983; Berridge, 1984a). The molecular mechanisms by which these extracellular signals are transduced across the plasma membrane to provoke a complex cellular response which eventually leads to mitosis are still not fully understood.

The proliferation rate of animal cells is largely determined by the relative times that they spend in the cell cycle as opposed to quiescence. An initiation event is required to propel a cell towards mitosis; in its absence cells enter a resting state in which they remain until an effector provides the necessary signal starting the chain of events leading to DNA synthesis and division once again (Pardee, 1974).

1.2 The Cell Cycle

The cell cycle is the period between the division of one cell and the subsequent division of a daughter cell. However this strict definition is normally broadened to include the cellular and biochemical aspects that regulate cell proliferation (Baserga, 1981; Ronot & Adolphe, 1986).

DNA synthesis (the S phase) occurs during a discrete period during interphase and is an essential prelude to mitosis (the M phase). Before and after the S phase, cells engage in growth and metabolic activity but not in chromosome replication. Cells preparing for DNA replication are in the G_1 phase whilst cells preparing for mitosis are considered to be in the G_2 period. The four consecutive phases G_1, S, G_2 and M first described by Howard and Pelc (1953) are collectively known as the cell cycle.

Subdivision of the intermitotic period into G_1, S and G_2 has generated many attempts to elucidate the process controlling the progression of cells from one mitosis to the next. The duration of S and G_2 do not vary greatly between populations since both phases are predetermined and specifically related to division (Smith and Martin, 1973; Ronot & Adolphe, 1986). However the greatest variation in duration of the interphase is found in the G_1 phase. When a cell cycle is long most of the prolongation is in the G_1 phase and when a cell cycle is short as it is in egg cells and in certain rapidly proliferating cell lines, there is no measurable G_1 phase at all (Mazia, 1974; Liskay and Prescott, 1978; Baserga, 1985). G_1 has therefore attracted particular interest as the phase in which proliferation is regulated. Hidden in the G_1 phase are the key processes that make

it possible for the cell to enter the S phase and thereby commit itself to division in the future. Restraints or 'blocks' prevent uncontrollable proliferation in non-neoplastic cells and normally occur in the G_1 phase, (Pardee, 1974; Pardee et al, 1978; Baserga, 1981; Whitfield, 1982; Murray, 1987a) but may also occur in the G_2 and G_0 phases of the cell cycle (Gelfant, 1981). Neoplastic growth occurs because cancerous cells circumvent these restriction points and grow autonomously (Walker, 1986).

The growth status of cell populations has been categorised in three ways, thus there are proliferating or 'cycling' cells, differentiated cells and quiescent cells. Proliferating cells continuously divide going from one mitosis to the next, through the four consecutive phases of the cell cycle.

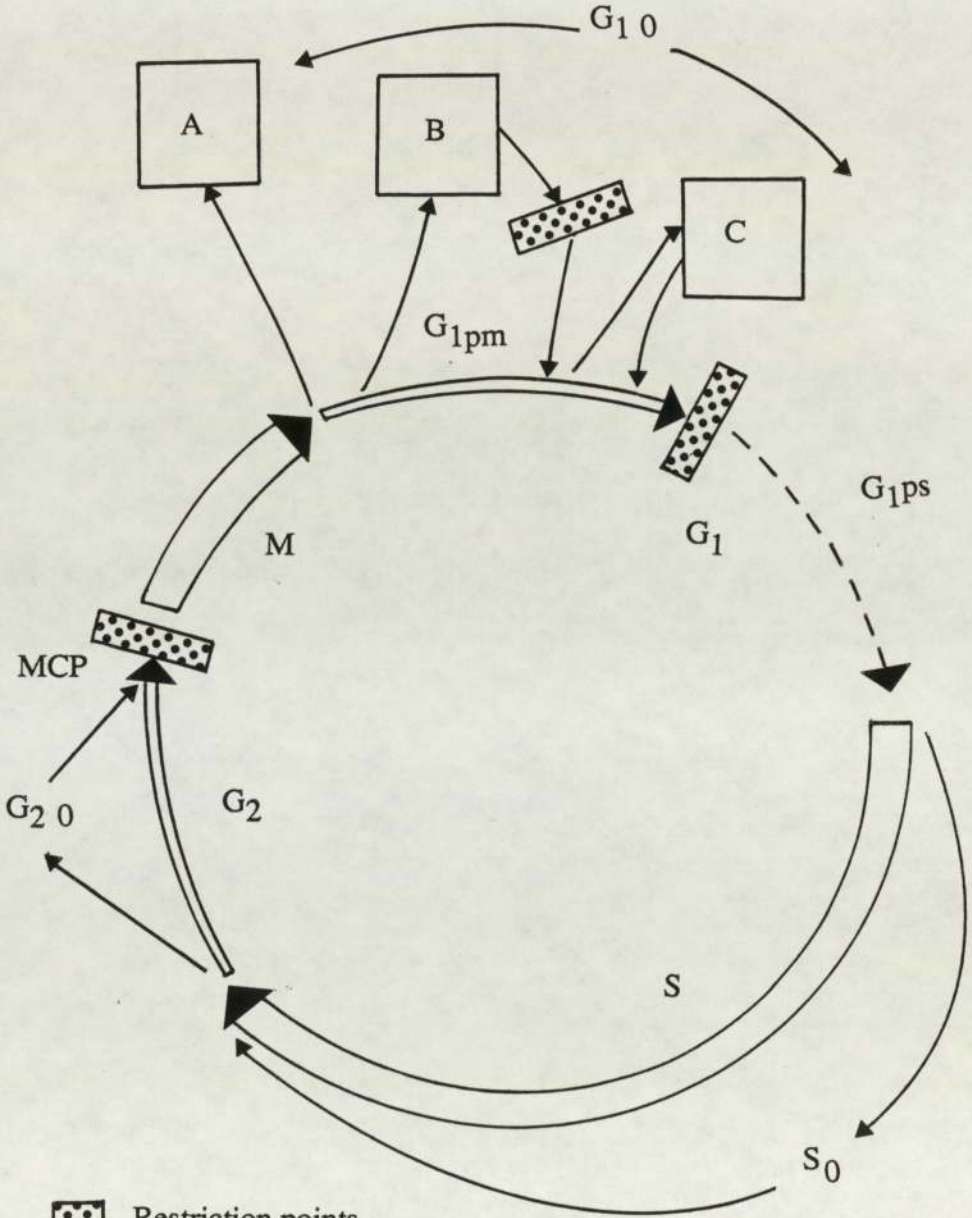
A further group of cells may temporarily leave the cell cycle and become quiescent because they are unable to pass through a critical control or restriction point in G_1 due to failure of key processes. They remain in a quiescent or G_0 phase until environmental conditions stimulate their re-entry into the cell cycle at G_1 (Fantes, 1986).

The G_0 phase may encompass cells with different degrees of readiness to resume cell division, eg. serum starved or nutrient deprived cells may pass the restriction point close to the G_1/S boundary fairly rapidly upon serum or nutrient addition whilst in other cells such as peripheral blood lymphocytes and hepatocytes the recruitment is more complex and protracted (MacManus et al, 1972; Klaus & Hawrylowicz, 1984). Recent work by Zetterberg and colleagues observing 3T3 cells under the microscope so that age related effects could be precisely analysed have determined that cells are only sensitive to serum deprivation for between 3 and 4 hours after mitosis. This period is referred to as

G_1 pm (post-mitotic). This infers that cells leave the cycle and enter quiescence from a discrete period of the G_1 phase. The remainder of the G_1 phase is termed G_1 ps (pre S phase). Commitment to mitosis is thought to occur at a control point at the end of G_1 pm (Larsson et al, 1985; Zetterberg & Larsson, 1985; Fantes, 1986) and is determined by appropriate growth factors in the serum.

Interestingly it appears that DNA synthesis is not initiated at an invariant time after the completion of the commitment process in G_1 pm. The time spent in G_1 ps varies considerably from cell to cell, thus the variation in cycle duration attributed to the G_1 phase is now considered to be in this latter part of G_1, G_1 ps. The timing of DNA synthesis and subsequent mitosis is thought therefore to be adjusted during the G_1 ps interval. G_1 ps variability is consistent with a transition-probability event (Smith and Martin, 1973; Brooks, 1980) but could equally reflect variability in cell size (Killander & Zetterberg, 1965; Shields et al, 1978). Thus although the cells are committed to mitosis in G_1 pm the time at which the S phase and subsequently division occurs may depend on the cells reaching a critical size in G_1 ps (Zetterberg & Larrson, 1985).

Moreover the possibility exists that there are separate commitment and division time regulatory points in mammalian cells comparable to those demonstrated recently in the fusion yeast *Schizosaccharomyces pombe*. In addition to a 'start' control point in G_1 there is a second regulatory point in the cell cycle between G_2 and mitosis (Hayles and Nurse, 1986). At this latter control position at the G_2/M boundary the cell monitors cell size and nutrient availability. Under favourable conditions the cell becomes committeed to immediate entry into



A: terminally differentiated cells
B: proliferatively inactivated cells, eg. peripheral lymphocyte cells
C: rapidly recruitable cells, eg. serum starved cells

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

mitosis, while if they are unfavourable it enters a quiescent state. Interestingly the same gene product cdc 2 (cell division cycle) is required for both these regulatory points in *S.pombe* (Murray, 1987a). Human homologues of cdc 2 gene may act at the G_1 restriction point in mammalian cells and also at a G_2/M transition point. A mitotic inducer, maturation promotion factor (MPF) is postulated to initiate mitosis in vertebrate cells at a mitotic control point at G_2/M comparable to that found in the fusion yeast, and may therefore be an activated form of the cdc 2 gene homologue protein (Murray, 1987a; Murray, 1987b; Lee & Nurse, 1987).

Thus, committment to enter the cell cycle occurs at a restriction point at the end of G_1 pm. Cells may be induced to pass through this control point by the appropriate chemical factors or other stimuli occurring at the critical time. Once committed to mitosis cells then pass through the G_1 ps phase. The timing of the subsequent S phase and mitosis may be adjusted by unknown critical factors during this interval. A second control point initiating mitosis occurs late in G_2 in which maturation promotion factor (MPF) is thought to be involved. If such critical factors are missing during G_1 or G_2 or even during the S phase the cells may become arrested and enter a quiescent phase ($G_{1\,0}$, $G_{2\,0}$ or S_0). They will remain there until the appropriate stimulus provokes them to re-enter the cell cycle (see Diagram 1).

1.3 Extracellular Growth Substances

Normal cells proliferate as an adaptive response to a sequence of external signals. A mitogen is a factor which acts as a primary signal to induce a cell to undergo DNA synthesis and proliferation.

Among the various agents involved in growth regulatory processes are certain classical protein or polypeptide endocrine hormones.

Perhaps the best example of this group of classical hormones which regulate growth is growth hormone (GH), which promotes longitudinal bone growth by inducing chondrocytes in the epiphyseal growth plate to divide. In addition to skeletal growth, GH also promotes cell proliferation in a variety of cells such as skeletal muscle, liver, kidney, heart, adipose tissue and intestinal epithelium (Goldspink and Goldberg, 1975; Isaksson et al, 1985). Many studies have shown that the effect of growth hormone on different growth processes, particularly skeletal growth is indirect, mediated by GH dependent plasma factors, the somatomedins. However there is evidence that GH may have direct effects on longitudinal bone growth *in vivo* (Isaksson, 1982; Isaksson et al, 1985) and on cultured chondrocytes *in vitro* (Madsen et al, 1983).

Thyroid hormones have also long been implicated in growth. Thus, thyroidectomy in young rats leads to a significant reduction in skeletal growth together with morphological and histological abnormalities of skin, brain and other organs. Upon administration of thyroid hormone, normal growth and development resumes (Gibson et al, 1977). There are indications that thyroid hormone synergises with growth hormone and that both are essential for normal skeletal growth and development (Tonna, 1973).

Another hormonal vector controlling mitotic activity and body growth may be parathyroid hormone via its ability to modulate plasma calcium concentrations (Whitfield et al, 1969; Perris et al, 1970; Rixon & Whitfield, 1972). In the absence of the parathyroid gland there is thymic atrophy and bone marrow hypoplasia and the heightened mitotic activity which normally follows haemorrhage and immunological challenge is reduced (Perris et al, 1971; Edwards et al, 1981). A functional parathyroid gland is also essential for the normal

regenerative response of liver cells after partial hepatectomy (Rixon & Whitfield, 1976).

Growth *in vivo* is dependent on the anabolic hormone insulin. In addition to its well characterised effects on metabolism insulin has for a long time also been implicated in the stimulation of growth and proliferation of cells *in vitro* (Gey & Thalhimer, 1924). Since then insulin has been shown to stimulate a variety of cells to grow (Straus, 1981). Insulin alone, under certain culture conditions, is able to stimulate DNA synthesis (Temin, 1967; Teng et al, 1976; Coppock et al, 1980; and Koontz and Iwahashi, 1981). Typically however its stimulatory effect on growth is enhanced by and in some cases requires the presence of other hormonal growth factors. Insulin is generally believed to belong to the class of factors which are required for progression of "competent" cells through G_1 and S (Scher et al, 1979; Stiles et al, 1979; Callahan et al, 1985).

The synergistic effects of insulin with other growth factors is remarkably well illustrated in 3T3 cells. Quiescent cells can be stimulated to reinitiate DNA synthesis by specific combinations of mitogens which act synergistically when added to serum free cultures. Maximal DNA synthesis occurs when a growth factor which raises cAMP levels is administered in combination with another mitogen which activates protein kinase C and enhances ion fluxes. Some substances such as platelet derived growth factor (PDGF) have the ability to activate both pathways and achieve maximal stimulation alone. Interestingly, insulin may act synergistically with a factor from either group to maximally promote DNA synthesis, and thus plays a central role in the generation of early signals leading to proliferation in 3T3 cells (Rozengurt & Collino, 1983; Rozengurt, 1985; Rozengurt & Mendoza, 1985).

Other peptide hormones which promote growth and maturation in their respective target tissues are adrenocorticotrophic hormone, thyroid stimulating

hormone, follicle stimulating hormone, luteinizing hormone and erythropoietin (Tata, 1976; Nandi et al, 1980). Placental lactogen, relaxin, thrombin, transferrin and vasopressin also exhibit growth promoting activity on occasions (James & Bradshaw, 1984).

Certain steroids are also extremely important in the normal growth process. Thus, pubertal growth in girls is driven by low levels of oestrogen (Cutler et al, 1986). Oestradiol and progesterone powerfully stimulate cell division in mammalian uterine, vaginal and mammary tissue. Oestrogen will also stimulate proliferation of other tissues such as cultured decidual cells (Peleg & Linder, 1980) and rat hepatocytes, (Pietras & Szego, 1979). Additionally, oestrous-cycle controlled cell proliferation is apparent in the adrenal cortex of female rats (Pappritz et al, 1977).

Oestrogens are thought to exert some of their mitogenic effects via specific growth factors termed oestromedins. Thus, it is proposed that oestrogens may induce certain target tissues (uterus, kidney and pituitary) to synthesize or secrete polypeptide growth factors that enter the circulation and promote the growth of distant target tissue tumours (Sirbasku, 1978; Sirbasku & Moo, 1982; Sirbasku et al, 1982). More recently it appears that in addition to their endocrine actions, oestromedins may exert potent autocrine activity toward normal or neoplastic cells, or both, from the tissue of origin (Sirbasku et al, 1985). In contrast to their growth promoting role, oestrogens exert an inhibitory effect on erythropoiesis (Dukes et al, 1961). The immune system is also modulated by oestradiol and other steroids. Steroid administration clearly produces thymic atrophy whilst removal of steroids by ovariectomy, castration or adrenalectomy increases the size of the gland (Dougherty, 1952; Kinoshita et al, 1974; Castro, 1974; Sobhon & Jirasttham, 1974; Fitzpatrick et al, 1985; Greenstein et al, 1986).

Androgens stimulate growth of the male reproductive tract; testosterone is the predominant androgen produced and is synthesised in the Leydig cells of the testes. Testosterone may be converted to an even more powerful androgen 5α dihydrotestosterone within some target cells (Mainwaring, 1977; Mooradian et al, 1987) other tissues aromatize testosterone to oestradiol. Androgens control the growth of diverse tissues such as kidney (Bardin et al, 1978) skin fibroblasts (Keenan et al, 1975), skeletal muscle and fibroblast cells in culture (Powers et al, 1975). Androgenic steroids also exhibit a stimulatory effect on erythropoiesis (Fried et al, 1964; Gurney & Fried, 1965; Fried & Morley, 1985). The adolescent growth spurt is under androgen control and this is superimposed on the effects of growth hormone and thyroxine (Gupta, 1977; Cutler et al, 1986). There is evidence to suggest that growth hormone is necessary for the growth promoting action of testosterone, thus, in the hypophysectomized rat, testosterone alone does not increase the growth of any tissues other than accessory sexual glands (Scow & Hagan, 1965). Interestingly, testosterone influences the production and release of growth hormone in intact animals (Birge et al, 1967; Macleod et al, 1969; Jansson et al, 1984; Jansson et al, 1985).

In addition to these classical growth hormones there exists a further group of growth regulatory agents, the polypeptide growth factors (PGFs). Numerous recent reviews are a testimony to their potential importance in normal and neoplastic growth (Rozengurt & Collins, 1983; James & Bradshaw, 1984; Heldin & Westermark, 1984; Paul, 1985; Burgess, 1986; Goustin et al, 1986; & Weinstein, 1987). PGFs are distinct from the more classical endocrine hormones in that little is known about their role in the animal. Many growth factors have been isolated from serum and other body fluids by assaying the growth promoting activity in cells cultured in medium containing little or no serum. Earlier studies illustrated that not only do animal cells in culture require essential nutrients and

maintenance at optimal pH and temperature mimicing conditions *in vivo* but also the presence of serum in the culture medium if proliferation is to occur (Holley & Kiernan, 1968; Holley, 1975). Arrest of growth in G_1 causing cells to enter the quiescent phase $G_{1\,0}$ occurs when cells are deprived of serum or when the growth promoting activity of the serum present is exhausted. On addition of fresh serum cells re-enter the cell cycle (Pardee et al, 1978; Rozenburt, 1980). However, not until nutrient media and purified growth factors were available, was the study of the mitogenic actions of individual growth factors under defined culture conditions possible (Bottenstein et al, 1979; Shipley & Ham, 1981).

PGFs have since been shown to represent a large family of regulatory agents with many subsets characterised by structural similarities suggesting common ancestries. No single definition can be used to describe the extensive collection of PGFs which have so far been characterised. However, all are polypeptides and each PGF has an individual specific high affinity receptor located on the plasma membrane of target cells. Receptor ligand binding results in molecular responses that generally increase anabolism of the cell and modulation in expression of specific genes. The transmission of the regulatory signal is mediated by one or more second messengers but no universal mechanism has yet been determined. Lastly, PGFs and their receptors are extensively internalized by receptor-mediated endocytosis which ultimately leads to degradation of the ligand and of receptor recyling (Rozengurt & Collins, 1983; James & Bradshaw, 1984 Paul, 1987). PGFs are unlike the more classical endocrine hormones, since the majority of them are thought to act in a paracrine mode. However, there are notable exceptions, such as insulin like growth factors I and II (IGFs) which are endocrine (Froesch et al, 1985) and transforming growth factors (TGFs) which are autocrine (Anzano et al, 1985; Sporn & Roberts, 1985; Burgess, 1986).

Growth factors found in serum are presumed to be largely derived from platelets released during the clotting process (Holley & Kreman, 1974; Vogel et al, 1978; Childs et al, 1982; Oka & Orth, 1983). In addition PGFs are present in many tissues, adult and embryonic, and are thought to be released by most cells in culture (Shields, 1978). Specific combinations of growth factors are often required by normal cells for maximal stimulation of DNA synthesis (Barnes & Sato, 1980; Walthall & Ham, 1981; Rozengurt & Collins, 1983), it is proposed therefore that most cells will have membrane receptors for more than one growth factor (Wrann et al, 1980; Bowen-Pope et al, 1983).

Growth factors are thought to operate at different points of the cell cycle. According to the model of growth control initially proposed by Pledger and co-workers (1977) for density arrested Balb/C 3T3 cells, PGFs could be subdivided into two groups, the 'progression' and 'competence' factors. "Competence" factors prime the cells and enable them to respond to "progression" factors which stimulate the cells to traverse G_1 and enter S phase, thus competence and progression represent two functionally distinct subphases of G_0/G_1 (Pledger et al, 1978; Stiles et al, 1979; Scher et al, 1982; Wharton et al, 1982; Leof et al, 1982). It is unclear how these two phases relate to the G_1 pm and G_1 ps subphases of the cell cycle (Zetterberg & Larsson, 1985), possibly "competence" factors enable cells to pass the control point at the end of G_1 pm and become committed to mitosis, whilst "progression" factors allow these cells to traverse through G_1 ps into the S phase of the cell cycle. Competence factors such as PDGF have a restricted target specifically and act predominantly in the region in which they are produced (Alexander & Currie, 1984; Olashaw & Pledger, 1985), whilst factors in the progressive category include the systemically active insulin

like growth factors and epidermal growth factor (EGF) which stimulates a wide variety of cell types, both epithelial and mesenchymal (O'Keefe & Pledger, 1983; Alexander & Currie, 1984; Goustin et al, 1986).

The multiplicity of growth factors found in various tissues, the different cell type specificity of PGFs and the requirement for more than one PGF generally for maximal stimulation of specific cell types allows for the fine tuning of relative proliferation rates necessary for the co-ordinated growth of cells to form tissues during development and for adult tissue maintenance.

Included in the category of competence factors is platelet derived growth factor (PDGF) one of the major mitogens found in serum. Early studies showed that it is released from platelets into serum during blood clotting and that it is a potent mitogen for cultured fibroblasts, smooth muscle cells and glial cells (Balk, 1971; Ross et al, 1974; Scher et al, 1974; Antoniades et al, 1975; Westermarke & Wasteson, 1976; Scher et al, 1979). In the intact animal, PDGF may play a role in wound repair and in maintenance of the vascular system (Balk, 1971; Ross et al, 1974; Deuel & Huang, 1984; Lipton, 1985). More recently PDGF has been used as an experimental probe in the study of cell cycle control in fibroblasts. Thus, PDGF confers proliferative "competence" on density arrested Balb/C 3T3 mouse cells, ie. they are potentially able to leave G_0 and enter the cell cycle (Pledger et al, 1977; Pledger et al, 1978; Stiles et al, 1979). It is thought that PDGF promotes the rapid synthesis of a family of cytoplasmic proteins within quiescent G_0 cells (Pledger et al, 1981). These proteins, gene products of the competence gene family which include c-*myc* and c-*fos*, confer "competence" and allow the cells to respond to "progressive" factors and traverse from G_1 into the S phase of the cell cycle (Cochran et al, 1983; Cochran et al, 1984; Callahan et al, 1985; Lipton, 1985; Ollashaw & Pledger, 1985). Other growth factors which

belong to the PDGF family are the osteosarcoma derived growth factor, the glioma derived growth factor and the transforming protein of Simian sarcoma virus (Scher et al, 1979; Westermark et al, 1983; James & Bradshaw, 1984; Deuel and Huang, 1984; Heldin et al, 1986).

Pituitary and brain fibroblast growth factor (FGF) has also been shown to be a "competence" factor for Balb/c and Swiss 3T3 cell lines (Jimenez de Asua, 1977; Stiles et al, 1979; Jimenez de Asua, 1980). FGF is a potent mitogen which is considered to have a role to play in blastema cell proliferation *in vivo* during regeneration of lost appendages in the newt (Gospodarowicz & Mescher, 1981). Additionally FGF demonstrates angiogenic activity (Gospodarowicz et al, 1979) and induces cartilage regeneration *in vivo* (Jentzsch et al, 1980). FGF has both short and long-term effects on the morphology and growth pattern of many cells *in vitro*. It increases the rate of cellular proliferation, stabilises their phenotypic expression, stimulates cell migration and extends the culture lifespan (Gospodarowicz, 1984; Gospodarowicz, 1985).

Many other types of growth factors with broad target tissue specificities have been described which are included in the "progressive" category according to the model proposed by Pledger and colleagues (1977). These include epidermal growth factor (EGF), insulin and insulin-like-growth factors (sometomedins). Thus, once density-arrested Balb/c 3T3 cells are rendered competent by transient exposure to PDGF; EGF and either insulin or insulin-like growth factor I (IGF I) enable the cells to progress through G_1 to the S phase. Moreover it appears that EGF is only required in addition to insulin or IGF I to induce the cells to leave G_0 and progress for the first 6 hours of the 12 hour G_0/G_1 lag and then IGF I alone is sufficient for progression through the last 6 hours of the lag period (Leof et al, 1982). Human fibroblasts, unlike 3T3 cells, only appear to require EGF for

progression through G_0/G_1 *in vitro*, probably due to autocrine secretion of IGF I (Clemmons et al, 1981; O'Keefe & Pledger, 1983). EGF has been isolated from murine submaxillary glands and visceral tissues (Cohen, 1959, 1962). It is mitogenic for a variety of cultured cells of ectodermal, mesodermal and endodermal origin (Carpenter & Cohen, 1979; Schlessinger et al, 1983). EGF promotes the proliferation of the basal cell layer of various epithelia of ectodermal origin *in vivo* (Cohen & Taylor, 1974; Cohen, 1984). The mitotic effect of EGF is strongly potentiated by insulin (Shipley et al, 1984), and EGF appears to be an important potentiator of one of the class of transforming growth factors (TGFß) (Roberts et al, 1981) illustrating once again that PGFs often act in combination to induce maximal DNA synthesis.

Other members of the EGF family are the group of transforming growth factors (TGFs) (Roberts et al, 1981; Marquardt et al, 1983; Brown & Blakeley, 1984; Roberts & Sporn, 1985). These polypeptides have the ability to confer the transformed phenotype on normal cells. Two types of TGFs have been well characterised, but only one of these, TGFα is in fact related to epidermal growth factor and binds to the EGF receptor (Todara et al, 1980; Marquardt et al, 1983; Keski-Oja et al, 1987). TGFα was originally observed in the conditioned medium of murine cells transformed by sarcoma virus as EGF competing activity that was associated with anchorage-independent growth of fibroblastic rat kidney NRK cells (Delarco & Todaro, 1978). Although TGFα is produced by different malignant cells including those transformed by viruses, it does not appear to be produced by normal adult cells, nevertheless it may have a benign physiological activity in developing tissues (Keski-Oja et al, 1987). By binding to authentic EGF receptors TGFα may permanently stimulate cell proliferation, thus transformed cells are able to synthesise, release and respond to their own growth

factors (Heldin & Westermark, 1984). TGFß is produced by a variety of normal and malignant cells, it is neither structurally nor functionally related to EGF and mediates its effects by distinct receptors (Roberts et al, 1983; Roberts & Sporn, 1985). Many of the mitogenic effects of TGFß are probably an indirect result of the activation of certain growth factor genes in the target cell (Keski-Oja et al, 1987). TGFß appears to have a bifunctional nature since depending on the cell types, growth conditions and the presence of other growth factors in culture it may exhibit growth stimulatory or growth inhibitory effects (Roberts et al, 1985; Nakamura et al, 1985; Roberts & Sporn, 1985). The apparently universal presence of TGFß in non-neoplastic tissues suggests that in addition to its possible action in mediating cell transformation, this factor might have an important role in controlling normal cell growth and function. Thus, it is thought that TGFß may function as a wound healing agent (Sporn et al, 1983; Assoian & Sporn, 1986). Evidently the possibility exists that TGFß in combination with other growth factors or inhibitors has the potential to play a key role in the control of normal and malignant cell proliferation (Keski-Oja et al, 1987).

In addition to the aforementioned "progression" factor, EGF, other growth factors which share the ability of stimulating competent cells to progress to the S phase of the cell cycle and ultimately mitosis include insulin and the insulin like growth factors (Stiles et al, 1979; O'Keefe & Pledger, 1983). IGFs were originally included in a group of factors termed sometomedins, a family of circulating peptides produced mainly in the liver which act as intermediaries in the stimulation of growth by growth hormone (Phillips & Vassilopoulou-Sellin, 1980; Hall & Sara, 1983). It is now believed that somatomedin C is chemically identical to IGF I (Spencer et al, 1983) and multiplication stimulating activity (MSA) has been shown to be the rat homologue of human IGF II (Marquardt et al, 1981). IGFs are capable of mimicing all of the biological effects of insulin on metabolism

and growth (Zapf et al, 1978) and are able to stimulate *in vitro* DNA synthesis and cell division not only in rat cartilage (Salmon & Duvall, 1970) but also in numerous other cell types, very often in combination with other PGFs (Van Wyk, 1984; Froesch et al, 1985; Moses & Pilistine, 1985; Zapf & Froesch, 1986; Flier et al, 1986). Recently it has been illustrated that IGF I stimulates growth in normal rats *in vivo* (Hizuka et al, 1986). Serum levels of IGF I are strongly dependent on growth hormone, reduced in states of hormone deficiency and increased in states of growth hormone excess (acromegaly) (Zapf et al, 1981). There is also evidence that IGF I levels may be modulated by sex steroids, thus in puberty an increase in IGF I is correlated with an increase in plasma oestradiol and testosterone. In young subjects low doses of these sex steroids enhance IGF I secretion, whilst oestrogen at higher doses and/or older subjects has an inhibitory effect on IGF I (Caufriez & Copinschi, 1986). The role of IGF II *in vivo* is unclear (Zapf et al, 1985) although it may play an important role in rat foetal growth and development (Moses & Pilistine, 1986).

Nerve growth factor (NGF) belongs to the insulin family of PGFs (Bradshaw, 1983) and although NGF was the first substance to bear the label "growth factor" (Levi-Montalcini, 1952) its role as a mitogen is controversial. NGF is essential for the survival and differentiation of a number of neural crest derivatives, including sympathetic and sensory neurones, it is essential for the maintenance of their specific functions and plays a role in regeneration (Levi-Montalcini & Angeletti, 1968; Thoenen & Barde, 1980; Calissano et al, 1984; Levi Montalcini, 1987). Early studies indicated that NGF might also have a mitogenic effect on these neurones (Levi Montalcini, 1960), but subsequent work implied that NGF promotes cell survival or differentiation rather than proliferation (Hendry, 1977; Thoenen et al, 1985). However recently it has been shown that NGF does indeed induce a mitotic response in cultured chromaffin cells from

young rats. These cells divide in the presence of NGF and become neuronal in the continued presence of NGF (Lillien & Claude, 1985). Thus, it is possible that NGF may have a potential mitotic effect on other neural crest derivatives at early stages of development *in vivo*.

Relatively well characterised growth factors have also been described which regulate the proliferation or differentation of haematopoietic progenitor cells, the haematopoetic growth factors (HGFs); these include colony stimulating factors (CSFs) (Burgess & Metcalf, 1980; Metcalf, 1985) and erythropoietin (Miyake et al, 1977). There are four well defined CSFs which differ in their effects within the hierarchial framework of haematopoietic progenitor cells and in the different cell lineages they affect (Yunis et al, 1983; Burgess, 1985; Arai et al, 1986). Multi-CSF also known as haematopoietic cell growth factor (HCGF) and interleukin 3 (Dexter et al, 1985; Ihle, 1985), has a very broad haematopoietic target specificity, GM-CSF stimulates granulocyte and macrophage progenitor cells whilst G-CSF and M-CSF restrict their effects to the granulocyte or macrophage cell lineages, respectively (Nicola & Metcalf, 1985). Characterisation of haematopoietic growth factors (HGFs) has been made from culture studies in which unfractionated bone marrow cells, thymocytes or spleen cells have been stimulated with growth factors produced by cell lines or tissues *in vitro*. Although haemopoiesis *in vivo* occurs within a stromal cell environment, indications are that haemopoiesis in the absence of stromal cells may be provoked *in vitro* if the appropriate growth factors are present in the culture medium (Metcalf, 1981). So far only two HGFs have been detected in animals, erythropoietin (Goldwasser, 1985) and GM-CSF (Metcalf, 1971; Metcalf, 1985). Erythropoietin controls the production of red blood cells (Goldwasser et al, 1985) and the correlation of erythropoietin levels and subsequent erythropoiesis is good. Indeed *in vivo* injection of this growth factor increases erythropoiesis in a dose dependent fashion

(Sherwood & Goldwasser, 1979).

Another group of regulatory proteins "the interleukins" have been described which regulate many aspects of the immune response. Many factors have been described, some of which either stimulate or inhibit lymphocyte cell division (Gillis & Mizel, 1981; Oppenheim & Gery, 1982; Duff & Durum, 1983; Schreier, 1984; Robb, 1984; Hemler et al, 1984; Nowell et al, 1985; Lachman , 1985; Smith, 1985; Smith & Rennick, 1986; Arai et al, 1986; Klaus 1986). The roles of the two most studied, interleukin 1 and interleukin 2, may be summarised, thus; interleukin 1 (IL1) a macrophage derived factor and the T cell derived T cell growth factor interleukin 2 (IL2) are known to take part in T cell activation and proliferation. Thus, T lymphocytes can be activated to move from the G_0 into the G_1 phase of the cell cycle by the synergistic action of IL1 and antigen (or lectin). The expression of interleukin 2 receptors on activated blasts allows them to undergo proliferative expansion under the influence of IL2 secreted by themselves or by another activated T cell subpopulation (Wagner et al, 1980; Klaus & Hawrylowicz, 1984; Robb, 1984; Pfizenmaier et al, 1984).

Thus, there is a good deal of evidence which suggests that proliferation of normal cells is under the control of exogenous growth factors and classical hormones, many of which, although by no means all have been described in this section. Nevertheless, there is an opposing view which holds that, in the natural state all well nourished cells engage in proliferation and that *in vivo* this mitotic exuberance is suppressed by some form of negative feedback inhibitor. Historically, these inhibitors have been named "chalones", (Bullough & Laurence, 1968; Bullough, 1975; Allen & Smith, 1979). Because of the inherent difficulties in measuring cell inhibition in a specific manner and problems in the purification of the various chalones, the biological evidence for the existence of "chalones" has yet to be unequivocably established (Patt & Houck, 1980).

Recently the isolation and purification of TGFß and a few other endogenous inhibitors of cell proliferation from several sources, including normal tissues, has provided some experimental basis for the hypothesis of negative regulation of cell growth (McMahon et al, 1982; Assosian et al, 1983; Roberts et al, 1985; Sporn & Roberts, 1985; Wang & Hsu, 1986). BSC 1 growth inhibitor was originally isolated from medium conditioned by BSC 1 cells (derived from African green monkey kidney cells) (Holley et al, 1980). It is now considered to be identical with TGFß (Holley et al, 1985). TGFß has been found in many normal tissues, and has been isolated from serum-free conditioned medium of normal and transformed cells. TGFß binds to specific cell-surface receptors in responsive cells and is established as a negative growth regulator that may function in the autocrine pathway (Sporn & Roberts, 1985).

Growth inhibitory activity has also been shown for a family of proteins, the "interferons" usually identified by their ability to protect cells against virus infections (Stewart, 1979). Cell growth inhibitory effects of interferons have been demonstrated both in cell culture and in tumour bearing animals (Taylor-Papadimitrou et al, 1982). Many cell types have shown varying degrees of sensitivity to the growth inhibiting properties of interferons. Nevertheless the proliferative activity of some normal cells including fibroblasts, epithelial cells and lymphocytes has been inhibited (Balkwill et al, 1978; Gurari-Rotman et al, 1978) in addition to inhibition of tumorigenic cells such as the Daudi line of Burkitts lymphoma-derived B lymphoblastoid cells (Broder et al, 1982). Furthermore, it appears that no single stage in the cell cycle is uniquely sensitive to interferon action (Clemens & McNurlan, 1985; Taylor-Papadimitriou et al, 1985).

Various soluble factors produced by leukocytes have been described that inhibit bone marrow colony formation *in vitro*, they include various types of interferons, prostaglandins, tumour necrosis factor and natural killer cell-derived

haematopoietic colony-inhibiting activity (Degliantoni et al, 1985). Recently the existence of an autocrine growth inhibitor has been postulated which inhibits the IL2 driven T cell proliferative response in a cloned T cell line D10 (Horowitz et al, 1986).

Rat platelets contain two inhibitors of growth for adult rat hepatocytes. These have been termed platelet derived growth inhibitors, PDGIα and PDGIß (Nakmaura et al, 1986). The latter has very similar properties to TGFß whereas PDGIα is distinct from TGFß and other cell growth inhibitors, such as "liver chalone" isolated from adult rat liver (Iype & McMahon, 1984).

A growth regulatory factor which reversibly inhibits DNA synthesis and proliferation of fibroblasts has also been isolated. This factor termed FGRs (fibroblast growth regulator), is found in medium conditioned by density-inhibited mouse 3T3 cells (Hsu & Wang, 1986; Blat et al, 1986).

An extremely potent growth inhibitor has been recovered and partially purified from the culture medium of density-inhibited U79 cells (tumorigenic chinese hamster cells). Medium conditioned by exposure to these cells was able to reversibly inhibit growth and DNA synthesis of proliferating cultures not only of the same cell line but also of the Balb/c 3T3 A31 murine cell line (Koga et al, 1986).

Most of the examples of growth inhibition by regulatory factors have been illustrated in tissue culture systems and it remains to be determined whether knowledge about growth inhibitors can be applied to the intact animal. A possible role for growth inhibitors *in vivo* may be the prevention of overgrowth of tissues during wound healing. Thus there is limited evidence to suggest that cell proliferation may be controlled by the balance between growth promoting and inhibiting factors.

1.4 Receptors

Control of growth is governed by a signalling system that translates external information into a limited variety of internal signals termed 'second messengers'. The majority of hormones and growth factors signal their target cells usually through the formation of a recognition complex at the exterior of the plasma membrane, although the steroids involved in growth are thought to have their initial recognition interaction in the cytoplasm or nucleus of the cell (Clark & Peck, 1977).

For non-penetrating ligands the receipt and transduction of extracellular signals is a cell surface phenomenon dependent upon the existence of membrane associated receptors. The definition of a receptor is still evolving, but in its limited sense can be thought of as the initial recognition site on the cell surface with which a ligand interacts or binds (Kohn & Shifrin, 1982). The criteria that make a binding site a receptor are tissue specificity, stereospecificity, saturability, reversibility and high affinity (Rozengurt & Collins, 1983; Laduron, 1984). Most of the receptors or recognition molecules are large protein species which appear to be in a dynamic rather than a static state, both in terms of the turnover of these membrane localised entities (eg. *de novo* synthesis, internalization and degradation) and in terms of the mobility of certain receptors within the plane of the plasma membrane (Hollenberg, 1982; Helmreich & Elison, 1984). The cell surface receptor proteins span the membrane; the ligand binding domain protrudes from the external side of the cell surface and bears sugar residues which are thought to protect against proteolysis. Stability is also achieved by disulphide bridges between cysteine residues in the extracellular portion. Groups of charged basic amino acids just outside the membrane domain on the cytoplasmic face impose a steep energy barrier against movement of the receptor out of the plane of the membrane (Geisow, 1986). With the exception of insulin and IGF1 receptors,

PGF receptors are composed of single polypeptide chains. The molecular weight and subunit structures of the PGF receptors (except those for insulin and IGF1) do not show any obvious relationships despite their common involvement in growth regulation. This may indicate independent evolution of each ligand/receptor pair, despite the clear ancestral relationship of some of the hormones themselves. Alternatively, the techniques of analysis may have obscured similarities in their structures (James & Bradshaw, 1984).

Theoretically, hormone and PGF action can be regulated at many stages before, during and after their interaction with the cell surface receptor. Clearly receptor regulation can play an important role in modulating the cells response to an agonist. Desensitization is the tendency of biological responses to decrease over time despite the continuous presence of hormone or growth factor. Mitogenic desensitization can be achieved by two means; thus homologous or heterologous ligands can reduce either the number of receptors or alter the affinity of the receptor for its ligand (Rozengurt & Collins, 1983; Sibley & Lefkowitz, 1985). There is increasing evidence that receptor phosphorylation plays a key role in the regulation of receptor function; thus a major mechanism for heterologous desensitization of adenylate cyclase appears to be feedback regulation of the receptors via phosphorylation by the cAMP-dependent protein kinase (protein kinase A). In avian erythrocytes, following stimulation of adenylate cyclase by any of several hormones, intracellular cAMP levels rise, protein kinase A is activated and the ß adrenergic receptors become phosphorylated exclusively on serine residues (Stadel et al, 1983, 1986; Sibley et al, 1984a; Nambi et al, 1985). Protein kinase C is also able to phosphorylate the ß adrenergic receptor, thus treatment of intact erythrocytes with phorbol esters directly activates protein kinase C leading to receptor phosphorylation and desensitization (Sibley et al, 1984b; Kelleher et al, 1984). The inference is that protein kinase C activated by

phosphatidylinositol (PI) hydrolysis promoted by various ligand/receptor interactions may also play a role in some cases of heterologous phosphorylation and desensitization of the ß adrenergic receptor (Sibley et al, 1987). Other receptors coupled to PI turnover also exhibit agonist-induced desensitization. For example treatment with phorbol esters leads to the desensitization of the α_1-adrenergic receptor (Leeb-Lundberg et al, 1985). A classical negative feedback loop may exist such that diacylglycerol, one of the second messengers generated by PI hydrolysis, activates protein kinase C. This kinase subsequently causes the response to wane by phosphorylating and thus, desensitizing the α_1 adrenergic receptor (Sibley et al, 1987).

Certain of the plasma membrane receptors for polypeptide growth factors are themselves tyrosine kinases, these include EGF (Ushiro & Cohen, 1980; Carpenter, 1983) PDGF (Ek et al, 1982; Nishimura et al, 1982), insulin (Kasuga et al, 1982; Roth & Cassell, 1983; Kasuga et al, 1983) and IGF1 (Jacobs et al, 1983; Rubin et al, 1983). The receptor proteins span the membrane, the external portion binds the growth factor and a catalytic domain inside the cell accomplishes phosphorylation. Thus, the binding of the growth factor and tyrosine phosphorylation are activities of a single receptor molecule (Hunter, 1982; Sefton & Hunter, 1984; Hunter & Cooper, 1985). Receptor function of these PGFs may be regulated both by autophosphorylation of tyrosine residues and by phosphorylation catalyzed by other kinases.

Insulin receptors are integral membrane glycoproteins composed of 2α and 2ß subunits linked covalently by disulphide bonds. The ß subunit has a cytoplasmic domain that is homologous with the EGF receptor and the *src* family of protein-tyrosine kinases (Hunter, 1982; Hampe et al, 1984; Sefton & Hunter,

1984; Ullrich et al, 1985). When insulin binds to the α subunits, the kinase activity of the ß subunits is enhanced. In cell free conditions, insulin-stimulated phosphorylation of the insulin receptor occurs exclusively on several tyrosine residues (Van Obberghen et al, 1983; Czech, 1985; Kahn, 1985; Pike & Krebs, 1986; White & Kahn, 1986; Goren et al, 1987).

The receptor autophosphorylation bestows kinase activity upon the cytoplasmic domain and several exogenous substrates may be phosphorylated. Once activated in this way the receptor kinase becomes insulin-independent (Klein et al, 1986; Yu & Czech, 1986). In intact cells receptors undergo phosphorylation on serine and threonine residues in addition to tyrosine residues implying that other protein kinases may also be involved in insulin receptor regulation. Indeed it has been demonstrated that activation of protein kinase A and protein kinase C in intact cells by other agonists leads to phosphorylation of the insulin receptor, decreased insulin binding and reduced protein-tyrosine kinase activity (Takayama et al, 1984; Haring et al, 1986). Thus insulin receptors appear to be under positive control, through autophosphorylation of the ß subunit leading to enhanced tyrosine kinase activity toward exogenous substrates and negative control though phosphorylation of serine and threonine residues (Sibley et al, 1987).

Activation of the EGF receptor also leads to enhanced protein phosphorylations on tyrosine residues on the receptor molecule itself (autophosphorylation) and on intracellular proteins (Sefton & Hunter, 1984; Hunter & Cooper, 1985; Pike & Krebs, 1986). In intact cells, treatment with EGF leads to the EGF receptor being phosphorylated on serine and threonine residues in addition to tyrosine residues and it appears likely that these phosphorylations are involved in the regulation of receptor function (King & Copper, 1986; Chinkers & Garbers, 1986). Protein kinase C is able to phosphorylate threonine residues on the EGF receptor (Hunter et al, 1984; Davis

& Czech, 1985). Protein kinase C phosphorylation of the EGF receptor inhibits receptor tyrosine kinase activity (Cocher et al, 1984; Downward et al, 1985), it decreases receptor affinity for EGF (Davis & Czech, 1986a) and appears to promote internalization of the receptor possibly by phosphorylating threonine 654 (Beguinot et al, 1985; Fearn & King, 1985). Not only does EGF stimulation promote rapid autophosphorylation of tyrosine residues, it also induces phosphorylation of threonine 654 leading to receptor internalization and tyrosine kinase inhibition (Chinkers & Garbers, 1986). This pathway thus provides a means of homologous and heterologous desensitization. Heterologous ligands that activate protein kinase C may exert negative regulation on the EGF receptor. Indeed, many heterologous ligands upon binding with their own specific receptors on 3T3 cells inhibit EGF binding indirectly by diminishing the apparent affinity of the EGF receptor population for EGF. The term "transmodulation" has been ascribed to this novel mechanism whereby structurally unrelated growth promoting agents such as phorbol esters, vasopressin, FDGF and PDGF are able to alter the affinity of the plasma membrane receptors for EGF (Rozengurt et al, 1981, 1982; Rozengurt & Collins, 1983) It is tempting to speculate that these transmodulating agents decrease the number or affinity of EGF receptors via a common mechanism; the activation of protein kinase C. Indeed, PDGF and vasopressin indirectly activate protein kinase C by inducing the formation of its endogenous activator diacylglycerol following PI hydrolysis, in 3T3 cells (Brown et al, 1984). However, recent evidence suggests that phorbol esters and PDGF modulate EGF binding via different mechanisms (Olashaw et al, 1986). PDGF causes a more protracted loss of EGF binding which requires preliminary protein synthesis (Olashaw & Pledger, 1987). A remarkable correlation exists between the agents that reduce EGF binding in 3T3 cells and those that act synergistically with EGF to promote proliferation of these cells (Brown et al, 1984; Collins &

Rozengurt, 1984; Davis et al, 1985). However, despite these correlations a causal relationship between heterologous receptor modulation and mitogenesis is not yet established (Olashaw et al, 1986).

In general then, it may be said that phosphorylation of tyrosine confers enhanced activity of receptors whereas serine or threonine phosphorylation diminishes receptor function. It should be pointed out that growth factor stimulated tyrosine kinase activity of the receptor might catalyse the phosphorylation and activation of serine and threonine protein kinases which could result in a cascade of phosphorylations some of which could be significant in proliferative control (Pike, 1983).

Fundamental to an understanding of how tyrosine kinase regulates cell proliferation is the identification of the primary substrate(s) of the growth factor receptor associated tyrosine kinase activity. A likely candidate appears to be a protein p42 which is phosphorylated on tyrosine residues by several mitogenic agents (Phorbol ester, PDGF, EGF and IGF1), although the precise function of this protein awaits identification (Bishop et al, 1983; Cooper et al, 1984; Foukes & Rosner, 1985; Hunter et al, 1985; Sefton, 1985).

Receptor phosphorylation may also play a role in the regulation of receptor distribution; an emerging paradigm is that receptor phosphorylation promotes receptor internalization. Receptor-mediated endocytosis is a mechanism by which cells internalize many macromolecules including certain growth factors such as EGF, PDGF, Insulin, Interleukin 1 and 2. (Hopkins, 1985; Goldstein et al, 1985; Hopkins 1986a; Hopkins 1986b; Dunn et al, 1986; Duprez & Dautry-Varsat, 1986; Mizel et al, 1987; Sibley et al, 1987).

The process is initiated following ligand binding when these receptors slide laterally and cluster in specialized regions of the membrane, "coated pits". These invaginations of the plasma membrane have a highly ordered lattice,

constructed in the protein clathrin, which may be involved in trapping the ligand receptor complex. These clathrin coated pits invaginate into the cell within minutes and form coated endocytic vesicles. After shedding their clathrin coats several vesicles fuse to form endosomes. The ligand may then be carried to lysosomes for degradation whilst the receptors may cycle back to the cell surface. Alternatively both receptor and ligand may be degraded or both be recycled (Goldstein et al, 1985).

Phosphorylation of receptors is proposed as a potential mechanism for signalling entry possibly by promoting receptor binding to clathrin. Protein kinase C activity has been demonstrated to be associated with the phosphorylation of transferrin (Klausner et al, 1984) insulin (Hachiya et al, 1987) and EGF receptors (Fearn & King, 1985; Beguinot et al, 1985; Chinkers and Garbers, 1986) and their internalisation. Protein kinase C activation may thus represent a general signalling mechanism that modulates the internalization of the membrane receptors.

Receptor mediated endocytosis functions in the rapid control of receptor number and in the removal of the growth factor or hormone from the circulation (Carpenter & Cohen, 1979; Terris et al, 1979). This form of receptor modulation, "down regulation" also plays a key role in reducing a target cell's sensitivity. Thus it is apparent that exposure to physiological concentrations of ligand induces a rapid and sometimes prolonged removal of receptors from target cell surfaces (Beguinot et al, 1984; Stoscheck & Carpenter, 1984). Down regulation can easily be demonstrated in tissue culture (Soll et al, 1974) and in chronic conditions *in vivo* where high levels of hormone are continuously present. In diabetic conditions, obese hyperinsulinaemic individuals have their insulin receptor populations reduced by more than 80% below normal, this process of 'down regulation' is thought to be the most likely cause of the disease (Archer et al, 1975;

Soll et al, 1975). In less abnormal physiological conditions this form of receptor modulation is thought to occur in systems such as the reproductive system where there are pulsatile surges of hormone release and cyclical changes in receptor number (Dockray & Hopkins, 1982; Mooradian et al, 1987).

Heterologous ligands may also affect the number of receptors, for example treatment with phorbol esters reduces the number of EGF receptors in Hela cells (Lee & Weinstein, 1978, 1979). PDGF is thought to promote a transient down regulation of the EGF receptor in murine 3T3 cells (Wrann et al, 1980) in contrast to reports from other laboratories which suggest that PDGF mediates its effects on EGF binding in these cells by altering the affinity of the EGF receptor (Rozengurt & Collins, 1983).

Cellular desensitization to mitogenic ligands has thus the potential to regulate the proliferative response since it is crucial in determining the ultimate level of response. The interaction of the processes involving mitogenic desensitization and cell committment to DNA synthesis may provide a delicate mechanism to regulate growth control (Rozengurt & Collins, 1983). The extent of the proliferative response may also be effected by an 'up regulation' of hormone receptors in some instances (Hollenberg, 1982). Thus IL2 'up regulates' both high and low affinity IL2 receptors on T lymphoblasts (previously stimulated with lectin) (Diamantstein et al, 1986). In addition, an increase in the affinity of a receptor for its ligand may be promoted by heterologous ligands. Thus, a ten fold increase in the affinity of the IGF II receptor for its ligand is induced by insulin in isolated adipocytes (Oppenheimer et al, 1983) and rat hepatoma cells (Massague et al, 1982) despite the fact that IGF II receptor has essentially no affinity for insulin (Czech et al, 1983). Insulin is also believed to induce a redistribution of IGF II receptors from a microsomal compartment (which under basal conditions, contains most of the receptors) to the plasma membrane (Corvera & Czech, 1985).

Oestradiol has been shown to increase the number of EGF receptors 3 fold in rat uterine membranes within 12 hours of administration, probably by *de novo* synthesis of the EGF receptor. This raises the possibility that events coupled to the EGF receptor may play a role in oestrogen stimulated growth (Mukku & Stancel, 1985). Alternatively or in addition oestradiol may enhance the effect of EGF on growth.

Some PGF receptors such as those for insulin, (Krupp & Livingston, 1978) EGF (King & Cuatrecasas, 1982), NGF (Landreth & Shooter, 1980) and Interleukin-2 (Diamantstein et al, 1986) are reported to show two classes of affinity, as judged by the binding of radioiodinated ligand to various receptor preparations. In the case of NGF, it has been shown that the phaeochromocytoma PC12 cell possesses receptors with two different affinities, NGF binds first to receptors of low affinity and this binding induces a conversion of a proportion of the receptors to a higher affinity state. The change in affinity may be due to conformational changes in the receptor, interaction of the occupied receptor with other receptors or interaction with effector proteins in the plasma membrane. This change in receptor affinity may be a prerequisite for the internalization of the bound NGF or a sorting mechanism to select for only that NGF which is to be translocated to the nucleus (Landreth & Shooter, 1980; Hosang & Shooter, 1987). Thus, NGF-NGF receptor interaction is compatible with the mobile receptor hypothesis, receptors in the plane of the membrane change the apparent affinity with which they bind hormone when they interact with other effector molecules (possibly receptors) in the membrane (Jacobs & Cuatrecasas, 1976).

Classically, polypeptide hormones and growth factors bind to their specific receptors at the cell surface and initiate a chain of intracellular events mediated by second messengers which are considered in more detail in the next section. There is evidence however that certain polypeptide hormones and growth

factors also interact with the nuclei of their target cells (Burwen & Jones, 1987). Nuclear binding sites satisfy the criteria for hormone receptors, and have been demonstrated in target cells for EGF (Marti et al, 1987), NGF (Rakowicz-Szulczynska et al, 1986) and PDGF (Rakowicz-Szulczynska & Koprowski, 1986) by antireceptor antibodies raised against plasma membrane receptors. The origin of nuclear receptors is not known, they may be present before the arrival of the ligand to the cell and be distinct from their plasma membrane counterparts. Alternatively, they may be delivered to the nucleus together with their ligands in vesicles formed during receptor-mediated endocytosis (Burwen & Jones, 1987). A physiological role for nuclear receptors remains to be elucidated but may represent a mechanism for the phosphorylation of nuclear proteins by tyrsoine kinase activity present in the receptor molecule. Phosphorylation of specific nuclear proteins may result in a cascade of nuclear events involved in the initiation of mitosis (Burwen & Jones, 1987).

Although association of polypeptide hormones and growth factors with nuclei of target cells represents a novel alternative mechanism of effecting a biological response, steroid hormones are classically considered to interact with the genome following receptor binding. The mechanism of action of steroid hormones has generally been considered to incorporate the following stages. The steroid enters the target cell by simple diffusion and combines with a high affinity cytoplasmic receptor. The steroid-receptor complex undergoes tansformation (activation) and is transferred to the nucleus where it binds to selective sites in the chromatin. The hormone-receptor interaction with the genome leads to a modulation of RNA and protein synthesis. The synthesis of specifically induced proteins is finally responsible for the physiological response to the steroid (Jensen et al, 1968, 1982; Feldman et al, 1972; Edelman, 1975; Baxter & Harris, 1975).

More than 90% of steroid hormones that circulate in the blood are bound to plasma proteins; however it is the unbound fraction that is endowed with biological activity (Mooradian et al, 1987). The lipophilic nature of the hormone facilitates easy permeation of the plasma membrane by simple diffusion. However, the mechanisms of translocation of steroids across the membrane, the processes on and in the membrane and inside the cell regulating the influx are still subjects of conjecture. Thus, there is increasing evidence indicating that steroid hormones interact with components in the plasma membrane resulting in the transport of the hormones into the cell (Rao, 1981; Rao et al, 1977; Pietras & Szego, 1984). Once inside the cell, the hormone binds to a high affinity receptor, specific to the steroid and not to the response (Baulieu, 1984). The hormone acts to induce receptor transformation to the active chemical form that is capable of penetrating the nuclei (Gorski et al, 1968; Jensen & DeSombre, 1972). Although the precise mechanism(s) of transformation are unknown the possibilities include association or dissociation of subunits, conformational changes, addition or removal of cytosol factors, limited site-specific proteolysis and phosphorylation or dephosphorylation (Grody et al, 1982; Sherman & Stevens, 1984; Walters, 1985). The location of non-transformed receptor is particularly controversial (Schrader, 1984; Walters, 1985; King, 1986; Wolff et al, 1987; Jensen, 1987). Recent observations using monoclonal antibodies suggest that the unoccupied oestrogen receptor does not reside in the cytosol in intact cells as formerly thought (Jensen et al, 1968, 1972) but is exclusively found in the nucleus (King & Greene, 1984; Welshons et al, 1984). Furthermore similar observations have been made with the progesterone receptor (Perrot-Applanat et al, 1985), indicating that there is no translocation of the receptor from cytoplasm to nucleus under the influence of the hormone. Rather than being a compartmental shift from the cytosol to the nucleus, the change in receptor (receptor transformation), induced

upon hormone binding might be more subtle. Thus, steroid receptors may be permanently associated with the genes they regulate, and have only to rotate or undergo a conformational change to induce their effects (Shrader, 1984).

Nevertheless, steroid-receptor complexes are nuclear regulatory elements wherever they originate in the cell. The mechanism by which hormone receptor complexes increase specific transcription is still largely unknown (Baulieu, 1984). The activated or transformed receptor has a greater affinity for chromosomal sites in the cell nucleus; receptor association has been reported with almost all nuclear components; (Yamamoto & Alberts, 1976; Anderson, 1984). The selectivity of steroid action on transcription might reflect receptor binding to specific DNA sequences within or near regulated genes called steroid response elements (REs). Interaction of steroid receptors with REs may enhance transcription of specific proteins by potentiating the activity of particular transcription factors at nearby promoters, leading to enhanced mRNA synthesis, altered protein synthesis and ultimately regulation of cell function (Yamamoto, 1985). Alternatively, the selectivity of hormone-receptor action could reflect site specific receptor binding to a non-DNA 'acceptor' molecule. The avian progesterone receptor is claimed to interact with a particular class of non-histone proteins. This acceptor molecule may mediate the effect of the steroid-receptor complex and cause gene derepression perhaps by preventing production of repressor molecules (Thrall & Spelsberg, 1980; Yamamoto, 1985).

The numbers of steroid receptors in a given cell may vary in an analogous fashion to plasma membrane receptors under physiological circumstances. Thus, variations of progesterone receptor number are apparent in the guinea pig uterus during the oestrous cycle; the synthesis of progesterone receptors is induced by oestradiol at proestrous whilst progesterone causes 'down regulation' of its own receptor during the luteal phase (Baulieu et al, 1984).

Furthermore, it has been demonstrated that heterologous ligands such as EGF and insulin are able to phosphorylate tyrosine residues on hen oviduct progesterone receptor. The significance of phosphorylation of progesterone receptors, regarding their cellular functions has not yet been delineated although, ligand affinity, receptor transformation and/or affinity for nuclear sites could all potentially be modulated (Ghosh-Dastidar et al, 1983; Woo et al, 1986). Phosphorylation on tyrosine of the purified oestradiol receptor by an endogenous tyrosine kinase *in vitro* confers hormone binding capacity to this receptor (Migliaccio et al, 1984). More recently it appears that the oestradiol receptor is phosphorylated on tyrosine *in vivo*(Migliaccio et al, 1986). These findings illustrate that like PGF receptors, steroid receptors also interact with a protein tyrosine kinase. The fact that certain PGF receptors exhibit tyrosine kinase activity themselves whilst steroid receptors depend on endogenous tyrosine kinases for phosphorylation does not detract from the functional similarities between these receptors (Migliaccio et al, 1986).

Recent cloning and sequencing of oestradiol receptors (Walter et al, 1985; Green et al, 1986; Krust et al, 1986), glucocorticoid receptors (Hollenberg et al, 1985; Weinberger et al, 1985a, 1985b) has shown that both contain a large degree of homology with the v-*erb*-A gene product of the avian erythroblastosis virus (AEV). The AEV genome contains two cell-derived genes termed v-*erb*-A and v-*er*b-B. AEV causes erythroleukaemia in chickens; the transformation is due to the product of v-*erb*-B and is potentiated by v-*erb*-A gene product (Frykberg et al, 1983). 'v-*erb*-B' encodes a protein that is remarkably similar to the cytoplasmic portion (which incorporates the tyrosine kinase domain) of the EGF receptor (Downward et al, 1984). These homologies imply that the interaction between v-*erb*-A and v-*erb*-B gene products might be analogous to that observed between oestradiol receptor and the endogenous 'oestradiol receptor' tyrosine kinase

(Migliaccio et al, 1984). It follows from this hypothesis that there is likely to be homology between oestradiol receptor kinase and v-*erb*-B gene product. Because of the homology between v-*erb*-A gene product and the oestradiol and glucocorticoid receptors it was suggested that the gene product of c-*erb*-A (the cellular counterpart of v-*erb*-A) was likely to be a receptor for a steroid-related ligand (Krust et al, 1986). Surprisingly, however it has been recently demonstrated that the product of c-*erb*-A gene specifically binds thyroid hormone and may therefore be the thyroid hormone receptor (Sap et al, 1986; Weinberger et al, 1986). It thus appears that steroid and thyroid hormones which are neither structurally nor biosynthetically related have receptors which have evolved from a common ancestor gene. This implies that there is a large superfamily of genes whose products are transcriptional regulatory proteins. This superfamily of receptor genes contains potential oncogenes encoding 'enhancer' factors (*erb*-A gene products) which can bind to response elements in the genome and induce ultimately the synthesis of specific proteins leading to the cellular response. 'Altered' enhancer factors, by interfering with the transcriptional regulation of crucial target genes might be important in certain types of carcinogenesis (Sap et al, 1986; Weinberger et al, 1986; Green & Chambon, 1986). Steroids are known to stimulate the growth of certain tumours, this may reflect an ability to enhance the effect of an oncogene, thus steroid receptors from neoplastic tissues may differ as v-*erb*-A and c-*erb*-A do (Parker, 1987).

Thus, there is an increasing body of evidence which does not fit with the current conceptual framework that a sharp demarcation exists between the mechanism of action of polypeptide hormones/PGFs and steroid hormones. This is further amplified and complicated by the recognition that the classical receptor mediated pathway of hormone action is not sufficient to account for all the known effects of steroids. Thus, Baulieu and co-workers (1978) demonstrated that

several steroid hormones are able to promote the maturation of *Xenopus laevis* oocytes by reinitiating meiosis although these cells do not contain classical steroid cytosolic receptors. Recently, a plasma membrane steroid receptor has been identified in *Xenopus laevis* (Sadler & Maller, 1982, 1984; Sadler et al, 1985). Steroid binding sites have been demonstrated on plasma membranes in several tissues (Harrison et al, 1976; Koch et al, 1977; Pietras & Szego, 1979, 1980; Towle & Sze, 1983; Haukkamaa, 1987). Thus, there is some evidence to indicate that steroids may mediate some of their effects via plasma membrane receptors rather than their classical mode of action which involves binding to intracellular receptors (Duval et al, 1983).

1.5 Signal Transmission

The importance of the internalization of certain PGFs involved in growth by receptor mediated endocytosis in signalling mitosis is still largely unknown. In contrast, the role of internalization in regulating the sensitivity of the cell to the mitogen is firmly established (see section 1.4). Many of the hormones/PGFs which have been shown to internalize have both short term and longer term trophic effects on their target cells. Thus, EGF binding elicits a series of rapid events including tyrosine kinase activation of its receptor and increases in ion flux (Sawyer & Cohen, 1981; Carpenter, 1984). These effects occur within seconds and precede internalization; DNA synthesis begins many hours later and could feasibly depend in part upon internalization of the ligand/receptor complex as well as on the generation of second messengers. After internalization the receptor and hormone are subsequently degraded, probably by lysosomal proteases. The role of cellular degradation in the effect of EGF stimulated DNA synthesis in 3T3 cells has been investigated by the use of pharmacological agents which inhibit peptide degradation. The EGF stimulated DNA synthesis is not affected by inhibition of

its degradation suggesting that intracellular processing of EGF is not necessary for its mitogenic effects (Greenberg & Rozengurt, 1982). In other studies to determine the role of internalization in EGF-stimulated mitosis, receptor specific antibodies have been developed which will bind to the receptor but are unable to stimulate DNA synthesis unless they are cross-linked by another reagent (Shechter et al, 1979; Schreiber et al, 1983). These observations have led to the hypothesis that microclustering of receptors (a prelude to internalization) may play a role in the early stages of the mitotic signal (Schlessinger et al, 1983). However, since the initial internalization of EGF is not sufficient for EGF induced mitogenesis, the major role of internalization is thought by some to be the adjustment of the cells sensitivity to EGF (Aharonov et al, 1978). No consensus of opinion has yet been attained regarding internalization and mitosis (Hopkins, 1985). Perhaps both the rapid generation of second messengers and internalization are necessary preludes in the stimulation of DNA synthesis by certain PGFs and hormones.

Another mechanism whereby growth promoting factors may initiate metabolic activities leading to DNA synthesis without the necessity of a second messenger has been proposed. A growth factor or hormone, on binding to its specific receptor may cause a conformational change allowing the complex to diffuse laterally in the plane of the membrane, the "mobile receptor hypothesis" (Jacobs & Cuatrecasas, 1976). This ligand/receptor complex is able to interact with membrane localised effector systems for which the complex has high affinity and change their functional properties (Cuatrecasas, 1974; Rozengurt, 1976). Since changes in the organisation of microtubules can alter protein-protein interactions (Berlin et al, 1974), the effect on DNA synthesis of agents known to dis rupt the microtubule network has been investigated (Friedkin et al, 1979; Friedkin & Rozengurt, 1981). These agents were shown to enhance the initiation of DNA synthesis to a variety of mitogens although the mechanism remains to be

elucidated (Friedkin et al, 1979; Wang & Rozengurt, 1983). Alternatively, signal transduction might depend on the ability of the cytomatrix to transmit forces from the plasma membrane to the nucleus and components such as actin fibres, and microtubules might be involved (Packard, 1986).

Since these 'direct' effects of PGFs in the promotion of DNA synthesis and mitosis remain nebulous a great deal of energy is still directed towards gaining an understanding of the mechanism(s) of action of growth promoting factors and hormones. A central problem being to elucidate how after binding to specific receptors on the plasma membrane such mitogens are able to induce metabolic responses in the cell leading to proliferation since, after all, internalization of PGFs/hormones is far from universal. There is evidence suggesting the opening of ion permeability pathways through the plasma membrane, mobilization of ions from intracellular stores, alterations in the concentration of cyclic nucleotides, and phosphoinositide metabolism may play a role in signal transduction (Rozengurt, 1980, 1985; Rozengurt & Collins, 1983; James & Bradshaw, 1984; Paul, 1985; Downes & Michell, 1985; Berridge, 1986).

It is unlikely that the mechanism of action for all PGFs and classical hormones will be identical since it has already been acknowledged that the mitogenic responses to PGFs may be subdivided into 'progression' or 'competence'. Cells rendered 'competent' to replicate their DNA by PGFs in the 'competent' category may be stimulated to enter the S phase and subsequently mitosis by 'progression' factors. Additional evidence that growth factors do not operate through a universal mechanism is that some agents are able to initiate mitogenesis in a relatively short time whereas others require long periods of exposure to accomplish the same effect (James & Bradshaw, 1984). A remarkable feature of many mitogenic agents added to quiescent cells in serum-free medium is that they demonstrate synergy when administered in specific

combinations. These synergistic effects may be explained by their ability to trigger the generation of separate internal signals which act in a complimentary manner. This synergy can be further enhanced by the depolymerization of microtubules which is thought to act at a later stage (Rozengurt, 1980, 1985; Rozengurt & Collins, 1983). The regulation of cell proliferation by low concentrations of interacting growth factors may illustrate a flexible mechanism for the fine regulation of growth of individual cells in the intact animal (Rozengurt, 1980).

Thus, it becomes apparent that there are likely to be several intracellular signals which can couple the stimulus to the proliferative response, for a variety of growth factors and hormones in different cells, under different circumstances. In what follows, the early signals induced by PGFs/hormones leading to DNA synthesis and mitosis will be described, together with the possible interactions of these signal transduction pathways.

1.5.1 Cyclic nucleotides

cAMP has been implicated as an intermediate in the action of many peptide hormones since its discovery. Ligand-receptor binding induces membrane bound adenylate cyclase to catalyse the formation of cyclic AMP (Rall & Sutherland, 1962). Interposed between the receptor and effector molecule (adenylate cyclase) are two transducing or coupling proteins the G proteins (Gilman, 1984; Bourne et al, 1987). Each of these proteins binds Mg^{2+} and guanine nucleotides. The Gs protein is responsible for mediating the effects of stimulatory hormone receptors, the other, Gi protein mediates the effects of inhibitory hormone receptors (Codina et al, 1984; Jakobs et al, 1984; Birnbaumer et al, 1985). In fact adenylate cyclase systems to which receptors couple are three component systems formed of a catalytic unit C (which forms cyclic AMP plus

Mg PPi from the substrate Mg ATP) and the Gs and Gi proteins (Birnbaumer, 1985). The catalytic unit serves as a monitor of the activity states of Gs and Gi. Addition of a stimulatory hormone upon binding to its receptor activates the Gs protein which subsequently results in increased C activity and the formation of cAMP. Phosphodiesterase located in the cytosol is the enzyme responsible for the degradation of cAMP (Wells & Hardman, 1977). The increased concentration of cAMP acts as a second messenger activating its protein kinase which potentially results in further phosphorylation reactions leading to the biological response.

The role of cAMP in the regulation of cell proliferation is highly controversial (Pastan, 1975; Friedman et al, 1976; Rozengurt et al, 1981; Ralph, 1983). Original studies regarding the role of cyclic AMP in cell proliferation demonstrated that the presence of the cyclic nucleotide in the culture medium of fibroblasts inhibited cell doubling, cultures of growing cells contained lower intracellular cAMP concentrations than cultures of non-growing cells and furthermore, adding serum to growth arrested cells promoted a decline in intracellular cAMP levels (Otten, 1971; Froehlick & Rachmeler, 1972; Rudland et al, 1974). Thus it was generally believed that cAMP was an inhibitory regulator of cell proliferation (Kram, 1973). However it now seems apparent that cAMP is stimulatory during the entire proliferative process in certain cell types and that temporal increases in cAMP have positive regulatory effects in other cell types even though the continued presence of cAMP might inhibit subsequent events necessary for division (Haddox & Greenfield, 1982).

In 3T3 cells and other fibroblast cells, increased levels of cAMP were generally believed to reduce the rate of growth and inhibit the stimulation of DNA synthesis promoted by serum addition to quiescent cells (Willingham et al, 1972; Burger et al, 1972; Bombik & Berger, 1973). An objection to these studies however was that the effects were elicited by high concentrations of cAMP

analogues and could be regarded as non-specific (Friedman et al, 1976).

Further support for a possible 'negative' control of cell proliferation by cAMP comes from observations that cAMP at high intracellular concentrations usually stops growth in the G_1 phase of the cell cycle (Ralph, 1983). Moreover, the addition of serum to quiescent 3T3 fibroblasts has been shown to rapidly decrease the cellular cAMP content and promote entry into DNA synthesis (Pastan et al, 1975). Thus, there is considerable support for cAMP as a negative or inhibitory regulator controlling the normal mammalian cell cycle (Pardee et al, 1978). More recently the idea that cAMP inhibits growth has been challenged on the basis that cAMP analogues or cAMP elevating agents such as cholera toxin or prostaglandin E_1 act synergistically with specific mitogens to stimulate DNA synthesis in quiescent 3T3 cells (Rozengurt et al, 1981; Rozengurt, 1982a,b; Rozengurt, 1985). In addition, cAMP stimulates DNA synthesis in a variety of other cells, such as adult rat hepatocytes (Armato et al, 1981), cultured guinea pig skin (Carney, 1982), cartilage (Kawashima et al, 1980) and MDCK kidney epithelial cells (Taub et al, 1979). T51B rat liver cells deprived of Ca^{2+} also exhibit a pre-DNA synthesis surge of cAMP following Ca^{2+} addition (Boynton & Whitfield, 1981). It is believed that this cAMP surge and a subsequent burst of cAMP-PK activity are required for events in the late G_1 phase of the cell cycle (Boynton et al, 1985). Furthermore many other cells including fibroblasts and lymphocytes generate a transient cAMP surge as they near the end of the G_1 phase (Boynton & Whitfield, 1983). This cAMP surge is believed to result from the activation of the c-*ras* proto-oncogene. There is evidence that c-*ras* protein mimics the action of adenylate cyclase's GTP binding G_S subunit. The implication is that cells may trigger this replication-related burst of adenylate cyclase activity by producing the highly conserved GTP-binding c-*ras* proteins. An increase in

cAMP and therefore cAMP-PK activity activates a cdc (cell division cycle) master gene(s) whose products lead to chromosome replication (Whitfield et al, 1985; Whitfield et al, 1987). cAMP may also have a regulatory role in G_2 since a transient cAMP surge precedes both mitosis and meiosis in many kinds of cells (Boynton & Whitfield, 1983; Whitfield et al, 1987). This G_2 surge like the early G_1 surge must subside before the cell is able to progress to mitosis (Boynton & Whitfield, 1983). An interesting hypothesis is that the G_2 cAMP surge might trigger the production of maturation promotion factor (MPF) (Whitfield et al, 1987).

The inconsistent reports of cyclic AMP metabolism in relation to the growth of cultured cells referred to earlier can perhaps be accounted for now. Thus, it is apparent that increased intracellular cAMP concentrations are stimulatory at one stage of the cell cycle and inhibitory at another; therefore, the administration of cAMP analogues to synchronized cells in different phases of the cell cycle may contribute to the contrasting observations.

The role of cGMP in the regulation of cell proliferation is probably even more controversial than cAMP; this is because there is little evidence of any receptor-mediated modulation of cGMP. Moreover, there is no real evidence that changes in cGMP have physiological effects within the cell (Hunt & Martin, 1980; Houslay, 1985) A negative regulatory role is implicated in some cases; addition of serum to 3T3 fibroblasts induces a rapid fall in cGMP concentration and promotes cell growth (MacManus et al, 1978). In contrast, cGMP has a stimulatory effect on DNA synthesis in cultured guinea pig skin (Carney, 1982) and primary neonatal hepatocytes (Armato et al, 1981).

The role of cGMP in proliferation of lymphocytes also remains hotly contested (Kaever & Resch, 1985). There are reports implicating cGMP as the

second messenger for B-cell activation; mitogenic agents were found to raise the concentration of intracellular cGMP but not cAMP (Coffey et al, 1977) and exogenous cGMP, its derivatives or cGMP elevating agonists are able to promote a mitogenic response in several cells (Seifert & Rudland, 1974; Hadden et al, 1979). On the contrary, other workers found that mitogenesis elicited small increases of cAMP rather than cGMP (Wedner et al, 1975; Parker, 1976). Moreover, other workers failed to correlate changes in either cyclic nucleotide concentration with the mitogen stimulated DNA synthesis (Watson, 1976). Haddox and co-workers proposed the 'Yin-Yang' hypothesis, which suggested that cell proliferation may be controlled by the relative concentrations of cAMP and cGMP within the cell. A transient increase in cGMP and an associated fall in cAMP was thought to trigger the initiation of cell division (Goldberg et al, 1975). More recently the role of cGMP has been implicated in mediating the action of atrial natriuretic factor in certain tissues (Leitman, 1985). cGMP is also considered to play a role in visual transduction in rod photoreceptors. The cGMP concentration transiently decreases following illumination, due to the stimulation of cGMP phosphodiesterase by rhodopsin. Transducin, a member of the family of G proteins mediates this signal-coupling (Kwok-Keung Fung, 1985). Perhaps in a similar fashion the role of cGMP in cell proliferation will be resurrected. This indeed seems likely since the tumor promoting phorbol esters increase cGMP concentration in a variety of cell types probably as a result of protein kinase C activation which ultimately phosphorylates and activates guanylate cyclase (Zwiller et al, 1985).

The role of cyclic nucleotides in the initiation of cellular proliferation is therefore difficult to interpret and made more complex by the observation that the cations, Ca^{2+} and Mg^{2+} are intimately connected with the cyclic nucleotides in several ways. Thus, Mg^{2+} is an absolute requirement for adenylate cyclase

activity and indeed may regulate its activity (Maguire, 1984). Ca^{2+} has many interactions with the cAMP system causing a rise or fall in cAMP concentrations in different cells by activating phosphodiesterase and regulating adenylate cyclase activity (Rasmussen & Barrett, 1984). Moreover, Ca^{2+} ions stimulate guanylate cyclase activity and exogenous cGMP will promote an immediate cAMP surge in thymic lymphocytes (Whitfield, 1979). Thus, the precise roles of the cyclic nucleotides may not be delineated at present and remain controversial, it is possible that cells may use precise, temporally distinct fluctuations in the local intracellular concentrations of the molecules or in their ratio, as signals serving to regulate cell cycle progression. Other cellular components such as cations and protein kinase C may initiate or modulate such fluctuations which ultimately control the cellular response.

1.5.2 Calcium and Phosphoinositide Metabolism

Calcium ions play an essential role in many key biological processes such as maintenance of cellular organisation, nerve conduction, bone formation, coupling of stimuli to their mechanical, secretory or metabolic responses (Rasmussen, 1970; Rasmussen & Goodman, 1977; Rasmussen & Barrett, 1984; Campbell, 1986; Rasmussen et al, 1986). There is also substantial evidence that calcium is implicated in stimulus-mitosis coupling (Rebhun, 1977; Swierenga et al, 1981; Durham & Walton, 1982; Whitfield et al, 1979, 1985, 1987). The role of calcium as a second messenger for regulation of numerous physiological processes linking stimuli from extracellular origins to intracellular environments is possible due to the steep 10,000 fold calcium gradient which is known to exist across the plasma membrane (Rasmussen, 1970; Rasmussen & Barrett, 1984). Therefore when a small quantity of calcium enters cytoplasm from the external medium or from internal membrane bounded stores there can be a transient change

of several orders of magnitude in Ca^{2+} concentration, these intracellular Ca^{2+} transients regulate a variety of biological processes. This steep gradient can exist because under resting conditions the plasma membrane is relatively impermeable to Ca^{2+} and extrusion of Ca^{2+} from the cell to counteract the inevitable slight passive influx of ions is achieved by two mechanisms associated with the plasma membrane, an ATP dependent Ca^{2+} pump (Ca^{2+} ATPase) (Schatzman & Vincenzi, 1969; Huggins & England, 1985; Schatzman, 1985; Pederson & Carafoli, 1987) and by Na^{+}/Ca^{2+} exchange (Dipolo & Beange, 1983, 1986). The relative importance of the Ca^{2+} pump and Na^{+}/Ca^{2+} exchange mechanisms appear to vary between tissues (Cavero & Spedding, 1983; Huggins & England, 1985). In addition Ca^{2+} may be sequestered into intracellular organelles such as mitochondria, (Fiskum & Lehninger, 1980) and endoplasmic reticulum (Rasmussen & Barrett, 1984) or sarcoplasmic reticulum in some tissues (Feher & Briggs, 1982). Ca^{2+} ions may also be immobilised by forming complexes with acidic residues of phospholipids or proteins (Gill, 1982).

A rise in intracellular free Ca^{2+} may be accomplished in several ways, by influx of extracellular Ca^{2+} ions, mobilisation of Ca^{2+} from intracellular stores or by the inhibition of calcium extrusion. Ca^{2+} entry into the cell normally occurs through specific Ca^{2+} channels in the plasma membrane, the conductance of which is controlled by gates that are opened by critical changes in cellular polarisation (Voltage operated channel: VOC) or by receptor activation (Receptor operated channel: ROC) (Bolton, 1979). In smooth muscle a third channel, generally referred to as a Ca^{2+} leak into the cell is thought to be responsible for allowing Ca^{2+} into the resting cell, the resting influx is largely through these and is not susceptible to blockade by Ca^{2+} entry blockers (Bolton, 1985). Na^{+}/Ca^{2+} exchange may also enhance Ca^{2+} entry when intracellular Na^{+} is high (DiPolo & Beagué, 1986). The intracellular message generated by external stimuli is

transferred to the response mechanism by calcium receptor proteins that bind Ca^{2+} with high affinity. There are two classes of Ca^{2+} receptor proteins: (1) true Ca^{2+} receptor proteins such as calmodulin, troponin C, parvalbumin and myosin light chain, which have no intrinsic enzymatic activity; on binding to Ca^{2+} they undergo a conformational change that alters their association with other proteins, response elements (REs), thereby changing the activity of these REs; and (2) Ca^{2+} regulated enzymes which bind Ca^{2+} directly and have no Ca^{2+} binding subunit, eg. protein kinase C (Rasmussen & Barrett, 1984). A further group of tissue specific calcium binding proteins, distinct from calmodulin which respond to the calcium signal, are the calcimedins and related proteins (Owens et al, 1984; Smith & Dedman, 1986 Geisow & Walker, 1986; Heizman & Berchtold, 1987). Nevertheless, it is well known that calmodulin (CaM) mediates many of the Ca^{2+} regulated events in the eukaryotic cell and is the most universally distributed of the true Ca^{2+} receptor proteins (Wang & Waisman, 1979; Means & Dedman, 1980; Klee et al, 1980; Cheung, 1982; Klee & Newton, 1985). It is felt by some that for CaM to activate an enzyme at least three of the four Ca^{2+} binding sites on CaM must be occupied resulting in the formation of Ca_3 CaMRE and Ca_4 CaMRE, the high activity states of the response element (RE) (Cox, 1984; Rasmussen & Barratt, 1984). However, other workers have detected three different conformers of calmodulin the Ca^{2+} free protein, $Ca^{2+}{}_2$ calmodulin and the fully liganded calmodulin ($Ca^{2+}{}_4$ calmodulin) (Manalan & Klee, 1984; Klee & Newton, 1985). The different conformers may allow calmodulin to translate quantitative changes in Ca^{2+} concentration into qualitatively different physiological responses if different enzymes have the power to recognise the various conformers (Klee et al, 1980). Calmodulin has been shown to interact with an ever increasing number of enzymes and proteins and is thought to mediate directly or indirectly the Ca^{2+}

regulation of such activities as glycogen and cyclic nucleotide metabolism, protein phosphorylations, microtubule assembly, Ca^{2+} flux and cell division (Chafouleas & Means, 1982; Veigl et al, 1984; Klee & Newton, 1985).

When a hormone or other extracellular messenger interacts with its specific receptor and raises the level of intracellular calcium ions, the flow of information through the CaM branch of the Ca^{2+} messenger system is initiated. The increase in free cytosolic calcium concentration leads to the association of Ca^{2+} with binding sites on CaM. The binding of Ca^{2+} to these sites leads to activation of a variety of REs, the physiological response of the cell results from these activations (Rasmussen & Barrett, 1984).

There is increasing evidence that phosphoinositide metabolism plays a vital role in the Ca^{2+} messenger system. Michell and colleagues in the mid-1970's found that mobilisation of Ca^{2+} in the cytoplasm of cells stimulated by hormones, neurotransmitters and other agents acting via cell surface receptors was nearly always associated with an increased turnover of inositol phospholipids (Michell, 1975; Michell et al, 1977). The link between the phosphoinositide response and the mobilisation of calcium from intracellular stores is attributed to the second messenger inositol trisphosphate ($InsP_3$) (Berridge, 1984b, 1987; Berridge & Irvine, 1984).

This hypothesis is supported by the finding that $InsP_3$ induces the release of Ca^{2+} ions from the endoplasmic reticulum (ER) of permeabilised pancreatic cells (Streb et al, 1983) and hepatocytes (Burgess et al, 1984; Joseph et al, 1984). Inositol bisphosphate, inositol phosphate and inositol 1,2 cyclic phosphate were unable to release Ca^{2+} from the ER of permeabilised pancreatic cells, suggesting that the release mechanism is specific for the trisphosphate (Streb et al, 1983). This has been substantiated by testing a range of inositol phosphates for their ability to mobilise Ca^{2+} from intracellular stores in other cells (Berridge & Irvine,

1984). The available evidence suggests that Ca^{2+} is mobilised from the ER rather than the mitochondria (Prentki et al, 1984; Biden et al, 1984) whilst $InsP_3$ induces calcium release from sarcoplasmic reticulum of skeletal muscle (Volpe et al, 1985). It is considered that $InsP_3$ acts by binding to a specific intracellular receptor on the ER to stimulate release of Ca^{2+} ions (Burgess et al, 1984; Hirata et al, 1985; Spat et al, 1986). Occupancy of this receptor releases Ca^{2+} into the cytosol via an unknown mechanism, which may however, involve a G protein (Dawson, 1985). Despite the evidence that points to a role for inositol lipid breakdown in receptor controlled Ca^{2+} mobilisation there is an opposing view that the inositol lipid breakdown may be a response to the Ca^{2+} mobilised in certain cells by receptor activation rather than a cause of the Ca^{2+} mobilisation (Cockcroft et al, 1981, Cockcroft, 1984).

The perturbation of membrane phospholipids following ligand receptor binding is thought to be the transducing mechanism that initiates a signal cascade, resulting not only in the mobilisation of calcium, but also the activation of protein kinase C, the release of arachidonic acid and the stimulation of guanylate cyclase leading to cGMP formation (Berridge, 1984b, 1986, 1987; Berridge et al, 1985; Downes & Michell, 1985; Downes, 1985).

The initial event following agonist-receptor binding is the hydrolysis of phosphatidylinositol 4,5, bisphosphate to inositol trisphosphate ($InsP_3$) and diacylglycerol (DG) by phospholipase C, both of which have roles as intracellular messengers. These two second messengers are produced very rapidly, act at low concentrations and are removed by specific mechanisms once the external signal is removed (Berridge, 1984b, 1987).

As in the adenylate cyclase and rhodopsin/cGMP phosphodiesterase systems there is increasing evidence that a G protein may function in signal

transduction by coupling the surface receptor to phospholipase C (Wallace & Fain, 1985; Verghese et al, 1985; Cockcroft & Gomperts, 1985; Bradford & Rubin, 1986; Uhing et al, 1986; Baldassare & Fisher, 1986; Litosch & Fain, 1986). Evidence suggests that this G protein, Gp whilst related to other members of the ubiquitous family of G proteins (Dohlman et al, 1987; Bourne et al, 1987) is distinct from those involved in receptor regulation of adenylate cyclase (Merritt et al, 1986). When a hormone which mediates its effects by PI hydrolysis interacts with its receptor, the exchange of bound GDP to GTP on the α subunit of the G protein is catalysed. This leads to dissociation of the heterotrimer to α and $\beta\gamma$ subunits. The released α subunit activates phospholipase C and this enzyme catalyses the hydrolysis of PIP_2. The α-subunit possesses GTPase activity and so hydrolyses the bound GTP, thus terminating the action of phospholipase C (Cockcroft, 1987) The p21 protein encoded by the *ras* oncogene is also a typical G protein and is able to activate adenylate cyclase (Sadler et al, 1986). It may also have effects on phosphoinositide metabolism since *ras*-transformed cells produce inositol phosphates more efficiently than do normal cells in response to carbamylcholine (Chiarugi et al, 1985) and transformation by *ras* of two fibroblast cell lines (NIH 3T3 and NRK cells) leads to enhanced levels of DG and inositol phosphates (Fleischman et al, 1986; Wakelam et al, 1986).

The hydrolysis of PIP_2 represents a bifurcation in the signal pathway because both products function as second messengers. Diacylglycerol (DG) stimulates protein kinase C (PKC) (Nishizuka, 1984, 1986; Takai et al, 1985) and decreases the apparent K_m a and b for the calcium-mediated activation of protein kinase C (Kishimoto et al, 1980). A prior elevation in cytosolic Ca^{2+} ions may enhance the activation of the enzyme by diacylglycerol illustrating the co-operative nature of PKC activation by both Ca^{2+} and DG (Wolf et al, 1985; Dougherty &

Niedel, 1986). During the activation of PKC, a process which also requires phosphatidylserine (Takai et al, 1979), the enzyme seems to move from the cytosol onto the membrane. Thus, there is evidence that IL2 and IL3 upon binding to their receptors promote a rapid and transient redistribution of PKC from the cytosol to the membrane (Farrar et al, 1985; Farrar & Anderson, 1985). Since IL3 does not appear to induce phosphoinositide turnover, it seems that certain PGFs might be able to activate PKC without generating DG (Berridge, 1987). Independent activation of PKC may be explained by the discovery of multiple forms of this enzyme (Coussens et al, 1986; Woodgett & Hunter, 1987). Although IL2 and IL3 share a common feature of transmembrane signalling with phorbol esters, ie. translocation of PKC, other growth factors do not appear to. In fibroblasts treated with phorbol ester, translocation of PKC was apparent whilst PDGF, FGF and Bombesin did not alter intracellular PKC localisation (Halsey et al, 1987).

The DG/PKC pathway is likely to play a significant role in cell proliferation in certain instances since the tumour promoting phorbol esters act by mimicking DG (Castagna et al, 1982) indeed PKC is believed to be the cellular receptor for phorbol esters (Fiyika et al, 1984; Parker et al, 1984). Activated PKC has been shown to phosphorylate many different proteins when studied *in vitro* , the identification of substrate proteins in the intact cell has been more problematical although vinculin (Werth & Pastan, 1984) is phosphorylated and the Na^+/H^+ exchanger is believed to be activated by PKC. Evidence for this is that not only do many growth factors promote an increase in pH when stimulating cells to divide but that phorbol esters have also been shown to induce an increase in pH presumed to be under the control of DG/PKC pathway (Moolenaar et al, 1983; Takai et al, 1985).

The two second messengers generated following hydrolysis of PIP_2 activate two separate ionic events both of which have been implicated in the control of cell proliferation (Michell, 1979, 1982ab; Berridge, 1984a; Downes & Michell, 1985). Further evidence for the role of inositol lipid turnover in the generation of signals that control cell proliferation is that PDGF a growth factor that stimulates PIP_2 hydrolysis is encoded by the proto-oncogene c-*sis* (Doolittle et al, 1983; Robbins et al, 1983; Waterfield et al, 1983). and two 'tyrosine kinase' oncogenes *src* and *ras* stimulate polyphoshoinositide synthesis probably by encoding inositol lipid kinases (Sugimoto et al, 1984; Macara, 1984). The possible role of oncogenes in encoding proteins implicated in the inositol lipid signalling system will be discussed in more detail in Section 1.6.

The concept that the two limbs of the phosphoinositide metabolism pathway act synergistically with each other to regulate cell processes including cell proliferation is based on the findings that the effects triggered by agonists could be induced by a combination of calcium ionophore and phorbol ester. The calcium ionophore mimicks the effect of $InsP_3$ in raising intracellular calcium whereas the phorbol ester activates PKC. Many different cellular processes including DNA synthesis in lymphocytes can be activated by a combination of these two agents (Mastro & Smith, 1983; Truneh et al, 1985; Guy et al, 1985; Klaus et al, 1986).

An emerging paradigm is that growth factors and other agents belonging to the "competence" category eg. (PDGF, thrombin, phytohaemagglutinin) are capable of stimulating rapid inositol lipid breakdown. Another group of agents such as vasopressin and bradykinin stimulate PIP_2 hydrolysis and synergise with insulin and/or EGF in stimulating fibroblast proliferation and therefore seem likely to act as competence factors (Macara, 1984). These 'competence' stimuli produce a rapid activation of c-*fos* proto oncogene and accumulation of the nuclear protein

that it encodes (Greenberg & Ziff, 1984; Kruijer et al, 1984; Muller et al, 1984). This is followed by similar activation and expression of the c-*myc* proto-oncogene (Kelly et al, 1983). Thus, PIP_2 hydrolysis might constitute a 'competence' signal that would allow cells to divide on exposure to an appropriate progression signal such as insulin and/or EGF. However, a cautious approach in accepting this hypothesis is necessary since enhanced PIP_2 hydrolysis is not restricted to the critical G_0/G_1 point where competence factors act. Thus ,stimulated inositol lipid turnover is sustained in dividing cells (Halenda & Feinstein, 1984) and rapid inositol lipid turnover has been demonstrated during the G_1 and S phases of the cell cycle (Rampini & Dubois, 1981) the period during which the 'progression' factors are thought to act.

A link between the turnover of phosphatidylinositol and mobilisation of intracellular calcium in signal transduction is fairly well established, but it is also believed that the hydrolysis of PIP_2 may have a role to play in the entry of calcium across the plasma membrane (Michell et al, 1977; Michell, 1982a). In several types of cells, stimulation by receptors which trigger an initial, rapid increase in the intracellular free Ca^{2+} concentration also give rise to a second, more prolonged elevated rise in Ca^{2+} concentrations which appears to be dependent on the concentration of extracellular Ca^{2+} (Putney, 1978; Joseph et al, 1985; Hesketh et al, 1985). Several possible molecular mechanisms underlying the control of receptor operated channels have been proposed. One idea advanced to explain how hydrolysis of these phospholipids might lead to a Ca^{2+} influx is that phosphatidic acid (PA) formed from DG may function as a calcium ionophore (Putney et al, 1980; Salmon & Honeyman, 1980; Mitchell, 1982a). Other workers consider that the Ca^{2+} influx which accompanies the phosphatidylinositol

turnover is not due to the ionophoretic action of PA but to Na^+/Ca^{2+} exchange. Thus, treatment of isolated canine cardiac sarcolemmal vesicles with phospholipase D (which converts phospholipids to phosphatidic acid) increases Na^+/Ca^{2+} exchange activity by up to 400% (Philipson & Nishimoto, 1984). The effect was specific for Na^+/Ca^{2+} exchange and the enhanced Ca^{2+} uptake was Na^+ gradient dependent. Electrogenic Na^+/Ca^{2+} exchange can transport Ca^{2+} in both directions across the plasma membrane (Aickin et al, 1984). Whether stimulation of Na^+/Ca^{2+} exchange by PA will result in a net influx or efflux of Ca^{2+} will be a function of the ionic gradient and membrane potential. PA stimulated Na^+/Ca^{2+} exchange might also be important in returning elevated cytosolic Ca^{2+} to its normal level following the phosphoinositide response (Philipson & Nishimoto, 1984). A more recent model for receptor-regulated entry of calcium proposes that the initial emptying of the intracellular Ca^{2+} pool by $InsP_3$ leads to an opening of a pathway from the extracellular milieu to the intracellular pool, this fills with Ca^{2+} from outside the cell and is subsequently released into the cytosol by the continued presence of $InsP_3$ (Putney, 1986).

Even more recently a number of novel inositol phosphates have been shown to be produced upon hormonal stimulation of cells, some of which may in the future be designated second messengers (Palmer et al, 1986; Michell, 1986). Indeed one of these, $Ins(1,3,4,5)P_4$ is thought to play an important function in modulating intracellular Ca^{2+} levels. Phosphorylation of $1,4,5IP_3$ a step that may be stimulated by the increased cytosolic Ca^{2+} concentration (following mobilisation of internal stores) (Michell, 1986b; Biden & Wollheim, 1986; Zilberman et al, 1987) gives $InsP_4$. $InsP_4$ is believed to regulate transfer of Ca^{2+} from the extracellular space to the intracellular ER pool. For as long as $1,4,5,IP_3$

(and/or 1,3,4IP_3) is present the ER Ca^{2+} gate is open and Ca^{2+} can enter the cytosol from the extracellular milieu via the ER (Irvine & Moor, 1986; Nahorski & Batty, 1986; Houslay, 1987; Taylor, 1987).

Despite the recent interest linking hydrolysis of membrane phosphoinositides with calcium gating and calcium mobilisation from intracellular stores following ligand receptor binding, in some instances agonists may be capable of opening calcium channels in the plasma membrane without any change in phosphoinositide metabolism. But no matter how an increase in $[Ca^{2+}]i$ is achieved, termination of the cell's response is the reduction in the cytosolic Ca^{2+} concentration which may be accomplished by Na^+/Ca^{2+} exchange and/or by energy dependent pumps which extrude Ca^{2+} from the cell or store it in subcellular organelles. Accumulated Ca^{2+} is stored largely in the mitochondrial matrix (Fiskum & Lehninger, 1982; Akerman & Nicholls, 1983). It is slowly lost from this pool into the cytosol and then into the extracellular environment without flooding the cytosol, so that reactivation of the cell does not occur (Rasmussen & Barrett, 1984). The role of mitochondria in regulating the cytosolic calcium concentration is questioned by some workers who feel that the major role of the Ca^{2+} transport system in mitochondria is to relay changes in cytoplasmic Ca^{2+} concentrations into the mitochondrial matrix. Ca^{2+} ions stimulate intramitochondrial oxidative metabolism, thus actively promoting the synthesis of ATP. The increase in ATP supply meets the enhanced demand of energy requiring processes in the stimulated cell. They consider that Ca^{2+} ions should be considered to act as a second messenger in 'stimulus-response-metabolism' coupling (Denton & McCormack, 1980, 1985; McCormack & Denton, 1986).

The evidence presented here illustrates that Ca^{2+} is a potential major second messenger for regulation of numerous physiological processes. Direct evidence implicates Ca^{2+} in the regulation of cell proliferation and transformation.

The evidence is clearest with regard to egg cells; following activation a rise in intracellular Ca^{2+} concentration has been directly observed using the photoprotein aequorin (Cuthbertson et al, 1987). Lymphocytes in culture can be activated into 'capping' and ultimately into DNA synthesis by a variety of agents; extracellular Ca^{2+} has been shown to be essential for activation and that a small rise in cytosolic Ca^{2+} ions ($[Ca^{2+}]i$) occurs in response to mitogenic doses of plant lectins (Tsien et al, 1982; Lichtman et al, 1983; Hesketh et al, 1983). Serum, PDGF & EGF immediately raise the $[Ca^{2+}]i$ in human fibroblasts (Moolenaar et al, 1984; Mix et al, 1984). The elevation of Ca^{2+} concentration in the external medium may under certain conditions trigger resting 3T3 cells to divide (Dulbecco & Elkington, 1975). Furthermore an increase in $[Ca^{2+}]i$ following serum stimulation has also been observed in swiss 3T3 cells and is thought to involve the phosphoinositide signal pathway (Lopez-Rivas & Rozengurt, 1983; McNeil et al, 1985). Many individual growth factors and other mitogens have also been demonstrated to cause rapid transient increases in $[Ca^{2+}]i$ in swiss 3T3 cells (Morris et al, 1984; Ives & Daniel, 1987).

There is also evidence that Ca^{2+} controls cell replication *in vivo*; cellular proliferation in rat liver following partial hepatectomy is prevented by hypocalcaemia induced by prior parathyroidectomy (Rixon & Whitfield, 1976). DNA snythesis may be restored by appropriately timed calcium chloride or parathyroid hormone administration post-hepatectomy. The isoproterenol-induced activation of the rat parotid gland cells is similarly inhibited by prior removal of the parathyroid glands to elicit hypocalcaemia and may be reversed by appropriate hormone administration (Tsang et al, 1981).

The evidence suggests therefore that in normal cells an adequate supply of calcium is necessary for cell proliferation; the Ca^{2+} sensitive event may be at the late G_1/S boundary (Whitfield et al, 1981, 1985) or early in the G_1 phase of the

cycle (Paul & Ristow, 1979). In resting lymphocytes it is believed that the transition of cells from the resting state G_0 into the cell cycle is regulated by an increase in the $[Ca^{2+}]i$ above a critical point, cells remain in the cell cycle unless $[Ca^{2+}]i$ falls below a lower limit in which case the cells enter G_0 quiescent phase. If the $[Ca^{2+}]i$ rises above an upper limit of about 1μM cells are inhibited and if prolonged the high concentration of $[Ca^{2+}]i$ may be cytotoxic (Metcalfe et al, 1980; Hesketh et al, 1982). Since it is now well established that calmodulin mediates many of the Ca^{2+} regulated events in the eukayotic cell (Wang & Waisman, 1979; Means & Dedman, 1980; Vergl et al, 1984) it is not surprising that calmodulin may also play a role in cell cycle progression. Indeed when calmodulin levels were determined in synchronised CHO-K1 cells throughout the cell cycle, it was found that during late G_1 and/or early S phases, calmodulin levels begin to increase and reach the maximum level at the beginning of the M phase (Chafouleas & Means, 1982; Sasaki & Hidaka, 1982). Although increased total calmodulin levels have been observed in these and other cultured cells prior to or just after the onset of DNA synthesis (Criss & Kakiuchi, 1982; Bourgeade et al, 1983; Boynton & Whitfield, 1983) the levels remain unchanged in certain NRK cells and parotid cells (Conner et al, 1983; Durkin et al, 1983; Gonzales et al, 1984). One hypothesis is that Ca^{2+} calmodulin must reach a critical concentration to enable progression from G_1 to S to occur. Specific functions that Ca^{2+} calmodulin performs which may be important at the G_1/S transition are regulation of the cytoskeleton, protein phosphorylations in general and histone phosphorylation in particular (Chafouleas & Means, 1982). One of the proteins that may be activated by calcicalmodulin is the cytoplasmic stimulatory factor which can induce DNA synthesis in isolated nuclei; this protein may represent the

molecular trigger for the G_0/G_1 —> S transition (Gutowski & Cohen, 1983).

In addition to acting in concert with the DG/PKC limb of the PIP_2 hydrolysis pathway, Ca^{2+} or Ca^{2+}. calmodulin often functions in concert with cAMP to couple stimulus to response (Rasmussen & Goodman, 1977; Rasmussen & Barrett, 1984). Likewise in stimulus-mitosis coupling, calcium does not always act alone, the co-trigger of the late G_1 events in some cells is a transient cAMP surge which precedes the initiation of DNA synthesis (Whitfield, 1982; Whitfield et al, 1985). A model has been proposed to account for their interaction in promoting DNA synthesis. A rise in intracellular calcium activates calmodulin which in turn stimulates phospholipase A_2, this releases arachidonate from various phospholipids. Arachidonate is converted to a prostaglandin which stimulates adenylate cyclase causing a transient increase in cAMP concentration. The cAMP binds to a type II cAMP dependent protein kinase which is translocated to the nucleus and pohosphorylates an unidentified protein or proteins leading to the initiation of DNA synthesis (Gillies, 1982). Interaction between the two second messengers may occur in controlling the phosphorylation state of proteins, thus a cAMP dependent protein kinase might control the phosphorylation of one protein in an enzyme cascade whilst a Ca^{2+} dependent protein kinase controls the phosphorylation of a subsequent enzyme in the cascade (Rasmussen & Barrett, 1984). In addition, in some circumstances both Ca^{2+} and cAMP dependent protein kinases may catalyse the phosphorylation state of the same substrate protein at different phosphorylation sites (Le Peuch et al, 1979, 1982).

Evidence has been presented which demonstrates the ability of some second messengers to act in combination. It appears that the hydrolysis of membrane phospholipids may represent a fundamental transducing mechanism that initiates a signal cascade comprising a variety of putative second messengers

which act co-operatively. Thus, hormones and growth factors which bind to receptors that hydrolyse phosphoinositides have the potential to produce Ca^{2+} signals, to activate protein kinase C which may lead to activation of a neutral Na^+/H^+ exchange carrier (Vicentini & Villereal, 1986). Both an increase in pH and in cytosolic Na^+ concentration have been implicated as signals which may induce proliferation (Cassell et al, 1983; Moolenaar et al, 1983). In some cells arachidonic acid may also be released (Irvine et al, 1982; Rittenhouse, 1982) which may be converted to prostaglandins, thromboxanes and leukotrienes (Berridge, 1984, 1987).

Receptors involved in PIP_2 hydrolysis also appear to be linked to the generation of cGMP (Michell, 1975; Berridge, 1981). It has been suggested that guanylate cyclase may be regulated by either arachidonic acid or one of its metabolites (Peach, 1981; Takai et al, 1982; Gerzer et al, 1983). Thus, the generation of several of these putative second messengers appears to be inexorably linked. Interaction of some or all of these second messengers may contribute to the final response, the different signals may also provide the versatility necessary to introduce subtle variations in the control mechanisms. These second messengers also interact at other stages in the signal pathway. Thus, although it is evident that PIP_2 hydrolysis leads to mobilisation of Ca^{2+} ions, it is also recognised that PIP_2 hydrolysis depends on Ca^{2+} ions. Phospholipase C (PLC) the enzyme responsible for the hydrolysis of PIP_2 and subsequent production of the two second messengers DG and $InsP_3$ requires at least the normal resting intracellular Ca^{2+} concentration (Smith et al, 1986). Indeed at lower concentrations of $[Ca^{2+}]i$ the activity of the enzyme is reduced or even abolished (Moscat et al, 1986; Renard et al, 1987). Moreover since Ca^{2+} ionophores

stimulate the phosphatidylinositol cycle in several types of cells (Laychock, 1983; Charest et al, 1985; Murayama & Ui, 1985) it appears that Ca^{2+} ionophores induce increases in $[Ca^{2+}]i$ which are capable of activating phospholipase C without acting through a specific receptor coupling system (Moscat et al, 1986; Cockcroft, 1987). Similarly, high Ca^{2+} concentrations (1mM) are able to activate PLC in polymorphonuclear leukocyte membrane preparations in the absence of another stimulus (Smith et al, 1985).

Calcium ions are also required for the activation of protein kinase C (PKC) in addition to phospholipids and diacylglycerol (Takai, 1979; Schwantke et al, 1985). It appears that when a hormone or growth factor activates a cell via the phosphoinositide signal pathway the initial transient rise in $[Ca^{2+}]i$ is important not only in activating the CaM branch but also in determining the amount of PKC that becomes membrane bound and activated (Rasmussen & Barrett, 1984). The emerging hypothesis is that when the calcium messenger system couples stimulus to sustained cellular response, the flow of information occurs via two distinct branches which have distinct temporal roles. The CaM branch is involved in initiating the response and the PKC branch in sustaining the response. The magnitude of the sustained response appears to depend on the amount of PKC associated with the membrane and the rate of Ca^{2+} cycling across the membrane (Rasmussen et al, 1986). cAMP is also capable of modulating the Ca^{2+} messenger system, a rise in cAMP can lead to either a negative or a positive change in the set point depending on the particular tissue (Rasmussen et al, 1986). In a similar vein, PKC activation also has effects on cAMP formation; in intact platelets activation of PKC with phorbol ester leads to inhibition of prostaglandin and forskolin stimulated cAMP formation (Williams et al, 1987), whereas in certain other cells (rat pinealocyte and phaeochromocytoma cells) activation of PKC enhances agonist induced cAMP formation (Sugden et al, 1985;

Hollingsworth et al, 1986).

1.5.3 Magnesium

Although calcium ions have been implicated in the control of cell division and the cell cycle for a long time it is only relatively recently that magnesium has also been recognised as a possible regulator of cell proliferation (Rubin, 1975; Sanui & Rubin, 1982a; Walker & Duffus, 1983; Walker, 1986). Magnesium is thought to be ideally suited to play a primary role in the co-ordinate control of metabolism and cell proliferation since it is required as a cofactor by more intracellular enzymes than any other inorganic cation.

In mammalian cells total intracellular magnesium is maintained at approximately 10 mM over a broad range of external Mg^{2+} concentrations suggesting an efficient homeostatic mechanism (Rink et al, 1982). Little is known about cellular magnesium homeostasis although a specific magnesium transport system has been described (Maguire & Grubbs, 1983). In addition an inwardly directed Mg^{2+} pump has been implicated but not characterised in mammalian cell membranes (Sanui & Rubin, 1982b). Studies with chicken erythrocytes have indicated that the regulation of intracellular free Mg^{2+} is achieved by a gating process at the cell surface which is active at increased concentrations of intracellular free Mg^{2+} allowing exchange of 1 Mg^{2+} for 2 Na^{+} until the physiological level of free Mg^{2+} is reached. This efflux is independent of calcium (Gunther et al, 1984; Gunther & Vormann, 1986). The measurement of free Mg^{2+}, performed by several different methods gives a value of approximately 1 mM (Rink et al, 1982), which is in the range of, or below, the concentration necessary for maximum activity of many magnesium dependent enzymes (Sanui & Rubin, 1982b). Thus, there is the possibility that small changes in intracellular Mg^{2+} levels may have far reaching effects on cellular metabolism and proliferation. Evidence which supports the hypothesis that Mg^{2+} may act as a

second messenger is that Mg^{2+} transport and therefore internal Mg^{2+} concentrations are subject to hormonal control. ACTH, adrenaline and noradrenaline cause an elevation in the accumulation of Mg^{2+} by adipocyte plasma membrane vesicles (Elliot & Rizack, 1974). Insulin specifically induces Mg^{2+} uptake in fibroblasts and adipocytes (Cech et al, 1980). Enhanced magnesium uptake has also been demonstrated in rat pancreatic islet cells by stimulators of ß-cell function (Henquin et al, 1983, 1986). A hormone sensitive Mg^{2+} transport system has been demonstrated in murine S49 lymphocytes wherein Mg^{2+} influx is inhibited by activation of ß adrenergic receptors. The hypothesis is that free Mg^{2+} has an important role in the regulation of hormonal responsiveness; following hormone-receptor interaction a subcytoplasmic pool becomes depleted of Mg^{2+} such that a second hormonal stimulation would elicit a decreased response both from a lack of Mg^{2+} to activate adenylate cyclase and from a lack of Mg^{2+} to form the initial high affinity agonist receptor complex (mediated by the G protein), (Maguire, 1984). A similar hormone sensitive Mg^{2+} transport system has been demonstrated in other cell lines and in response to other hormones (eg.PGE_1) and adenosine, and it may be that hormonal modulation of Mg^{2+} transport leading to desensitisation is a general property of all receptor cyclase systems regardless of the hormone involved (Erdos & Maguire, 1983).

There is evidence that Mg^{2+} can modulate hormone stimulated responses in two distinct ways; firstly by interfering with the Ca^{2+} homeostatic system and thus mediating its effect by ultimately altering the intracellular Ca^{2+} concentration and secondly by influencing ligand-receptor interaction. Magnesium influences intracellular calcium homeostasis in several ways, it can regulate both calcium exit and entry processes and influence intracellular distribution. Thus, magnesium stimulates Ca^{2+} influx into sarcoplasmic reticulum and inhibits calcium dependent efflux (Stephenson, 1981). Magnesium may also retard calcium efflux into the

extracellular space (Altura & Altura, 1981) or when the Mg^{2+} concentrations are lower, promote efflux (Turlapaty & Altura, 1978). Thus, in this manner, Mg^{2+}may act as a physiological regulator of cytosolic Ca^{2+} levels within the cell so that Ca^{2+} ultimately assumes the role of second messenger.

Extracellular magnesium ions can induce an increase in the number of functional surface receptors available to an agonist (Pearlmutter & Soloff, 1979) and thus it is feasible that magnesium may help to stimulate the cellular response by causing the hormone to bind to more receptors. Physiological levels of Mg^{2+} induce a high affinity state of the ß receptor in many cellular systems including S49 cells (Maguire & Erdos, 1978). The role of Mg^{2+} in stimulus response coupling in systems utilising adenylate cyclase/cAMP formation is very important. A Mg^{2+} site on the G protein is responsible for altering the ability of the GTP regulatory unit to modulate both agonist affinity for the hormone receptor and the interaction of the catalytic subunit with the GTP regulatory unit (Cech & Maguire, 1982; Higashijima et al, 1987). A further Mg^{2+} site on the catalytic subunit is responsible for Mg^{2+} activation of catalytic activity (Maguire, 1984). In most cells the free Mg^{2+} concentration is insufficient to promote this activity, however the mechanism of hormonal activation of adenylate cyclase involves a marked increase in enzyme affinity for free Mg^{2+} and allows a higher rate of cAMP synthesis (Cech et al, 1980; Iyengar & Birnbaumer, 1982).

It thus appears that free Mg^{2+} has the ability to perform important roles in the regulation of cell function following ligand receptor interaction (Grubbs & Maguire, 1987). Indeed there are those who consider that the intracellular regulation of magnesium ions may represent a plausible mechanism for the control of events leading to the initiation of DNA synthesis and cell division (Rubin, 1975, 1977; Rubin et al, 1979; Sanui & Rubin, 1982b). Rubin (1975, 1977) proposed that this cation can regulate the 'co-ordinate' response reactions which

follow growth stimulation in animal cells. The hypothesis is that small changes in intracellular Mg^{2+} concentration alter the kinetics of many enzymes which in turn determine the rate of cell proliferation. Evidence of an important role for Mg^{2+} in cell proliferation is illustrated by the dependence of various cells on the availability of extracellular magnesium (Wyatt, 1961; Kamine & Rubin, 1976; McKeehan & Ham, 1978; Rubin & Chu, 1978; Abboud et al, 1985). A number of studies have correlated intracellular magnesium levels with rates of cell proliferation. Thus, it appears that elevated intracellular magnesium concentrations are needed to maintain rapid rates of normal cell division (Cameron et al, 1980). Rubin (1975) has demonstrated that intracellular magnesium rather than calcium is closely linked to growth of fibroblasts and believes that magnesium may be the major factor in governing proliferation rate in these cells. More recently studies with lymphocytes show that cells in different phases of growth contain varying amounts of cell magnesium such that low concentrations of Mg^{2+} correspond with low proliferative activity (Hosseini & Elin, 1985). It is believed by Rubin and co-workers that the effects of calcium on cell proliferation may be indirect, mediated by altering Mg^{2+} availability within the cell (Rubin, 1975; Rubin & Doide, 1976). An alternative view is that the observed effects of magnesium on cell proliferation may be due to alterations in calcium homeostasis (Levine & Coburn, 1984). Regulation of intracellular Ca^{2+} by the outwardly directed plasma membrane pump, mitochondrial and ER sequestration are all subject to Mg^{2+} alterations (Nicholls, 1978; Black et al, 1980; Pershadsingh et al, 1980).

Although the relative importance of Ca^{2+} and Mg^{2+} in the regulation of cell proliferation is the subject of much controversy (Berridge, 1975; Rubin, 1976; Sanui & Rubin, 1982b), there is evidence that in some cells at least, both of these cations are important. Calcium is believed to be the trigger for late G_1 progression with more of a permissive role for magnesium throughout the cell cycle

(Whitfield, 1982).

Human diploid 'fibroblast' like cells derived from fetal lung have an absolute requirement for both Ca^{2+} and Mg^{2+} even when the other is present in saturating amounts. Initiation of DNA synthesis and mitosis is mediated by a Ca^{2+} dependent process followed by a magnesium dependent process (McKeehan & Ham, 1978). NGF stimulated neurite outgrowth in phaeochromocytoma PC12 cells is also regulated by both Ca^{2+} and Mg^{2+} (Koika, 1983). Both cations are also believed to have regulatory roles in lectin stimulated DNA synthesis in human lymphocytes (Abboud et al, 1985).

1.5.4 Monovalent Ions

A significant literature has developed linking fluxes not only of divalent cations, but also of monovalent ions with initiation or maintenance of cells in a state of active replication. Changes in ion pumping activity may be an important regulatory device in linking surface and intracellular events, since the asymmetric distribution of $K^+$and Na^+ profoundly affects the transport of non-electrolytes, membrane potential and protein synthesis (Rozengurt, 1980; Lubin, 1982). Marked alterations in fluxes of monovalent cations are among the earliest changes that occur in mitogen stimulated lymphocytes (Quastel & Kaplan, 1970; Prasad et al, 1987), EGF and NGF induced proliferation in PC12 cells (Boonstra et al, 1983) and mitogen stimulated quiescent 3T3 cells (Rozengurt et al, 1983; Rozengurt & Burns, 1984). A number of reports have demonstrated correlations between the activity of Na-K-ATPase and cell growth (Rozengurt et al, 1979, 1980; Rozengurt & Mendoza, 1980; Varon & Shaper, 1983; Moore, 1983; Rozengurt, 1985; Takagi et al, 1986). Na^+ efflux via the ouabain-sensitive Na^+/K^+ pump leads to K^+ accumulation, this increase in K^+ may activate cell metabolism (Lubin, 1982) and cell K^+ concentrations could therefore be important

in growth control (Lopez-Rivas et al, 1982; Takagi et al, 1986).

The possibility that K^+ions are important in growth control is also implicated in another way since voltage gated K^+ channels are thought to play a role in T lymphocyte mitogenesis. Voltage-dependent potassium channels have been found to be the predominant ion channels in human T lymphocytes and phytohaemagluttinin (PHA) (at concentrations that induce proliferation) was found to alter K^+ channel gating within 1 minute of addition to the bathing solution. [^{3}H]-thymidine incorporation by T lymphocytes following PHA stimulation was inhibited by classical K^+ blockers (De Coursey et al, 1984). However, the K^+ conductance in human lymphocytes may be in part due to the presence of Ca^{2+}activated potassium channels (Matteson & Deutch, 1984).

Increased Na^+ entry is one of the earliest responses to mitogenic agents in many quiescent cells including human fibroblasts (Owen & Villereal, 1983), hamster fibroblasts (Poussegar et al, 1982), rat liver cells (Koch & Leffert, 1979), neuroblastoma (Moolenaar et al, 1981), lymphocytes (Felber & Brand, 1983), BSC-1 epithelial cells (Rothenburg et al, 1982) rat phaeochromocytoma cells (Boonstra et al, 1983) and glial cells (Benos & Sapirstein, 1983).

Mitogen activated Na^+ influxes are amiloride sensitive and are mediated via the ubiquitous Na^+/H^+ antiporter (Schuldiner & Rozengurt, 1982; Paris & Pousségur, 1983; Leffert & Koch, 1986; Frelin et al, 1986). It is believed that in the presence of growth factors the Na^+/H^+ antiporter operates at more alkaline pHi values, due to increased affinity of the pHi-sensor in the allosteric H^+ binding site (Paris & Pousségur, 1984; Grinstein et al, 1985). Recent studies have shown that synthetic diacylglycerol and phorbol ester (activators of PKC) both activate Na^+/H^+ exchange and it is now believed that PKC is a transducer of growth factor-mediated activation of the Na^+/H^+antiporter (Pousségur, 1985; Rozengurt, 1985). However since the activity of the Na^+/H^+ antiport is also enhanced by

some mitogens that do not activate PKC it is likely that other mechanisms may be involved (Vara et al, 1985), possibly a phosphorylation step activated by Ca^{2+}. calmodulin (Villereal et al, 1985). The fact that an increased influx of Na^+ is an early event following ligand-receptor binding does not necessarily imply that the increase in $[Na^+]i$ is the mitogenic signal since the interactions of ion concentrations, exchanges and pumps are complicated, but certainly the increase in Na^+ permeability in mitogen stimulated cells sets in motion an array of ion distributions and metabolic events associated with proliferation (Leffert & Koch, 1985; Moolenaar, 1986). The stimulation of Na^+ influx is believed to enhance the activity of the Na^+/K^+ pump (Lopez-Rivas et al, 1982) and thus an increase in K^+ concentration may be the important signal in the initiation of cell proliferation. Concurrent with an influx of Na^+ ions is an efflux of protons via the Na^+/H^+ antiporter leading to a rise in intracellular pH (pHi) which can then serve as an intracellular messenger (Frelin et al, 1986). An interest in intracellular pH as a possible ionic signal for cell activation arose from studies with sea urchin eggs; a rapid rise in pHi occurred after fertilisation (Johnson et al, 1976). Increases in cytoplasmic pH following mitogenic stimulation have also been demonstrated in other cells (Moolenaar et al, 1983; Burns & Rozengurt, 1984; Grinstein & Goetz, 1985; Villereal et al, 1985).

Many mitogens cause both rapid chances in intracellular pH and Ca^{2+}, Hesketh and colleagues (1985) suggest a highly conserved sequence of ionic events take place in the mitogenic stimulation of eukaryotic cells. In both thymocytes and fibroblasts a pHi response invariably follows a Ca^{2+} signal, it is believed that the two signals are uncoupled; thus the pHi increase is not a direct consequence of Ca^{2+} signal, but rather the two signals are generated independently. Although phosphoinositode metabolism can account for these two ionic responses in many instances there are cases where other mechanisms must

be responsible, for example EGF generates both signals in 3T3 fibroblasts without causing PIP_2 hydrolysis (Besterman et al, 1986). In some circumstances increased $[Ca^{2+}]i$ is sufficient to activate the Na^+/H^+ exchanger (Owen & Villereal, 1982), the Ca^{2+} induced activation of the antiporter in lymphocytes is thought to be mediated by changes in cell volume (Grinstein, 1987). In 3T3 fibroblasts an increased intracellular Ca^{2+} is neither necessary nor sufficient for activation of the Na^+/H^+ exchanger (Ives & Daniel, 1987). The precise roles of Ca^{2+} and PKC in activation of the Na^+/H^+ antiporter await further clarification.

1.5.5 Ornithine Decarboxylase and Polyamines

Polyamines and new polyamine biosynthesis have also been associated for some time with the traverse of cells through the cell cycle. Ornithine decarboxylase (ODC) is the first enzyme in the biosynthetic pathway of polyamines and is rate limiting (Pegg, 1986). Cell growth is always accompanied by high ODC activity, for reviews see (Janne et al, 1978; McCann, 1980; Heby & Anderson, 1980; Pegg & Williams-Ashman, 1981; Pegg & McCann, 1982). Polyamines are low molecular weight substances and because of their flexibility and cationic nature interact and support the synthesis of nucleic acids, proteins and phospholipids (Algranati & Goldenberg, 1977). ODC not only acts as an important regulatory enzyme in the generation of polyamines but is also believed to regulate ribosomal RNA synthesis and thus, protein synthesis (Russell et al, 1976; Russell, 1981). The induction of polyamine synthesis in lymphocyte activation, requires gene expression and occurs several hours after mitogen stimulation with peak ODC activity occurring at eighteen hours (Fidelus et al, 1984).

More recent evidence suggests that ODC may also take part in growth signal transduction. Thus, treatment of T lymphocytes, with mitogenic

monoclonal antibodies or lectins induces a rapid activation of ODC within minutes (Scott et al, 1985; Mustelin et al, 1986). This early activation is independent of *de novo* protein synthesis but requires an intact cytoskeleton and energy metabolism (Scott et al, 1985). This early induction of pre-existing ODC molecules appears to be specifically linked to the initiation of T lymphocyte proliferation and since non hydrolysable GTP analogues demonstrate rapid ODC activation a G protein is thought to be an important link in the transduction of the mitogenic signal (Mustelin et al, 1986). Recent work has demonstrated that ODC is covalently linked to the cytoplasmic surface of the cell membrane by phosphatidylinositol. An endogenous phospholipase C enzyme is thought to cleave the phosphotidylinositol bearing ODC into diacylglycerol and ODC linked to inositol-1-monophosphate, once cleaved, the activity of ODC rises several fold (Mustelin et al, 1987). The rapid activation of ODC in T cells also requires an elevation in the free cytosolic calcium (Scott et al, 1984). This suggests that ODC activation follows a Ca^{2+} signal in inducing T lymphocytes to divide. The exact function of the rapid ODC activation as opposed to the long term cycloheximide-sensitive ODC activation is unknown. The fact that ODC is attached to the inositol group of PI adds a new dimension to inositol phospholipid turnover and suggests the possibility that attachment and liberation of ODC from phosphatidylinositol may be an important mechanism of growth regulation (Mustelin et al, 1987).

Another intriguing facet in phosphoinositide turnover is that in A431 cells polyamines stimulate the phosphorylation of phosphatidylinositol to phosphatidylinositol-4-phosphate and might therefore have a role to play in replenishing the pool of polyphosphoinositides to be used in signal transduction (Vogel & Hoppe, 1986). Rapid ODC activation following mitogenic stimulation might serve to initiate polyamine synthesis, the polyamines then phosphorylate

phosphatidylinositol to phosphatidylinositol-4-phosphate, a further phosphorylation produces PIP_2 the hydrolysis of which leads to the generation of $InsP_3$ and DG, both of which function as second messengers to initiate the signal cascade.

Another model for signal transduction which involves the polyamines suggests that these polycations serve as intracellular messengers to increase free cytosolic Ca^{2+} by stimulating Ca^{2+} influx and mobilising Ca^{2+} from intracellular sites via a cation exchange reaction. A transient increase in ODC activity and a subsequent rise in polyamine levels are early events following receptor activation by testosterone (Koenig et al, 1983a) isoproteronol (Koenig et al, 1983b) thyroid hormone (Koenig et al, 1984a) and insulin (Koenig et al, 1984b). Thus polyamine synthesis has been found to be essential for hormone induced Ca^{2+} fluxes and the mediation of several Ca^{2+}-dependent membrane transport processes including endocytosis, hexose transport and amino acid transport. ODC and polyamines may also have an important part to play in the transduction and transmission of voltage-mediated signals and in stimulus-secretion coupling in nerve cells (Iqbal & Koenig, 1985).

Unravelling the network of second messengers involved in the control of cell proliferation is a daunting task. The number and variety of putative intracellular messengers under consideration is large arguing against the likelihood of a universal second messenger. The interaction of the proposed signal pathways presents a complex puzzle, making it difficult to consider second messengers in isolation (see Diagram 2) (Bauer et al, 1987). Moreover it is by no means clear how so relatively few second messengers are able to couple so many external signals to diverse cellular responses (Lichstein & Rodbard, 1987). Nevertheless many early events have been characterised which are assumed to couple extracellular signals and mitosis. The mechanisms by which these second

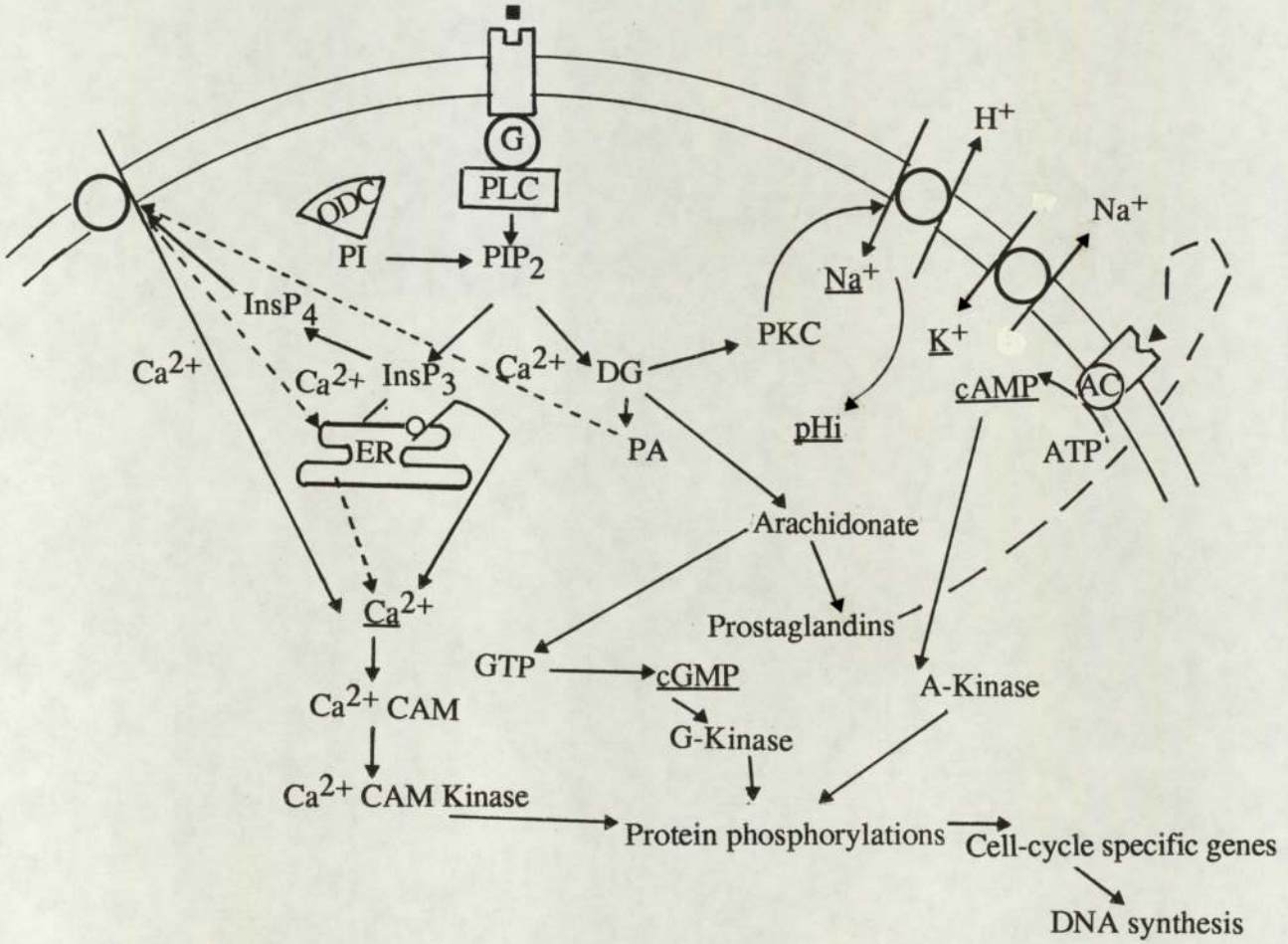


Diagram 2: A diagrammatic representation of the generation of putative second messengers and some of their possible interactions during stimulus-mitosis coupling (see Section 1.5 for details). The putative second messengers are underlined.

messengers cause alterations in gene expression still await much clarification and the expression of the so called cell cycle dependent genes inducible by different mitogens is an intensive field of study.

1.5.6 Cell Cycle Dependent Genes

The search for genes (and gene products) that regulate cell division aims at elucidating genes that are preferentially expressed in a specific phase of the cell cycle and to understand their significance in the mitogenic response. A number of genes have already been identified whose expression is thought to be cell cycle-dependent although this does not necessarily imply that they regulate cell cycle progression.

Transcription of the nuclear proto-oncogenes, c-*fos* and c-*myc* are among the earliest events following activation of quiescent cells (Taylor, 1986; Sassone-Corsi & Verma, 1987, March 1987) and they are thought to act as molecular switches, which may trigger the transition of cells from a quiescent to proliferating state (Curran et al, 1985). These two proto-oncogenes are members of the 'competence' gene family induced by PDGF in Balb/c-3T3 cells (Callahan et al, 1985). The induction of c-*fos* and/or c-*myc* by growth factors has been demonstrated in a variety of cells, including 3T3 cells (Greenburg & Ziff, 1984; Curran et al, 1985), A431 cells (Curran et al, 1985; McCaffrey et al, 1987), thymocytes (Moore et al, 1986), T-lymphocytes (Kelly, 1983), B lymphocytes (Kelly et al, 1984) and phaeochromocytoma cells PC12 (Morgan & Curran, 1986). Because the transcripts from c-*myc* increase approximately 20 fold shortly after cells are stimulated to proliferate and decline before the onset of DNA synthesis (Kelly et al, 1983, 1984) it has been suggested that the expression of c-*myc* may be specific to the G_1 phase of the cell cycle (Kelly et al, 1984). However other work indicates that the increase in c-*myc* mRNA following the

stimulation of quiescent cells is the result of an activational event which renders the cells competent to enter the cell cycle. The level of c-*myc* oncogene mRNA then remains invariant throughout the remainder of the cell cycle (Thompson et al, 1985). This is in agreement with other reports which suggest that c-*myc* protein synthesis is dissociated from the cell cycle in human and avian cells (Hann et al, 1985) and in *Xenopus* oocytes (Godeau et al, 1986).

Two other genes whose expression has been associated with cellular proliferation (Johnson et al, 1982; Sittman et al, 1983; Gibson et al, 1986) are thymidine kinase (TK) and histone 2b (H2b) and they do demonstrate consistent variations in the cell cycle. The mRNA levels of these two genes increase during the S phase in continuously growing cells and decrease when cell proliferation stops in density-arrested cultures (Thompson et al, 1985).

Although much attention has been focussed on the expression of two proto-oncogenes c-*myc* and c-*fos*, another proto-oncogene Harvey c-*ras* has also been labelled cell-cycle dependent (Goyette et al, 1984; Campisi et al, 1984). NIH 3T3 cells induced to divide by adding serum to the culture medium are unable to enter the S phase of the cell cycle after microinjection of anti-*ras* antibody implying that the protein product of the *ras* proto-oncogene is required for the initiation of the S phase in NIH 3T3 cells (Mulcahy et al, 1985).

The cellular oncogene p53 is also thought to be expressed in a cell cycle dependent manner; p53 is barely detectable in quiescent cells but is markedly increased in 3T3 cells stimulated by serum (Reich & Levine, 1984). The protein product levels of p53 increase dramatically in mouse lymphocytes stimulated with concanavalin A (Milner & Milner, 1981).

The inducibility of the four proto-oncogenes *c-fos*, *c myc*, Harvey *c-ras* and p53 by different mitogens in various cell types from different species implies that these genes are involved in cell cycle progression (Gibson et al, 1986).

1.6 Neoplastic Growth

Cells within the body are normally under strict control to ensure that proliferation only occurs when required for development or for body maintenance. According to the prevailing environmental conditions a normal cell can either be growing and proceeding through the cell cycle or be quiescent. The decision to proceed through another cycle or enter a quiescent phase G_0, takes place at the restriction point 'R' in the cycle (see Section 1.2). Growth control can be considered as a switching mechanism dependent upon external factors that act at this point 'R'. Normal cells can be transformed into malignant cells by a variety of chemical carcinogens, RNA or DNA viruses and radiation. Growth control in transformed cells is relaxed, these cells can grow under conditions that would restrict the growth of normal cells (Pardee et al, 1982).

The characteristics of transformed malignant cells are many and varied. A number of these features may be characteristic of rapidly proliferating normal cell populations and some transformed malignant cells may not exhibit all of the characteristics (Ruddon, 1981). These changes include: cytological changes (eg, increased nuclear: cytoplasmic ratio, formation of clusters and cords of cells), alterations in growth characteristics (immortality, loss of 'contact inhibition', loss of 'anchorage dependence', loss of 'restriction point' control) and a lower serum requirement. Malignant transformation of cells such as fibroblasts abolishes their requirements for specific serum growth factors such as PDGF (Currie, 1981). Indeed the loss of requirement for exogenous growth factors appears to be the paramount feature defining the malignant phenotype (Alexander & Currie, 1984). The simplest explanation for autonomous proliferation in the absence of exogenous growth factor stimulation is the autocrine secretion of transforming growth factors (TGFs) (see Section 1.3). Transformed cells cultured in serum

free medium produce and release growth factors with the ability to confer the transformed phenotype on normal fibroblasts (Keski-Oja et al, 1987). Upon removal of TGF normal cells resume normal growth patterns and the permanence of the transformed phenotype in malignant cells was therefore attributed to their capacity to produce TGF. However, since TGFs have been extracted from normal tissues (Roberts & Sporn, 1985) and authentic growth factors such as EGF and PDGF can in some circumstances confer upon normal cells many of the features associated with a transformed phenotype (Kaplan & Ozanne, 1983), the malignant phenotype does not appear to depend solely upon the synthesis of special growth factors by cancer cells. A hypothesis to explain malignant growth is that neither the growth factor nor the receptor for this factor is different from those in normal cells but it is their simultaneous presence which may distinguish malignant from normal. Thus, an essential characteristic of malignancy may be the production of a TGF and its receptor by the simultaneous expression of two genes, one for TGF and the second for its receptor (Alexander & Currie, 1984).

Uncontrolled activation or expression of any regulatory component in the signal pathway that conveys information from the cell surface to the site of DNA replication in the nucleus has the potential to promote uncontrolled cell proliferation (see section 1.5) . Thus, an alteration in cyclic nucleotide levels has been implicated in transformed malignant cells although it isn't clear whether changes in cAMP levels, or the cAMP response system are responsible for the appearance of the transformed phenotype. A number of transformed cell types have lower cAMP levels than their non transformed counterparts (Hunt & Martin, 1979; Elgebaly et al, 1982).

Likewise, an alteration in the concentration of Mg^{2+} may be responsible for many of the phenotypic traits of transfomed cells according to some. A spontaneously transformed clone of Balb/c3T3 cells could be induced to resume

the characteristics of normal cells when the extracellular concentration of Mg^{2+} was sharply reduced (Rubin, 1981). It is possible that transformation is brought about at least in part by a defect in regulating the distribution of Mg^{2+} in cells, such that transformed cells have higher Mg^{2+} levels than normal cells which correlates with their higher rate of DNA synthesis (Rubin, 1982; Walker, 1986). The loss of intracellular Mg^{2+}, in low concentations of extracellular Mg^{2+} restores density-dependent inhibition of DNA synthesis, a characteristic feature of non-transformed cells (Rubin, 1982).

In contrast, others suggest that neoplasticity may be associated with a gradual loss of sensitivity to calcium ions. In some cells there is a good correlation between loss of Ca^{2+} control and progression towards tumorigenicity (Boynton & Whitfield, 1978). There are also several observations that growth of normal cells is controlled by extracellular calcium levels whilst that of tumour cells is more independant of calcium (Hickie et al, 1983). Furthermore the concentration of the calcium regulatory protein calmodulin has been reported to be higher in transformed cells than in their normal counterparts (Chafouleas et al, 1981). For example, transformed chick embryo fibroblasts have a 2-4 fold increase in calmodulin levels compared to normal chick embryo fibroblasts (Van Eldik et al, 1982). However other workers do not support the contention that transformed cells obligatorily contain elevated levels of calmodulin, although a role for calmodulin-mediated regulation may be reflected by the inability of transformed cells to down regulate this protein (Veigl et al, 1984b). Nevertheless, calmodulin remains attractive as a mediator of changes induced by transformation, since transformation results from pertubation of major cascade regulation systems many of which may be controlled by calci-calmodulin (Veigl et al, 1984a).

Calmodulin levels may increase in conjunction with the *de novo* appearance of a tumour specific calcium binding protein oncomodulin.

Oncomodulin is related to the parvalbumins and unlike calmodulin has only two binding sites per molecule, a high affinity calcium/magnesium site and a calcium specific site (Williams et al, 1987). Oncomodulin has been extracted from oncogenic and foetal tissue (MacManus et al, 1985), its function is unknown although it has been shown to mimic calmodulin in its ability to activate several enzymes (MacManus & Whitfield, 1983). The appearance of oncomodulin may be responsible for the ability of tumour cells to bypass the restriction point at the G_1/S boundary and to grow in medium deficient in calcium (Boynton et al, 1982).

Protein kinase C may also be involved in neoplastic growth since potent tumour promoters such as phorbol esters are able to directly activate this enzyme both *in vitro* and *in vivo* (Castagna et al, 1982). The evidence available suggests that many of the pleiotropic actions of tumour promoters are mediated through this protein kinase. An uncontrollable production of an active form of protein kinase C, whether the product of a cellular or a viral gene has the potential to promote carcinogenesis in the same way that tumour promoting phorbol esters do (Nishizuka, 1984b). Recent work shows that TPA increases cellular diacylglycerol (the endogenous activator of protein kinase C) through a mechanism not involving phosphoinositide hydrolysis in Swiss mouse 3T3 fibroblasts. There is a close correlation between the effect of phorbol ester on DG formation and mitotic activity in 3T3 cells, although it is not known how the enhanced DG formation contributes to the activation of protein kinase C by TPA (Takuwa et al, 1987).

Cancer genes have been discovered in the chromosomes of tumour cells; these so called oncogenes represent the driving force behind the uncontrollable growth of many cancer cells and they become activated in the conversion of a normal cell into a cancer cell. Oncogenes isolated from tumours are highly homologous to benign genes in normal cells, the proto-oncogenes (Weinberg,

1983). The proto-oncogenes were discovered initially because of their great resemblance to certain retroviral genes responsible for the malignant transformation of their host cells. These oncogenic retroviral genes are called viral oncogenes (v-onc). Retroviruses carry a single gene which is responsible for the induction of cancer and they are structurally altered versions of the proto-oncogenes (c-onc) from which they were initially derived (Weinberg, 1983). Expression of viral oncogenes in cells is thought to cause cancer by mimicking the actions of the corresponding proto-oncogenes in an abnormal fashion (Alitalo et al, 1987). Since chemical carcinogens in contrast to retroviruses cannot introduce new genetic information into target cells they must distort the function of proto-oncogenes. There are three principal ways leading to oncogenic activation of proto-oncogenes, point mutations, chromosomal rearrangements and gene amplication (Spandidos, 1986; Nishimura & Sekiya, 1987; Alitalo et al, 1987).

Growth factors have been shown to increase the transcription of certain proto-oncogenes, eg. (c-*myc*, c-*fos*) (see section 1.5.6), the products of which in turn regulate the transcription of other genes necessary for stimulation of cell proliferation. The emerging paradigm is that a set of proto-oncogenes may encode for the various components of the growth factor-receptor-response pathway, and indicate points at which alterations may arise inducing cancer (Berridge, 1984a; Macara, 1985; Goustin et al, 1986; Alitalo et al, 1987; Nishimura & Sekiya, 1987). Thus, each of the sequential steps following binding of growth factor or other mitogen to their receptors may represent a site where oncogenes might mediate their effects (see diagram 3). The viral oncogene of Simian sarcoma retrovirus, v-*sis* codes for a protein that is almost identical to PDGF suggesting that cells carrying a genetic alteration of the c-*sis* gene may produce excessive amounts of PDGF leading to transformation through autocrine stimulation

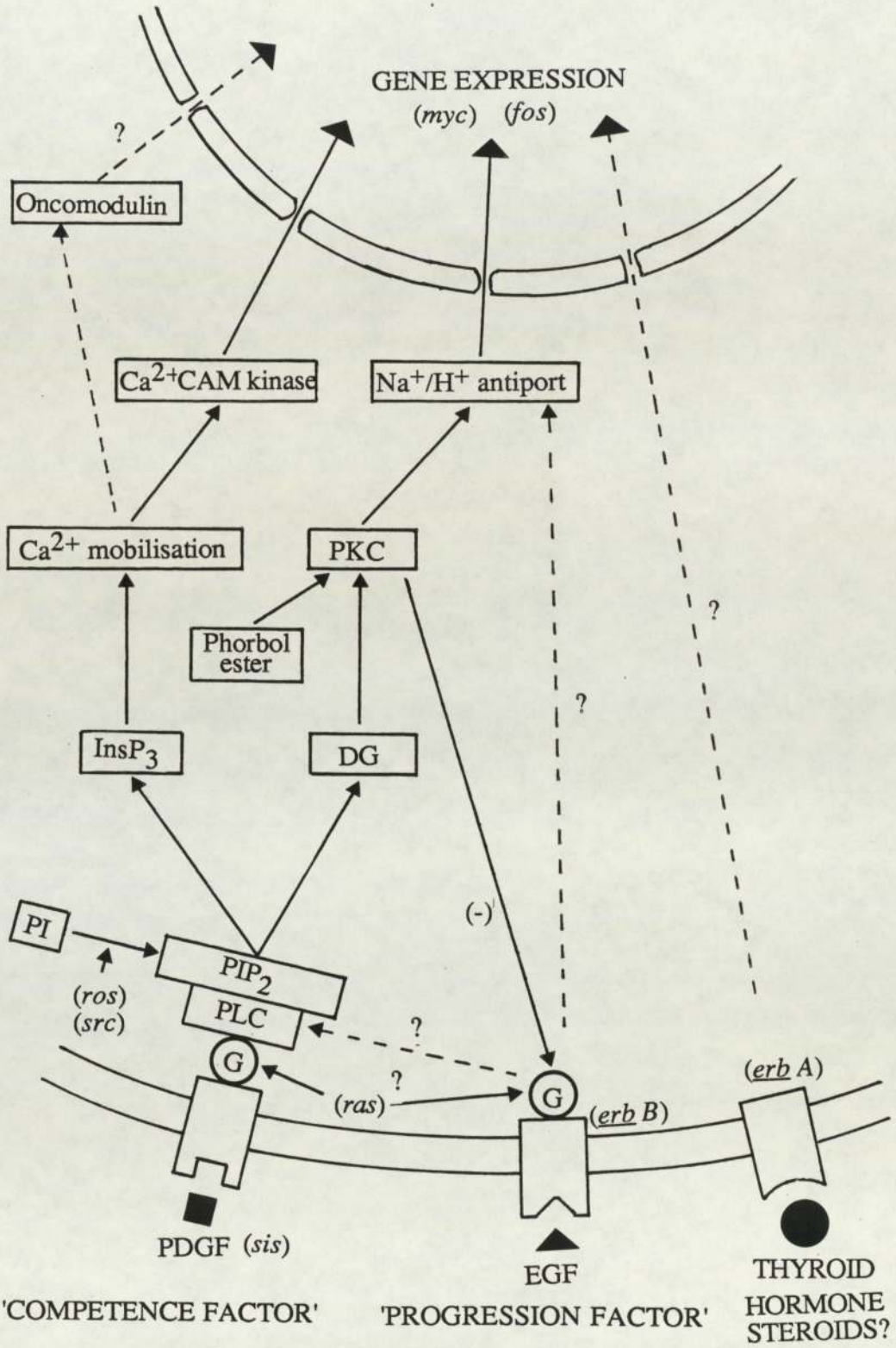


Diagram 3: A diagrammatic representation of the proposed mechanism for regulation of cell proliferation by proto-oncogenes.

Abbreviations are: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; G, G-protein; PLC, phospholipase C; PI, phosphatidylinositol; PIP_2, phosphatidylinositol-4,5-bisphosphate; $InsP_3$, inositol-1,4,5-triphosphate; DG, diacylglycerol; PKC, protein kinase C; Ca^{2+} CAM, calci calmodulin; identified oncogenes are shown in brackets; (-), negative regulation; ? unknown routes to gene expression. See text for further details.

(Doolittle et al, 1983; Waterfield et al, 1983).

Certain genetic defects may result in overproduction of receptors for growth factors, indeed recent studies have shown that *erb*-B oncogene of avian erythroblastosis virus encodes a protein that is remarkedly similar to the internal domain and transmembrane part of the EGF receptor. This truncated receptor may be capable of initiating the signal pathway independently of EGF (Downward et al, 1984).

The p21 protein encoded by the c-H-*ras* gene has been demonstrated to have GTP-binding activity and GTPase activity and is related to the G proteins that regulate adenylate cyclase activity (Nishimura & Sekiya, 1987). The viral p21 Ki-*ras* protein appears to stimulate adenylate cyclase activity in rat kidney cells and it is possible that this ability results in oncogenesis (Franks et al, 1987).

The existence of a functional relationship between growth factor receptors and oncogene products is further supported by the findings that certain oncogene products have transmembrane and tyrosine kinase domains like certain PGF receptors (eg. PDGF, EGF and insulin receptors). Thus the products of a family of oncogenes *src*, *yes*, *abl*, *fps*, *fes*, *ros* and *fgr* possess tyrosine kinase activity and are associated with the cell membrane (Hunter, 1984). Since their discovery, a number of cellular substrates for viral tyrosine kinases have been identified although their role in cell proliferation remains hypothetical (Marcara, 1985). An alternative mechanism of action for these oncogenic products is that they control PI metabolism. A phosphoinositide kinase activity is associated with two retroviral gene products, pp60 v-*src* (Sugimoto et al, 1984) and pp68 v-*ros* (Macara et al, 1984) and transformation of chick embryo fibroblasts by these retroviruses is associated with increased phosphoinositide turnover *in vivo*. Regarding v-*ros*, the presence of the oncogene product results in increases in the second messengers resulting from phosphoinositide turnover, IP_3 and DG

(Macara et al, 1985). The evidence for an intimate link between proto-oncogenes and PI turnover is increasing (Whitman et al, 1986) and models have been proposed demonstrating these links, one of which incorporates the idea of 'competence and progression' factors (see Section 1.3) (Berridge, 1984a; Macara, 1985).

The conversion of a normal cell to a malignant one is thought by some to be a process involving transformation by at least two oncogenes (Lacal et al, 1986). Oncogenes can be divided into two groups, those functioning in the nuclei and those functioning in the cytoplasm (including the cell membrane) (Weinberg, 1985). Activation of an oncogene from each group may be necessary for carcinogenesis (Nishimura & Sekiya, 1987).

In conclusion then, in normal cells proliferation is under the strict control of a set of proto-oncogenes. Certain environmental factors (eg. chemical carcinogens, radiation) may cause an abnormal expression of these genes (ie. point mutations, chromosone alterations and gene amplification), the resulting interference with the normal flow of information along the signal transduction pathway manifests itself in uncontrolled proliferation - cancer. In addition, viral oncogenesis may result from infection with a retrovirus, activation of the v-onc leads to neoplastic growth. Other viruses may cause abnormal growth due to the insertion of viral DNA within the host cells genome leading to mutation and abnormal gene expression.

1.7 The Control of Splenic Lymphocyte Proliferation

The purpose of this study is to investigate the mechanism(s) of T lymphocyte proliferation. It will attempt to elucidate the role of putative second messengers and any interaction amongst them following stimulation with mitogens. The ability of sex steroids to modulate lymphocyte mitogenesis will

also be examined. The experimental system employed has been that of cultured lymphocytes isolated from the mouse spleen. The main antigen recognising cells of the immune system are the T and B lymphocytes which operate in conjunction with accessory cells derived from the myeloid lineage such as macrophages. In very broad general terms T lymphocytes mediate cellular immunity and B lymphocytes become antibody forming cells responsible for humoral immunity. The spleen is a secondary lymphoid organ containing a mixture of T and B lymphocytes in approximately equal proportions (Sprent, 1977). The two components of splenic parenchyma are red pulp, consisting of splenic cords lined with macrophages and venous sinusoids and white pulp containing lymphoid follicles. T- and B-cell areas are segregated in these follicles such that T lymphocytes enclose a follicular arteriole whilst B lymphocytes surround them. The lymphoid tissue of the spleen has several functions; it traps blood borne antigen in the macrophage lined cords and this trapped antigen is then transported to the lymphoid interior by phagocytic migration, T cells exported from the thymus collect here and the lymphoid tissue provides both cellular and humoral immune responses to trapped antigens.

Immunity to those infectious organisms which have developed the capacity for living and multiplying within the cells of the host, eg. viral infections, is the responsibility of T lymphocytes. T cells may be long lived and persist for many years without dividing, however in response to an antigen which binds to a specific receptor on the T lymphocyte (the T cell receptor) the lymphocyte enlarges and divides rapidly. The T cell responds to an antigen by clonally dividing and differentiating into one of several kinds of T cell specific to the antigen. Thus, cytotoxic T lymphocytes bind to antigen displayed on the surfaces of the infected cell and kill the cell. Suppressor T lymphocytes act to inhibit the immune response to an antigen some time after the response has been set in motion.

Helper T lymphocytes bind to antigen on the surface of a B cell that has already bound to the antigen. The helper T cell then releases lymphokines which enable B lymphocytes to divide and differentiate (Swain & Dutton, 1983; Reinherz & Schlossman, 1983). Each functional lymphocyte class bears a distribution of T cell specific surface antigens characteristic of that class; thus mouse cells bear various Ly (Lyt) antigens (Plaut, 1987).

The recognition of antigen by T cell receptors involves the phenomenon of major histocompatibility (MHC) restriction. Thus, helper cells proliferate in response to antigen only when the latter is presented on the surface of macrophages in conjunction with Class II products of the major histocompatibility complex (Rosenthal & Shevach, 1973). Cytotoxic T cells will likewise only lyse targets bearing allogeneic Class I MHC antigens (Zinkernagel & Doherty, 1977). It is felt that the MHC cell surface glycoproteins act as a guide to ensure that the correct T cell can interact with the correct antigen or target (Klein & Nagy, 1982). The T cell receptor is believed to be a molecular complex comprising at least five polypeptide chains, two of these chains are highly variable disulphide linked T_1 α and ß subunits which together mediate antigen recognition. They are non-covalently associated with three CD3 subunits which are believed to mediate signal transduction when the T cells are activated by receptor binding (Mener et al, 1986; Reinherz, 1987). The mechanism by which resting T cells are activated in an antigen-specific response may be summarised thus; the T cell receptor recognises the antigen/MHC encoded protein complex on the antigen presenting macrophage, this bonding stimulates the release of IL1 by the macrophage and that in turn activates the T cell, which expresses IL2 receptors, the activated blasts undergo proliferative expansion under the influence of IL2 produced by another activated T cell subpopulation (Zanders, 1985; MacDonald & Nabholz, 1986;

Meuer et al, 1986). Preceding activation, T cells are in G_0, the quiescent phase of the cell cycle, the first activation signal is provided by the interaction between the antigen specific T receptor and antigen associated with MHC proteins, IL1 also plays a role in this event. As a result of this signal, IL2 receptors appear. Transition of T cells from G_1 and progression into the mitotic cycle then occurs as a result of the second signal, the production of IL2 and its binding to high affinity receptors (Klaus & Hawrylowicz, 1984; Isakov et al, 1987).

The proliferation of lymphocytes in response to plant lectins is often taken as a model for the proliferative aspect of immune responses (Paetkau et al, 1976). The advantage of using plant lectins in a model system to examine signal transduction and intracellular events in T-lymphocyte activation is that they stimulate a very large proportion of T lymphocytes to divide; hence they are described as "polyclonal activators". Concanavalin A (Con A) is the best characterised of the plant lectins and was isolated many years ago (Sumner, 1919). Con A binds specifically to α-D-glucopyranosides and α-D-mannopyranosides and to polysaccharides or glycoproteins containing such residues (Sumner & Howell, 1936). These properties of Con A were known for a long time before it was first reported to be mitogenic (Powell & Leon, 1970). The binding of Con A to sugar molecules on cell surface receptors is an irreversible phenomenon (Powell & Leon, 1970) and although binding occurs on both T and B lymphocytes, Con A preferentially stimulates T cells to divide (Hickel et al, 1985). Con A has been chosen in this study in preference to phytohaemagglutinin (PHA) another T cell mitogen since it is more potent than PHA and causes less agglutination and cell damage (Stobo et al, 1972).

Although the interaction of ligands with T-cell surface receptors and the subsequent division of T cells has been widely studied, the biochemical events

occurring between these two extreme points still await much clarification. Thus, it is unknown whether the signal transduction pathways set in motion by various exogenous stimuli (antigens, lectins or growth factors) act via distinct cell surface receptors or whether T-cell subsets, each with their own genetically programmed functions have differing signal requirements to activate these functions (Isakov et al, 1986). Several signal transduction pathways have been suggested to play a part in T cell activation (for reviews see Zanders, 1985; Gelfand et al, 1987; Linch et al, 1987). In what follows the early signals induced by antigen, lectin and interleukin leading to T cell activation will be described.

Calcium ions have long been implicated in T lymphocyte activation. Initial evidence came from the demonstration that Ca^{2+} ions were necessary in the external medium for T cells to proliferate (Whitney & Sutherland, 1972). Further support came from the observation that an increase in $^{45}Ca^{2+}$ uptake can be triggered by T cell mitogens (Freedman et al, 1975; Whitney & Sutherland, 1983) and that in certain instances the calcium ionophore A23187 is mitogenic (Luckasen et al, 1974). Following the advent of a series of fluorescent dyes (Tsien et al, 1982; Grynkiewicz et al, 1985), increases in cytosolic calcium $[Ca^{2+}]i$ have been monitored following the interaction of T lymphocytes with lectins (Tsien et al, 1982; Hesketh et al, 1985; Gelfand et al, 1984); specific antigen (Nisbet-Brown et al, 1985), or monoclonal antibodies that react with certain T cell surface proteins (O'Flynn et al, 1984; Imboden & Stobo, 1985; Oettgen et al, 1985). IL2 secretion but not IL2 receptor expression appears to require an increase in $[Ca^{2+}]i$ (Mills et al, 1985a; Gelfand et al, 1986a). However, the role of $[Ca^{2+}]i$ in the interaction of IL2 with its high affinity receptor, the second signal in T cell activation is controversial. Changes in $[Ca^{2+}]i$ following IL2 binding have not been detected in human or murine T lymphocytes or IL2 dependent cell lines by some workers (Mills et al, 1985b; Le Grue, 1987) whereas others have detected a rise in $[Ca^{2+}]i$

in rat thymocytes and IL2 dependent cell lines stimulated with IL2 (Gearing et al, 1985a; Rossio et al, 1986). The other interleukin involved in T cell activation IL1, does not appear to use an increase in $[Ca^{2+}]i$ in its transmembrane signalling (Abraham et al, 1987).

Evidence favouring a role for Mg^{2+} ions in T lymphocyte proliferation is sparse, although lectin induced DNA synthesis in T cells is inhibited by about 30% in Mg^{2+} free media, suggesting that Mg^{2+} ions in addition to Ca^{2+} ions are important in T cell proliferation (Abboud et al, 1985). Magnesium is also thought to mediate the response of rat thymic lymphocytes to high concentrations of various mitogens including adrenaline, isoprenaline, dopamine, glucagon and acetylcholine (Perris & Morgan, 1976; Morgan et al, 1984). Further evidence for a role for magnesium in T cell proliferation is the diminished immune response observed in Mg^{2+} deficiency (Gaudin-Harding, 1981). Con A stimulation of DNA synthesis in thymocytes from Mg deficient rats is reduced by 40% (Gunther & Averdunk, 1979), however the diminished immune response in Mg^{2+} deficiency is not caused by a defective Con A induced Ca^{2+} signal (Vormann & Gunther, 1987) inferring that other biochemical mechanisms must be responsible for the reduction of humoral and cellular immunity and in addition, that Mg^{2+} plays an important part in Con A stimulated proliferation.

Monovalent ion fluxes have also been linked with T lymphocyte proliferation. Thus, elevated rates of Na^{+} influx have been reported in lectin stimulated T cells (Averdunk, 1976). Potassium channels are thought to open more rapidly after lectin stimulation and because K^{+} channel blockers (eg. quinine) inhibit mitogenesis in activated T cells, K^{+} ions may have a role to play in signal transduction (Matteson & Deutsch, 1984). However since several investigations have implied that these K^{+} channels may be calcium dependent, it may be that Ca^{2+} mobilisation is the important primary event, the opening of

monovalent cation channels and membrane potential changes then results in secondary processes ultimately leading to cell division (Zanders, 1985).

Cyclic nucleotides are established as mediators of many rapid activation processes in different cells and have been implicated in the initiation of lymphocyte activation. However, as indicated previously (see Section 1.5.1) there are considerable contradictions in the data so that it is by no means clear whether cAMP or cGMP signals are stimulatory or inhibitory or whether they are indeed generated at all by T cell mitogens. More recent work presents evidence that cGMP does not play a role in the mitogenic activation of human peripheral lymphocytes and rabbit thymocytes stimulated with Con A whilst cAMP may be involved as an inhibitory signal (Kaever & Resch, 1985).

Many of the aforementioned putative second messengers which are thought to be implicated in T lymphocyte proliferation may be linked to the hydrolysis of membrane phospholipids following ligand/receptor interaction (see Section 1.5.2). The role of phosphatidylinositol breakdown in T lymphocyte proliferation has been under intense examination. Thus, stimulation of T cells with antibodies to the CD3/T-cell receptor complex causes turnover of phosphatidylinositol to form $InsP_3$ and DG, this signal transduction pathway which leads to calcium mobilisation and the activation of PKC can lead to T cell activation when other required signals are provided by accessory cells and Interleukin 1 (Imboden & Stobo, 1985; Imboden et al, 1985; Cockcroft et al, 1985; Ledbetter et al, 1987). Several investigators have used T lymphocytes treated with mitogenic lectins as models for cellular proliferation, in each case formation of $InsP_3$ and DG from PIP_2 hydrolysis was observed (Fisher & Mueller, 1968; Allan & Michell, 1977; Hui & Harmony, 1980; Moore et al, 1984; Metcalfe et al, 1985). Diacylglycerol activates PKC by increasing its affinity for Ca^{2+}. Following activation, PKC translocates from the cytosol to the membrane

and has the potential to catalyse the phosphorylation of numerous protein substrates at serine and threonine residues by using ATP as a phosphate donor. One of the substrates for PKC may be the Na^+/H^+ exchanger, the activation of which results in an increase in intracellular pH (pHi), an event that accompanies and seems to be causally related to cell proliferation (see Section 1.5.4). Early measurements of pHi using fluorescent probes failed to detect a significant alkalinization in lectin-stimulated T cells (Rink et al, 1982; Rogers et al, 1983). However more recent measurements have detected an increase in pHi in Con A-stimulated thymocytes (Hesketh et al, 1985). In mouse splenic lymphocytes a cellular alkalinization has also been detected following mitogenic stimulation with Con A (Gerson et al, 1982). Further evidence for the role of PIP_2 hydrolysis and the resulting generation of a dual signal pathway in T cell activation comes from studies using tumour promoters and calcium ionophores which mimic the effects of the physiological signals $InsP_3$ and DG respectively (Isakov et al, 1986; Gelfand et al, 1987; Isakov et al, 1987). In addition, the enzyme responsible for PIP_2 hydrolysis, phospholipase C has been isolated in the plasma membrane fractions from murine splenocytes (Kamisaka et al, 1986) inferring that PIP_2 hydrolysis is indeed a possibility in T cell activation. Attempts to correlate IL2 binding to its high affinity receptor with phosphatidylinositol turnover have however given ambiguous results. Thus, although an IL2 induced cytoplasmic alkalinization through stimulation of the Na^+/H^+ antiport has been demonstrated (Mills et al, 1985a), no changes could be detected in labelled inositol phosphates (Mills et al,1986) or in the intracellular distribution of PKC (Gelfand et al, 1987). In contrast other workers believe that the IL2 signal does involve the activation of inositol phospholipid turnover (Bonvini et al, 1986; Farrar et al, 1986), Ca^{2+} mobilisation (Rossio et al, 1986) and PKC translocation (Farrar & Anderson,

1985) which is inferred to be the result of DG generation/activation. Hence much confusion persists as to the nature of the secondary mediators in IL2 signalling. The mechanism of IL1 action is even more nebulous but does not appear to be associated with PIP_2 hydrolysis (Abraham et al, 1987).

The early induction of ornithine decarboxylase activity is also thought to be linked to the initiation of human T lymphocyte proliferation. The T cell mitogen, Con A and monoclonal antibody OKT3 cause a rapid activation of ornithine decarboxylase (ODC) (Scott et al, 1985). When T cells are selectively depleted of guanine nucleotides by treatment with mycophenolic acid, the early mitogen-induced activation of ODC is completely inhibited suggesting that a G-protein(s) is involved in the transduction of the proliferation signal in human T cells (Mustelin, 1987). The intracellular target system for this G protein may include phosphoinositide breakdown and ODC activation (Mustelin et al, 1987) (see Section 1.5.5).

Thus it is apparent that controversies exist in the delineation of intracellular signals involved in T lymphocyte proliferation. Therefore an important feature of the current study has been to attempt to clarify certain facets of stimulus-mitosis coupling in murine T lymphocytes, aiming towards a better understanding of this complex phenomenon. Another aim of the present work has been to gain an insight into the mechanism(s) of sex steroid modulation of the immune system.

There is a great deal of evidence to suggest that the endocrine system has the capacity to directly influence the development and function of the immune system (for a recent review see Kiess & Belohrodsky, 1986). Of the numerous hormones known to regulate the immune response, gonadal steroids have long been implicated (Dougherty, 1952). Observations from these and more recent studies demonstrate that gonadectomy increases the size of the thymus

(Glucksmann & Cherry, 1968; Castro, 1974) whilst sex steroid administration causes involution (Slijivic & Warr, 1973). Most studies suggest that the thymus is an important organ during development but that once sexual maturity is reached, involution occurs (Simpson et al, 1975; Tosi et al, 1982). Endogenous oestrogens or androgens may be causal factors in this naturally occurring process although some workers have shown that ageing of grafted thymi in young mice is independent of the systemic hormonal conditions of the host (Metcalf, 1966). More recent studies suggest that sex steroids are indeed implicated in thymic involution, since the thymus of 12-15 month old rats which is grossly atrophied can be markedly restored in size, 30 days following orchidectomy (Fitzpatrick et al, 1985). Moreover the regeneration of the thymus in old rats can be inhibited by testosterone administration (Greenstein et al, 1986) and oestradiol (Fitzpatrick & Greenstein, 1987). In contrast the age-related involution of the human thymus is not considered by some, to be related to endocrinological changes taking place at puberty (Steinmann et al, 1986). Intriguingly there are still others who believe that age related thymic involution is a myth (Simmons, 1987). Thus there is some controversy over the roles of sex steroids in thymic involution, and it is likely that it is a more complex phenomenon than originally imagined. Indeed there are many possible mechanisms which may lead to thymic involution; cells may die intrathymically, fail to arrive from the bone marrow, may be exported at a faster rate, develop longer cell cycles or simply cease to divide (Clarke & MacLennan, 1986).

Despite these ambiguities surrounding the effects of sex steroids on thymic involution, there is a large amount of clinical and experimental evidence supporting the contention that gonadal steroids interact with the immune system at both physiological and pharmacological concentrations. This is based on several observations; thus sexual dimorphism exists in the immune response, the immune

response is altered during pregnancy and the organs responsible for the immune response contain specific receptors for gonadal steroids (Grossman, 1985).

Many studies have suggested that the female of various mammalian species out performs the male when measured in terms of immune responsiveness (Eidinger & Garrett, 1972). The mechanisms underlying this sex difference are imperfectly understood. Two possibilities include a direct control exerted by X or Y chromosomal genes and/or an indirect modulatory influence of sex steroids. Indeed the majority of studies have shown that immune responses can be altered by sex hormones (Ahmed et al, 1985). Immunoglobulin production is greater in females than in males, particularly IgM (Myers & Petersen, 1985). A possible mechanism for this stimulation of antibody production proposes that oestradiol inhibits suppressor T cell activity. Since suppressor T cells prevent B cells from producing antibody it follows that inhibition of suppressor T cell function will enhance B-cell maturation and increase antibody production (Paavonen et al, 1981). Another report suggests however that the increase in antibody titres is the result of a direct action by oestradiol on B cells to specifically increase the synthesis of the IgM class of antibodies (Myers & Petersen, 1985). The supposed sex difference in cell-mediated immunity is surrounded by controversy. In some studies females are reported to have a greater capability for cellular immunity than males, (Eidinger & Garrett, 1972). Thus, females reject tumours and homografts with greater efficiency than males (Graff et al, 1969) and cells derived from females perform better in mixed lymphocyte cultures than cells derived from males (Lieber et al, 1969). Other reports suggest that females have decreased cellular immune responses as compared to males due in part to the presence of oestradiol (Huber et al, 1982; Myers et al, 1986), which prolongs graft survival time (Waltman et al, 1971), reduces delayed type hypersensitivity responses (Kalland & Fosberg, 1978) and decreases natural killer activity (Kalland, 1980).

A differential sex susceptibility to various autoimmune diseases has long been recognised. Although the aetiology of these disorders is not well understood, a large body of evidence indicates that the progression of autoimmune diseases may be influenced by steroid hormones (Lahita, 1985). For example, 90% of patients suffering systemic lupus erythematosus are female (DuBois & Tuffanelli, 1964). A similar sex related expression of disease is known to occur in animal models of autoimmune diseases (Roubinian et al, 1977; Ansar Ahmed et al, 1985). Even though there are ambiguities in the reported sexual dimorphism of the immune response many studies reveal that pharmacological and physiological levels of oestrogens do modulate immune functions. Thus, immunological effects associated with administration of pharmacological doses of natural and synthetic oestrogen include thymic atrophy (Dougherty, 1952), myelotoxicity (Boorman et al, 1980), stimulation of the reticulo-endothelial system (Nicol et al, 1964) suppression of cell-mediated immunity (Myers et al, 1986) and depression of natural killer cell activity (Kalland, 1980). Physiological levels of oestrogen provide some degree of immune regulation as clearly evidenced by the association of oestrogens with the pathogenesis of autoimmunity (Roubinian et al, 1978). There is also a correlation between increased serum oestradiol levels during pregnancy and depression of cell-mediated immunity and lymphopenia (Hamilton & Hellstrom, 1977; Mathur et al, 1979).

Testosterone has been implicated in the weaker immune responses of males compared to females (Cohn, 1979a). However the precise effects of androgens on lymphomyeloid function remain poorly understood due in part to conflicting observations. For example, in healthy animals androgen treatment decreases antibody responses to dinitrophenol (Oettgen et al, 1966) but has no effect on immune responses to sarcoma cells (Batchelor, 1968), on graft rejection or on graft versus host responses (Fujii et al, 1975). Similarly, administration of

testosterone suppresses (Fujii et al, 1975) enhances (Healy & Neeman, 1967) or has no influence (Frey-Wettstein & Craddock, 1970) on antibody responses to sheep red blood cells. Testosterone also has inconsistent effects on autoimmune diseases, ie.testosterone inhibits development of autoallergic thyroiditis (Kappas et al, 1963) and retards murine lupus in the NZB/W (Shear et al, 1981) but not the NZB strain (Steinberg et al, 1981). Effects of androgens on bone marrow such as erythropoiesis, granulopoiesis and stem cell division are generally accepted (Mooradian et al, 1987). The relationship between androgens and the immune response has been studied in healthy intact mice which demonstrate high or low androgen responsiveness according to a seminal vesicle bioassay. In these mice genetically controlled high sensitivity to androgen correlates with a low immune performance. High androgen responder (HAR) strains demonstrate lower polyclonal, proliferative responses to lipopolysaccharide suggesting a fundamental B cell weakness, alternatively T cell suppression could play a role in the low immune response. The proliferative responses of HAR males to Con A indicate that the mitogenic capacity of their T cells is not reduced (Cohn, 1979a & b, 1986). Orchidectomy induces thymic hypertrophy and increases in the mass of peripheral lymph nodes and spleen (Castro, 1974) whilst administration of androgens leads to thymic atrophy (Dougherty, 1952) and inhibition of growth and development in the bursa of Fabricius in chickens (Norton & Wira, 1977; Verheul et al, 1986). In many early studies, high doses of steroids were used such that confusion exists between the effects of steroid replacement in gonadectomized animals and the pharmacological effects on castrated or intact animals. In a more recent study a dose of dihydrotestosterone, which maximally suppresses the thymic hypertrophy which follows castration does not alter thymic weight in intact animals. This suggests a potential physiological role rather than a toxic effect (Pearce et al, 1981). Similarly, physiological concentrations of

testosterone inhibit the regeneration of atrophied thymus in orchidectomised mice (Fitzpatrick et al, 1985).

Both oestrogens and androgens have been reported to be both immunoinhibitory and immunostimulatory. However, despite all the confusions alluded to earlier, most agree sex steroids act mainly to suppress the cell-mediated immune system. Oestrogens and androgens may suppress either the same or different T lymphocyte subpopulations. Oestrogens can also act as stimulators of the humoral immune system, this may be a direct action on the B lymphocytes or regulated through the cell-mediated immune system by stimulation of helper and inhibition of suppressor T lymphocytes. Androgens may also stimulate the humoral immune response since they can be converted to oestrogens *in vivo*. The ratio of oestrogen to androgen may determine whether the circulating hormones will be immunostimulatory or immunoinhibitory (Grossman, 1985). Not only can the sex steroids regulate the immune system, but it is apparent that the circulating levels of these steroids may be modulated by the immune system. A hypothetical scheme to account for the interaction of the gonadal steroids on T lymphocyte function via a bidirectional hypothalamic-pituitary-gonadal-thymic axis (HPG-thymic axis) has been proposed (Grossman, 1985).

Evidence has been presented which suggests that the function of the cellular immune system is depressed by sex steroids. The mechanism of action of sex steroids on immune responses *in vitro* has been poorly investigated. The objective of the present study is to determine the mode of action by which sex steroids modulate the cell mediated immune system, specifically through an effect on lectin induced T lymphocyte proliferation (an experimental model considered to represent the proliferative aspect of the immune response). Several reports demonstrate that sex steroids do indeed have effects on *in vitro* human lymphocyte transformation, but this steroid induced inhibition of proliferation occurs at

unphysiological concentrations (Schiff et al, 1975; Mendelsohn et al, 1977; Niefield et al, 1977; Wyle & Kent, 1977). In a further study using mouse thymus and spleen cell populations, sex steroid hormones were shown to inhibit lymphoblast transformation but only at pharmocological concentrations. Since sex steroid receptors are believed to be located in the epithelial reticulum of the lymphoid organ rather than in lymphocytes themselves, (Grossman et al, 1979a,b) it has been proposed that the inhibitory effect of high concentrations of sex steroids *in vitro* is a non-specific, non-receptor-mediated event (Homo et al, 1980). An objective of this study is to discern the mechanism(s) by which sex steroids inhibit lymphocyte transformation and to elucidate whether it has any relevance to the modulation of the immune system *in vivo* by gonadal hormones. Additionally the effects of orchidectomy and ovariectomy on *in vitro* T cell function will be investigated since gonadectomy, in addition to causing hypertrophy of lymphoid organs (Dougherty, 1952; Castro, 1974) also results in improved *in vivo* immune function (Graff et al, 1969; Castro & Hamilton, 1972).

CHAPTER TWO

MATERIALS AND METHODS

The experimental techniques employed in this study have been chosen to investigate the effect and mechanism of action of several mitogenic stimuli with a view to gaining a better understanding of T cell proliferation. In addition, attempts have been made to elucidate the nature of the sex steroid modulation of the proliferative aspect of the cellular immune response. The interrelationships between mitogens, sex steroids, ionic environment and mitotic activity in the mouse splenic T lymphocyte have been specifically studied. Thus, methods for the estimation of DNA synthesis, determination of intracellular calcium ion concentration and intracellular pH using fluorescent dyes and measurement of $^{45}Ca^{2+}$ fluxes are described in this section.

2.1 Experimental Animals

Postpubertal male albino mice of the TO strain (Bantin and Kingman Ltd) weighing approximately 25g have been used unless otherwise specified to avoid unnecessary fluctuations resulting from age-related changes in lymphoid function (Walters & Claman, 1975) and oestrous cycle endocrine periodicity (Smith et al, 1975). The animals were maintained under constant laboratory conditions, which included a standard Heygate's rat and mouse breeding diet (Heygate and Sons Ltd) and tap water *ad libitum*.

The animals were sacrificed by cervical dislocation after being lightly anaethetised with diethyl ether. This method is preferential to the use of barbiturates since they have an antimitotic action (Baserga & Weiss, 1967) whereas ether has no short term effects on total red and white blood cell levels or plasma corticosteroid concentration (Besch & Chou, 1971).

2.2 Lymphocyte Proliferation

The small lymphocyte can be forced out of quiescence into an active state by a variety of stimuli both *in vivo* and *in vitro* with consequent synthesis of RNA, protein, and DNA and eventual cell division.

Concanavalin A (Con A) a potent T cell mitogen is capable of binding to the lymphocyte membrane and stimulating a sizeable proportion of T lymphocytes to divide (Larsson & Coutinho, 1979). Hence, it offers a simple *in vitro* system for studying the mechanism of induction of cell cycling in normally quiescent lymphocytes and as such may help elucidate apparently similar events which occur in the cellular immune response.

2.2.1 Preparation of Splenocyte Cultures

The cell suspensions were prepared using a modification of the techniques described by Ford (1978). Spleens were aseptically removed and rinsed in two changes of culture medium to remove superficial debris. Splenic lymphocytes were released into sterile culture medium by gently teasing the spleen avoiding breaking it up into fragments. Large aggregates of cells were subsequently removed by sedimentation and the cell suspension thus remaining centrifuged at 200g for 10 minutes. The cell pellet was resuspended in fresh culture medium and the cell viability estimated by trypan blue exclusion (see Appendix A) was consistently greater than 95%. The cell concentration was adjusted to 5×10^6 cells ml^{-1} and 0.5 ml aliquots were dispensed into sterile plastic culture tubes (Sterilin Ltd) to which the additions of Con A (Sigma Chemical Company Ltd) and/or other drugs were performed as desired in small aliquots from stock solutions made up in sterile 0.9% saline or 0.9% saline-ethanol/dimethyl sulphoxide. Cultures were set up in triplicate in sterile tissue culture microtitre plates (Flow Laboratories Ltd) with each well receiving 100 µl of cell suspension (5×10^5 cells). Cultures were maintained for 48 hours unless stated otherwise at 37°C in a

humidified 5% CO_2 atmosphere.

The culture medium consisted of RPMI 1640 medium (Sigma Chemical Company Ltd) supplemented with penicillin (5000 IU/ml) and streptomycin 5000 μg ml^{-1} (Flow Laboratories Ltd) and 10% foetal calf serum (FCS) (Flow Laboratories Ltd). No reduction in the viability of the total cell population was observed over the 48 hour incubation period (as assessed by Trypan Blue exclusion).

2.2.2 Estimation of DNA Synthesis

The measurement of the rate of DNA synthesis is the basis for the majority of assay systems used in the quantitation of the lymphocyte response to mitogens. The degree to which radioactivity from ^{3}H-thymidine is actually incorporated into the DNA of lectin stimulated lymphocytes is taken as a measure of the proliferation, and thus of the immuno-competence of the cells (Oppenheim, 1969). Despite the popular use of this technique to estimate DNA synthesis there are potential pitfalls. The amount of exogenous thymidine in the culture system is critical and needs to be sufficiently great to maintain adequate intracellular pools for up to 4 hours whilst not causing radiation damage and reduction of the rate of DNA synthesis (Waite & Hirschorn, 1978).

Two pathways exist in lymphocytes for the utilisation of thymidine, the "salvage" pathway utilises exogenous or preformed thymidine while the *de novo* pathway provides thymidine by synthesis from formate and serine. Thus, the possibility must be appreciated that the incorporation of exogenous labelled thymidine may vary with different conditions and may be different in various lymphoid cell types (Wolberg, 1971). Indeed, tritiated thymidine incorporation into DNA is not thought to reflect DNA synthesis in the rat thymocyte since the production of *de novo* thymidine is considered to dilute out the isotopic pool and specific activity within the cell (Youdale & MacManus, 1975). Another factor outlined in a recent report suggests that the incorporation of radioactivity in human lymphocytes cannot be used as a quantitative

indicator of cell proliferation since several components of blood catabolise nucleosides, including thymidine, rendering it unincorporable (Bodycote & Wolfe, 1986). Nevertheless, tritiated thymidine incorporation into DNA is considered to be a useful assessment of the rate of DNA synthesis in many lymphocytes when measurement of the incorporation of the label is made using short pulses of relatively high concentrations of thymidine (Ling & Kay, 1975).

Since DNA synthesis is both temporally and physiologically far removed from the initial event of activation the quantitative nature of the response may be obscured. For instance, in cultures incubated for more than 48 hours cell death and simultaneous cell division complicate the analysis of the response (Wilson & Thompson, 1968).

2.2.3 Proliferative Assay: ^{3}H Thymidine Incorporation

Proliferative responses were assayed by incorporation of 50 µl of ^{3}H methyl thymidine (1 µ Ci/well) (Specific activity 5 Ci/mmol, Amersham International plc) for the last four hours of incubation. Cultures were harvested using a Titertek Cell Harvester (water wash model) (Flow Laboratories Ltd) and ^{3}H-thymidine incorporation measured by liquid scintillation counting (Packard tri-carb counter, model no 2660) using a toluene based scintillant made up thus, 750 ml toluene, 250 ml 2-methoxyethanol, 0.1g POPOP (1,4, Di 2-(5-phenyloxazolyl-benzene) and 3.0g PPO (2,5, Diphenyloxazole) (BDH Chemicals Ltd).

2.2.4 Interleukin 2 Assay/Proliferative Model

Stimulation of splenocytes with mitogenic plant lectins induces a subpopulation of lymphocytes to become responsive to IL2. Hence, preparations of lectin activated cells can be used to assay the lymphokine and an experimental model subsequently developed to investigate IL2 induced proliferation, in particular, signal

transduction and the effect of sex steroids. Mouse splenocytes can be easily activated using Con A to provide lymphocyte preparations which respond to IL2, the major disadvantage being that these cells may also respond to other stimulators (eg. IL-1, and residual plant lectin) (Gearing et al, 1985b).

2.2.5 Preparation of Con A Blast Assay

Splenocyte cultures were prepared (see Section 2.2.1) at a cell density of 2 x 10^6 cells ml^{-1} in culture medium containing 5 x 10^{-5}M 2-mercaptoethanol and 2.5 μg ml^{-1} Con A and incubated for 72 hours. The blasts were washed 3 times and resuspended at 1 x 10^6 cells ml^{-1} in culture medium containing 5 x 10^{-5} M 2-mercaptoethanol and 25 mM α methyl mannoside (Sigma Chemical Company Ltd) which inhibits the mitogenic effect of any residual Con A. 50 μl aliquots of doubling dilutions of recombinant Human IL2 (a gift from Dr AJH Gearing) prepared in RPMI 1640 medium were plated out in triplicate in sterile tissue culture multi-well trays (Flow Laboratories Ltd), 50 μl of cell suspension was added to each well and cultures incubated for 18 hours. 50 μl (1 μ Ci) of ^{3}H-Thymidine (Specific Activity 5 Ci/mmol Amersham International plc) was then added to individual wells. Cells were harvested 4-6 hrs later, and incorporated radioactivity determined as before (Section 2.2.3).

2.3 Measurement of Intracellular Ca^{2+} Levels

There is substantial evidence consistent with a role for cytoplasmic Ca^{2+} as a key regulator in the proliferative response of lymphocytes and many other cells (see Section 1.5.2). However, much of the evidence for calcium's role as intracellular regulator comes indirectly, from experiments involving manipulation of extracellular calcium, measurement of Ca^{2+} fluxes and studies of Ca^{2+} on isolated enzymes and systems. Direct evidence requires that the initial stimulus should cause an increase in intracellular Ca^{2+} which is necessary for the proliferative response. Measurement of

cytoplasmic free Ca^{2+} could provide evidence for intracellular Ca^{2+} as the mediator of the cell's response to external stimuli and differentiate this role from other passive roles for Ca^{2+} in the cell. In addition, it would enable the source of Ca^{2+} for stimulus-mitosis coupling to be delineated, ie. intracellular and/or extracellular. Moreover it would allow $^{45}Ca^{2+}$ fluxes to be accurately interpreted. Thus, techniques for monitoring free Ca^{2+} levels in intact cells are crucial in determining the role of Ca^{2+} in stimulus-mitosis coupling.

An ideal intracellular free Ca^{2+} indicator should be sensitive within a range of 10nM —> 10 μM and be selective for Ca^{2+} in relation to other ions. The response of the indicator to free Ca^{2+} should be linear so that quantitation is simple. Additionally, the change in signal should be great for a small fraction of Ca^{2+} bound. Ideally, the response of the indicator should be quicker than the changes in $[Ca^{2+}]i$ being measured. Incorporation of the indicator into intact cells must be adequate to monitor free Ca^{2+} concentrations without perturbing intracellular Ca^{2+} homeostasis or disrupting the cell. Once incorporated the indicator must not leak out nor be taken up by intracellular organelles. The ideal indicator must obviously not be toxic to the cells in question.

Indicators which are currently used on the basis of the above criteria may be categorised into distinct groups.

(a) Chemiluminescent Indicators

Ca^{2+} activated photoproteins such as aequorin and obelin contain a covalently bound chromophore which emits light when Ca^{2+} binds to the protein. They have found wide applicability in measuring free Ca^{2+} changes in large cells but have proved inappropriate for small cells including lymphocytes because of the difficulty of incorporation without perturbation of cell function (Snowdowne & Borle, 1984; Campbell et al, 1985).

(b) Ca^{2+} Selective microelectrodes

These microelectrodes are based on the principle of separating the cytosol from a constant reference solution with a membrane which is selectively permeable for Ca^{2+} ions. The activity of Ca^{2+} ions is measured by a potentiometric signal. The major disadvantage of these microelectrodes is that they sense the chemical environment only at their tip and may therefore miss a localised change in free $[Ca^{2+}]i$ which may be functionally very important (Tsien & Rink, 1980). Moreover their use requires large robust cells which are not damaged by penetration of the microelectrode.

(c) Metallochromic indicators

Indicators such as Arzenazo III and Antipyralazo III change colour upon binding Ca^{2+}, but since Arzenazo III is susceptible to interference from Mg^{2+} and causes significant Ca^{2+} buffering and Antipyralazo III is not sensitive enough to detect resting $[Ca^{2+}]i$ they fail to meet the requirements of an ideal indicator (Dormer et al, 1985).

(d) Nuclear Magnetic Resonance Indicators

These indicators, 4-fluoro-BAPTA and 5-fluoro-BAPTA, show a shift in resonance upon binding to Ca^{2+}. They have been used to investigate free Ca^{2+} levels in lymphocytes but since the spectrum accumulates over a long period (~ 20 mins) it would be unsuitable for the measurement of Ca^{2+} transients (Metcalfe et al, 1985).

(e) Fluorescent Dyes

Quin 2 belongs to a new family of fluorescent Ca^{2+} indicators and has been a popular choice for the measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$). 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 (Tsien, 1980; Tsien et al,1982). Quin 2 is loaded into intact cells by incubating them with a membrane-permeant ester derivative. Cytosolic esterases split off the ester groups and leave the membrane-impermeant quin 2 trapped in the cytosol. Increases in quin 2 fluorescence then signal increased $[Ca^{2+}]i$. Although quin 2 has disclosed a lot of important biological information, including increases in $[Ca^{2+}]i$ in lymphocytes following lectin stimulation (Tsien et al, 1982), it has always had severe and acknowledged limitations (Tsien et al, 1982, 1984). Indeed earlier studies performed with rat thymocytes showed that high loading concentrations of quin 2/AM were necessary to produce an adequate signal, these higher concentrations of quin 2 appeared to be toxic, to buffer intracellular Ca^{2+} transients and furthermore to leak out of the cells in substantial amounts. Recently, a new generation of Ca^{2+} indicators with greatly improved fluorescent properties have been developed (Grynkiewicz et al, 1985) and the preferred dye for the majority of applications is now considered to be fura-2. Fura-2 has some major advantages over quin 2 including a sixfold higher extinction coefficient and five fold greater quantum efficiency, such that a given intensity of fluorescence requires approximately thirty times less fura-2 than quin 2, a decrease in intracellular dye loadings can be achieved and buffering of cytosolic $[Ca^{2+}]i$ transients can be avoided. Fura-2 bleaches much less rapidly than quin 2 and is therefore the dye of choice for fluorescence microscopy (Tsien et al, 1985). Fura-2 shifts its excitation peak from about 340 nM to 360 nM upon binding Ca^{2+}, both free and bound species have fairly high quantum efficiencies and pairs of excitation wavelengths may be used for ratio operation. The ratio of the fluorescences at two suitably chosen wavelengths can signal calcium whilst cancelling out most, if not all of the possible variability due to instrument efficiency and content of effective dye. Another advantage of fura-2 over quin 2 is its better selectivity for Ca^{2+} over divalent cations such as Mg^{2+}, Mn^{2+} and Zn^{2+}.

The main disadvantage of fura-2 compared to quin 2 is the need for dual wavelength instrumentation to take full advantage of the wavelength shifts. Nevertheless, fura-2 is still considered a better choice for monitoring cytosolic calcium even if the ratio method is prohibited due to lack of the sophisticated instrumentation required. Since fura-2 seems eminently suitable for measuring transient changes in $[Ca^{2+}]i$ in lymphocytes (Tsien et al, 1985) and has many advantages over quin 2 it was selected for use in this study with mouse splenocytes.

2.3.1 Measurement of Intracellular Ca^{2+} by the Fluorescent Indicator Fura-2

Since fura-2 is membrane impermeant it has to be incorporated into cells as an acetoxymethyl ester fura-2/AM. Once inside the cell, it is hydrolysed by cytoplasmic esterases which effectively trap the free acid, fura-2. Fura-2/AM supplied as a freeze-dried oil (Calbiochem) was dissolved in dry dimethylsulphoxide (DMSO) and desiccated at -20°C in small aliquots prior to use.

Fura-2 has a dissociation constant (Kd) of 224 nm at 37°C and shows optimal fluorescence at an emission wavelength of 510 nm using an excitation wavelength set at 340 nm. Fura-2 does not appear to be effected by pH changes within the physiological range and neither does it bind to membranes (Grynkiewicz et al, 1985) and thus is an effective method of monitoring changes in $[Ca^{2+}]i$.

Loading Cells with Fura-2

Mouse splenocytes were isolated as described in Section 2.2.1 and adjusted to 5×10^7 cells ml^{-1} in RPMI 1640 medium supplemented with 10% FCS. Fura-2/AM was added to the cell suspension at a final concentration of 5 μM (shown to give optimal fluorescence signals without showing signs of cytotoxicity) and this was then incubated in stoppered culture tubes (Sterilin Ltd) in a roller drum assembly to allow full and even uptake of the dye for 10 minutes at 37°C. The suspension was then

diluted 10 fold with culture medium and incubated for a further 20-30 minutes. The cells were then washed, resuspended and stored in RPMI medium prior to use.

Fluorescence Measurements

Freshly loaded cells were spun for 30 seconds in a Beckman microfuge and resuspended in 3 mls of physiological saline at 2.5 x 10^6 cells per ml and loaded into a 1cm square quartz cuvette. This procedure minimises any fura-2 carry over. Physiological saline is preferred to the usual RMPI 1640 medium since substances such as phenol red may filter excitation and emission wavelengths and/or contribute their own fluorescence. Physiological saline comprised 145 mM NaCl, 5mM KCl, 1mM Na_2HPO_4, 5mM Glucose and 10 mM HEPES adjusted to pH 7.4 at 37°C with NaOH. Mg^{2+} (1.0mM) and Ca^{2+} (0.6 mM) were added from stock solutions of their respective sulphate and chloride as required.

For fluorescence measurements a Perkin-Elmer LS-5 spectrofluorimeter was used with excitation wavelength set at 340 nm (2.5 nm slit width) and emission measured at 510 nm (5 nm slit width). Cells were continuously stirred and maintained at 37°C whilst the fluorescence signal was plotted in arbitrary units. Additions of small aliquots (< 30 µl) of concentrated reagents were made to the cell suspension (giving a final volume of < 3.030 mls) and any change in fluorescence monitored. Maximum fluorescence values (F_{max}) were obtained by adding 40 µl of a 10% solution of Triton X-100 R (reduced) (Sigma Chemical Company Ltd) thus permeabilising the cells to calcium and ensuring that essentially all fura-2 was complexed with calcium. Minimum fluorescence values (F_{min}) were then achieved by the addition of 50 µl of 0.5M EGTA in the presence of 25 µl of 10% NaOH to achieve a pH value > 8.3. A typical trace is presented in Diagram 4.

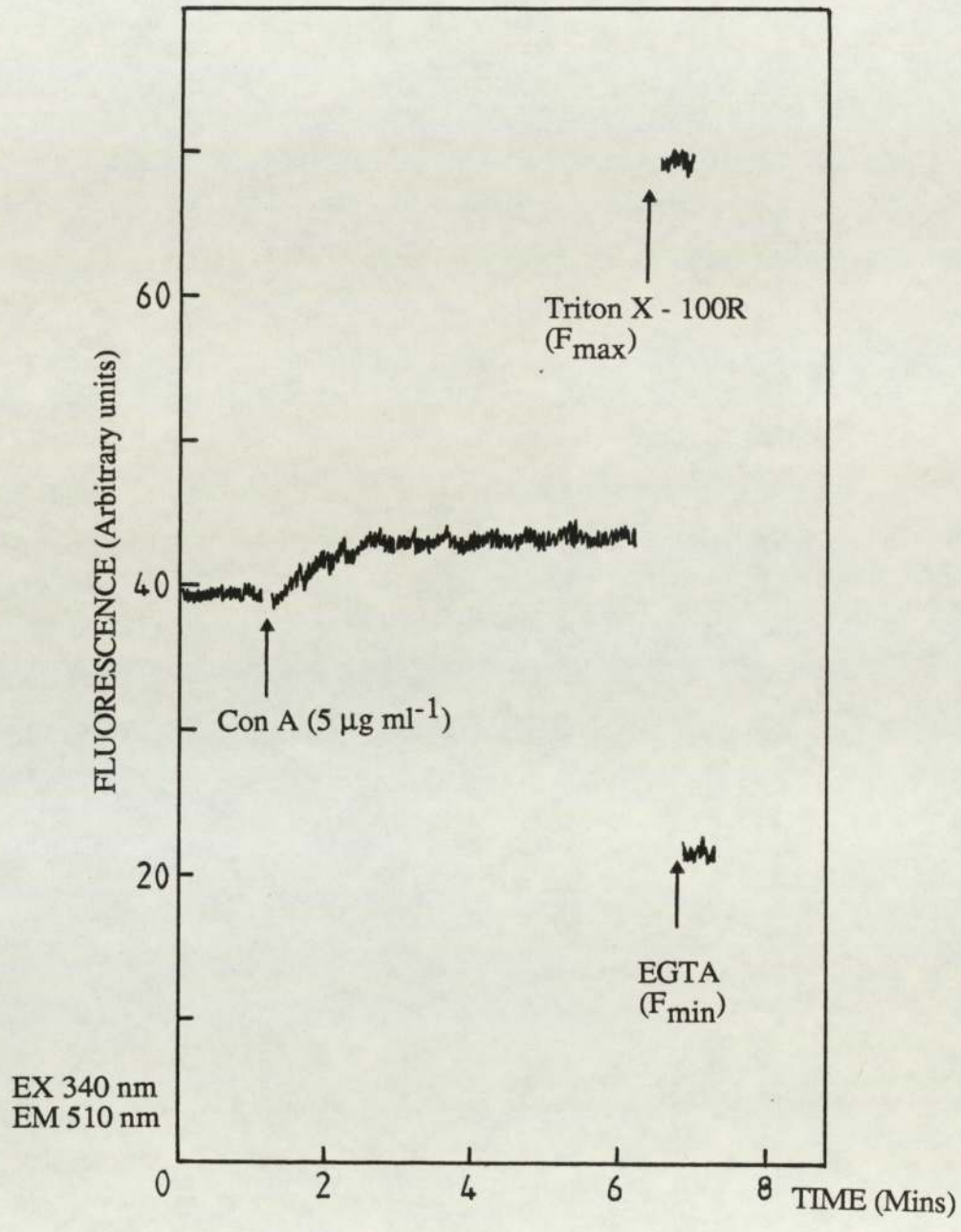


Diagram 4: Measurement of cytosolic calcium levels in fura-2 loaded murine splenocytes.

The fluorescence values correspond to resting calcium levels of 143 nM (R) and stimulated levels of 193 nM (S) which represents an increase of 1.35 (S/R). (See Section 2.3.1 for details).

$[Ca^{2+}]i$ Concentrations

Intracellular free calcium concentrations were calculated according to methods described by Grynkiewicz et al (1985). Thus:

$$[Ca^{2+}]i = \frac{F - F_{min}}{F_{max} - F} \times 224 \text{ nM}$$

where F is the fluorescence reading. F_{max} is the F for saturated fura-2 whilst F_{min} is the F for free fura-2. This method assumes that there is minimal quenching of fura-2 by heavy metal ions within the cells and little leakage of fura-2 from the cells during the experiment. Fura-2 leakage did not appear to be a problem during the course of experiments since they were invariably short-lived (~ 5 mins). However since batches of cells had to be prepared for four sequential experiments at one time it was necessary to have the cell suspensions incubating at 37°C in the spectrofluorimeter for about 10-20 minutes prior to use. Fura-2 leakage did appear to contribute to a gradual increase in the calculated resting value during the series of four experiments. For example on one occasion the resting value appeared to gradually increase from 124 nM in the first experiment to 218 nM in the last even though the cells originally came from the same freshly loaded stock. Under the circumstances it seems that qualitative changes in $[Ca^{2+}]i$ could accurately be demonstrated whilst quantitation of the results might be misleading, thus increases in $[Ca^{2+}]i$ have been expressed such that the resting or basal $[Ca^{2+}]i$ is equal to unity and an increase in $[Ca^{2+}]i$ is represented by a figure greater than 1 (see Results section, Chapter 3). Since there are some uncertainties in the interpretation of fura-2 data and it is unknown whether changes in $[Ca^{2+}]i$ observed by mitogen application to fura-2 loaded cells are comparable to those which occur in cells unperturbed by the presence of the dye it seemed appropriate to combine these studies with an indirect technique which could quantify transient transmembrane fluxes of radiolabelled Ca^{2+} during mitogenic stimulation.

2.4 Calcium Uptake Studies

Many calcium uptake studies have used procedures involving extensive centrifugation, washing and resuspension of cells to remove unincorporated isotope from the extracellular fluid. These procedures are not fast enough to prevent a trans-plasmamembrane readjustment of cell calcium and therefore tracer exchange. Furthermore, such procedures are likely to result in metabolic alterations when pelleted cells are resuspended. Not surprisingly, the reported values of $^{45}Ca^{2+}$ uptake in mitogen-stimulated cells using such techniques are conflicting (Deutsch & Price, 1982). These problems may be overcome by using a modification of the centrifugation technique developed by Freedman, Raff and Gomperts (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 column of oil. In the original technique cells were lysed in formic acid at the bottom of the microfuge tube, to release $^{45}Ca^{2+}$ to be counted. However, preliminary studies showed that elimination of this layer produced more consistent results. Thus, polypropylene microcentrifuge tubes (Beckman PRO-22) were prepared by addition of 175 µl MS 550 silicone oil (BDH Chemicals Ltd) and subsequently spun to ensure an air bubble-free column.

Mouse splenocyte cultures were prepared as described (see Section 2.2.1) and cell density adjusted to 10^8 ml^{-1}. Cultures were preincubated at 37°C for at least thirty minutes before isotope addition to allow recovery from the preparative procedures. Cell suspensions were subsequently added to prewarmed tubes containing an equal volume of medium comprising isotope (final concentration 5 µCi ml^{-1}, specific activity, 31.61 m Ci mg^{-1}, Du Pont de Nemours, Germany) and other agents of interest (resulting in a cell suspension of 5 x 10^7 cells ml^{-1}). Triplicate 200 µl aliquots were removed at appropriate intervals thereafter and placed on top of the column and immediately centrifuged for 1 1/2 minutes in a Beckman microfuge. The

incubation time for each tube was taken from the time of addition of cells to isotope to the beginning of the centrifuge run, since the microfuge achieves maximum force within five seconds.

The contents of the tube were then frozen by immersion in liquid nitrogen and the bottom (~ 1 cm) containing the cell pellet was cut off and placed in scintillation vials containing NE-260 scintillation fluid (Nuclear Enterprises Ltd). The inherent properties of the oil present potential errors to this technique but previous studies revealed that silicone oil (MS 550) did indeed have the required characteristics. Thus, the oil is chemically inert, highly water impermeable and allows maximal cell passage with minimal supernatant carry over. 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. Controls (blanks) were run during the course of each experiment by centrifuging 200 µl of the cell free isotope (5 µi Ci ml^{-1}) through the oil to monitor any changes in the physical properties of the oil, the subsequent counts were always found to be negligible. Radioactive calcium was measured using liquid scintillation spectroscopy (after 24 hours in contact with the scintillant to allow maximum dispersal) 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 $^{45}Ca^{2+}$ comprises both transmembrane influx of the ion and non-specific adsorption to the plasma membrane. Although some workers have chilled cells to eliminate 'active' transmembrane Ca^{2+} transport and used cell-associated $^{45}Ca^{2+}$ values at 4°C to represent non-specific membrane binding (Freedman et al, 1975) preliminary investigations suggested that this assumption was invalid. Thus, membrane-bound $^{45}Ca^{2+}$ at 4°C estimated by this technique was invariably greater than or equal to cell associated $^{45}Ca^{2+}$ for control cells incubated with isotope for 30

minutes at 37°C (results not shown). The act of chilling cells possibly alters plasma membrane properties with the effect of increasing non-specific adsorption of $^{45}Ca^{2+}$. Therefore it seemed more appropriate to consider membrane bound $^{45}Ca^{2+}$, to be cell associated calcium at zero time, ie. immediately after mixing cells with isotope and rapid centrifugation. The Ca^{2+} taken up into the cells was then calculated from the following equation, all values were precorrected for counting efficiency and background activity.

$$\text{Ca}^{2+}\text{ uptake at time (t) (ng per } 10^7 \text{ cells)} = \frac{\text{Sample dpm at time (t)} - \text{Membrane bound dpm at time } (t_o)}{\text{Total dpm}} \times 4800 \text{ ng}$$

where total dpm represents counts of a 200 µl aliquot of isotope-laden cell suspension without spinning through the oil layer. Since the basal medium contains 0.6mM Ca^{2+} (equivalent to 24 µg ml^{-1}) total dpm represents counts in a 200 µl aliquot and is therefore equivalent to 4.8 µg (4800ng).

2.5 Measurement of Intracellular pH (pHi)

An increase in pHi following a rise in cytoplasmic free Ca^{2+} ($[Ca^{2+}]i$) may occur during the stimulation of cell growth in a variety of cells. Thus, serum, growth factors, mitogenic lectins and other agents that induce an increase in $[Ca^{2+}]i$ also activate Na^+/H^+ exchange. Since the change in $[Ca^{2+}]i$ generally precedes the activation of the antiport, it has been suggested that there may be a common sequence of changes in $[Ca^{2+}]i$ and pHi when different types of cells enter the cell cycle (Hesketh et al, 1985) (see section 1.5.4). It therefore seemed appropriate to combine the studies on intracellular Ca^{2+} measurement with determinations of pHi. The pHi of lymphocytes from different sources and of cultured lymphoblasts has been determined by a variety of techniques, including weak acid and base partition (Gerson & Kiefer, 1982), ^{31}P and ^{19}F nuclear magnetic resonance (NMR) (Rink et al, 1982) and direct titration of

freeze-thawed cells (Rink et al, 1982). Since, these techniques are unable to resolve the time course of any changes in pHi which may occur within seconds of stimulation (Rogers et al, 1983) the use of fluorescent indicators to measure pHi changes was considered more promising.

2.5.1 Measurement of Intracellular pH by a Fluorescent Indicator, Quene 1

Quene 1, a fluorescent intracellular pH indicator is related to quin 2 and is designed to respond rapidly to changes in normal pHi values and to have negligible affinity for Ca^{2+} at normal levels of $[Ca^{2+}]i$ (Rogers et al, 1983). Quene 1 has excitation and emission maxima at 390 and 530 nm respectively, and shows a 30-fold increase in fluorescence between pH 5 and 9. The fluorescence is insensitive to Ca^{2+} and Mg^{2+} at free concentrations up to 10^{-4}M and to the proportions of Na^+ and K^+ at total concentrations of Na^+ and K^+ from 100 to 200 mM. The indicator is loaded into cells using the tetraacetoxymethyl ester derivative which is hydrolysed in the cells to give the membrane impermeant tetracarboxylate anion. Once incorporated quene 1 does not perturb glycolysis or the ATP level in resting cells at concentrations up to 0.8 mM (Rogers et al, 1983). Thus, the properties of quene 1 are well suited to its use as an intracellular indicator of pH.

Loading Cells with Quene 1

Mouse splenocytes were prepared as described in Section 2.2.1 and adjusted to 1 x 10^7 cells ml^{-1} in RPMI 1640 medium supplemented with 10% FCS. The cells were incubated for 30 minutes at 37°C with 1 μM quene 1 acetoxymethyl ester (shown to give optimal fluorescence signals without showing signs of cytotoxicity) (Amersham International plc) from a stock solution in DMSO (final concentration < 1% (v/v)) before washing by centrifugation and resuspension in fresh culture medium at a cell density of 5 x 10^6 cells ml^{-1}, for a further 30 minute incubation period prior to use.

Fluorescence Measurements

Cell aliquots (1.5 ml) were spun for thirty seconds in a Beckman microfuge and resuspended in 3 mls of physiological saline (2.5×10^6 cells ml^{-1}) and loaded into a 1cm square quartz cuvette. Cells were allowed to equilibrate prior to fluorescence measurements which were performed in a Perkin-Elmer LS-5 spectrofluorimeter (excitation 390nm, emission 530 nm). Cells were continuously stirred and maintained at 37°C whilst the fluorescence signal was plotted in arbitrary units. The agents of interest were added from stock solutions and any change in fluorescence monitored. The fluorescence of lymphocytes loaded with quene 1 may be calibrated by the addition of 0.05% Triton X-100R in 0.5 mM EGTA and 0.5 mM EDTA to lyse the cells and release quene 1 into the extracellular medium. Serial additions of 1 M Tris and 0.5M HCL solutions calibrate the fluorescence of the released quene 1 as a function of pH (Rogers et al, 1983) Although these steps were performed and changes in fluorescence recorded (see Diagram 5) no correlation has been made to pHi values.

Preliminary studies showed that quene 1, whilst recording changes in fluorescence (and therefore pHi) to a weak base (5 mM NH_4 Cl) (Diagram 5) did not monitor any change following stimulation with Con A (n = 3) or TPA (12-0-tetradecanoyl phorbol 13-acetate) (Sigmal Chemical Company Ltd) (n = 3) during the thirty minute period following addition.

The absence of any effect on pHi of these two agents is in agreement with other studies where quene 1 failed to monitor pHi changes in lectin stimulated thymocytes (Rogers et al, 1983). However, since lectin stimulation of mouse splenocytes does lead to an increase in pHi when measured with weak acid DMO (5,5-dimethyloxazolidine-2,4-dione) (Gerson et al, 1982) further studies were undertaken with another fluorescent indicator to eliminate the possibility that quene 1 might be insensitive to pHi changes in mouse splenocytes.

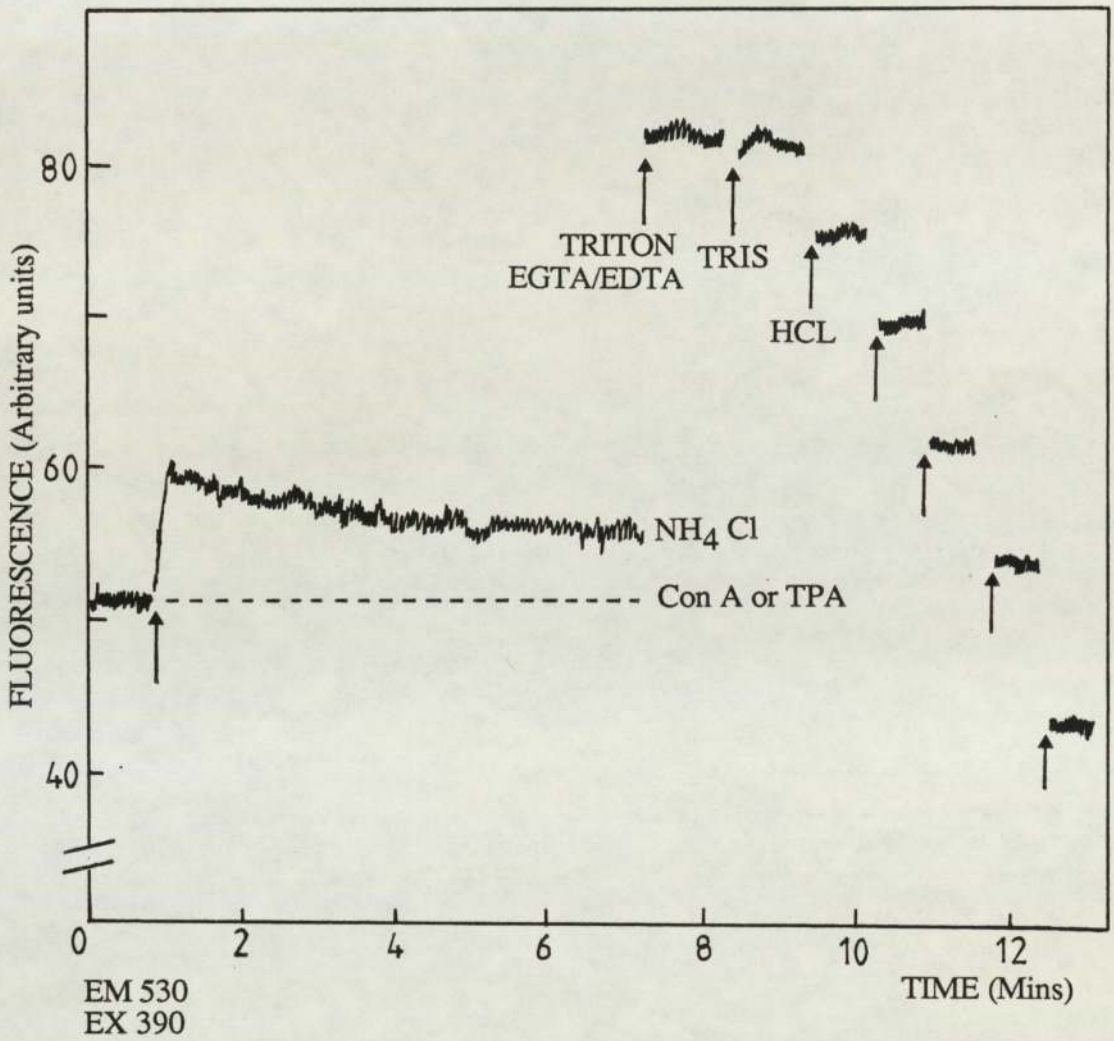


Diagram 5: The effect of a weak base (5 mM NH_4 Cl) on the pHi of quene 1 loaded murine splenocytes.

The fluorescence intensity of quene 1 loaded murine splenocytes in physiological saline prepared as described in Section 2.5.1. Arrows indicate additions of (i) NH_4Cl 5 mM (15 µl of 1M NH_4 Cl) or Con A (5 µg ml^{-1}) or TPA (10^{-9}M) (ii) 0.05% Triton X-100R with 0.5 mM EGTA and 0.5 mM EDTA (15 µl of 10% Triton X-100R + 15 µl 100 mM EGTA + 15 µl 100 mM EDTA) (iii) 2.5 mM Tris (7.5 µl of 1M Tris) and (iv) sequential additions of 1.25 mM HCl (7.5 µl aliquots of 0.5 M HCl).

2.5.2 Measurement of Intracellular pH by the Fluorescent Indicator BCECF (2', 7'bis (Carboxyethyl) - 5 (6)-Carboxyl fluorescein)

BCECF a fluoresent pH indicator originally used by Rink, Tsien and Pozzan (1982) is superior to the carboxyfluoresceins from which it is derived. It allows measurement of a nearly linear relationship of fluorescence emission vs pH in the physiologically important pH range of 6.5 to 7.5 and does not leak out of cells as rapidly as the carboxyfluoresceins. BCECF has excitation and emission maxima at 500nm and 530nm respectively. The indicator whilst having a high sensitivity to small changes in pHi does not appear to be toxic nor affect $[Ca^{2+}]i$ (Rink et al, 1982).

BCECF may be loaded into cells in the form of the membrane-permeant, substantially non-fluorescent ester derivative BCECF/AM. Once BCECF/AM penetrates the cell membrane it is cleaved by cytoplasmic esterases to yield free fluorescent BCECF. This indicator system has been used successfully to monitor intracellular pH changes in mammalian lymphocytes (Hesketh et al, 1985; Metcalfe et al, 1985; Grinstein & Cohen, 1987), fibroblasts (Moolenaar et al, 1983) and gastric cells (Paradiso et al, 1984). It therefore seemed desirable to investigate its ability to detect reputed pHi changes in stimulated mouse splenocytes (Gerson et al, 1982).

Loading Cells with BCECF

Mouse splenocytes were isolated as described in Section 2.2.1 and adjusted to 3×10^7 cells ml^{-1} in RPMI 1640 medium supplemented with 10% FCS. Cells were loaded with 1 μM BCECF (a gift from Dr Mike Thompson) from a stock solution in DMSO (final concentration <0.05% v/v) shown to give optimal fluorescence signals without adversely affecting cell viability (determined by Trypan Blue exclusion). Following incubation for thirty minutes at 37°C the cells were centrifuged and resuspended in fresh culture medium at a cell density of 6×10^6 cells ml^{-1}.

Fluorescence Measurements

Aliquots (1.5 ml) of freshly loaded cells were spun for thirty seconds in a Beckman microfuge and resuspended in 3 mls of physiological saline (3×10^6 cells ml^{-1}) and loaded into a 1cm square quartz cuvette. Following equilibration, fluorescence measurements were recorded in a Perkin Elmer S-5 spectrofluorimeter (excitation 500 nm, slit width 2.5 nm, emission 530 nm, slit width, 5.0 nm). Cells were continuously mixed with a magnetic stirrer and maintained at 37°C whilst the fluorescence signal was plotted in arbitrary units. Small aliquots (< 30 µl) of concentrated reagents were added (final volume < 3.030 ml) and any change in fluorescence monitored. The simplest calibration can be achieved by releasing the dye into the medium after lysing the cells and then recording the fluorescent signal at known pHs (Rink et al, 1982). However since the dye behaves differently in the physiological saline than inside the cells (excitation peak ~ 5 nm red shifted in intracellular dye) an appropriate correction on the calibration scale has to be made for each batch of cells by the method of Thomas et al (1979), using nigericin. Since the main purpose of using BCECF was to detect changes in pHi, this elaborate calibration method was not performed. Preliminary studies revealed that BCECF was able to record pHi changes following stimulation of mouse splenocytes (see Results section, Chapter 3).

2.6 Statistical Analysis

Groups of data have been compared using Anovar one way analysis of variance. The least significant difference (LSD) has been determined by substituting the value of 't' at a given probability level. Thus, in each of the figures (Chapter 3) asterisk(s) denote the degree of significance * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Values have been expressed as the means ± the standard error of the mean (SEM) and n represents the number of separate experiments in each group.

CHAPTER THREE

EXPERIMENTAL RESULTS

Preliminary studies demonstrated that Concanavalin A (Con A) induces an increase in DNA synthesis in murine splenocytes which reaches a maximum by 48 to 72 hours (results not shown). The dose-response curve of Con A stimulation for spleen cells incubated for 48 hours (Figure 1) shows that mitogen-induced stimulation increased with Con A concentrations up to 5 $\mu g\ ml^{-1}$ and then decreased with increasing concentrations of Con A. The stimulation indices proved to be rather variable as witnessed by the large SEM's (standard error of mean) although the trends were similar for individual experiments in which cells are derived from a single male TO mouse. With a view to achieving greater consistency in the Con A-induced lymphocyte transformation model, the effect of Con A on murine splenocytes derived from highly inbred male C_{57} BL/6 mice was examined (Figure 2). Having established that the variability could not be reduced by employing a different murine strain all subsequent experiments were performed using splenocytes from male TO mice stimulated with the optimal concentration of Con A.

Among the earliest responses common to a variety of mitogen stimulated cells is an increase in cytosolic Ca^{2+} concentration ($[Ca^{2}+]i$) (see Section 1.5.2). Calcium influx may thus mediate stimulus-mitosis coupling of the cells in this study. If calcium movement into murine splenocytes is involved in signal transduction then Con A induced DNA synthesis should be compromised by the addition of calcium antagonists to the culture medium. Three specific calcium antagonists thought to have different sites of action upon the so-called long or L-calcium channel were employed in this study (see Discussion, Chapter 4 for further details). The effects of the drugs verapamil, nifedipine and diltiazem on Con A (5 $\mu g\ ml^{-1}$) induced splenocyte proliferation are illustrated in Figures 3, 4 and 5 respectively. To assess the effects of drugs on Con A induced stimulation the level of $[^{3}H]$-thymidine incorporation with

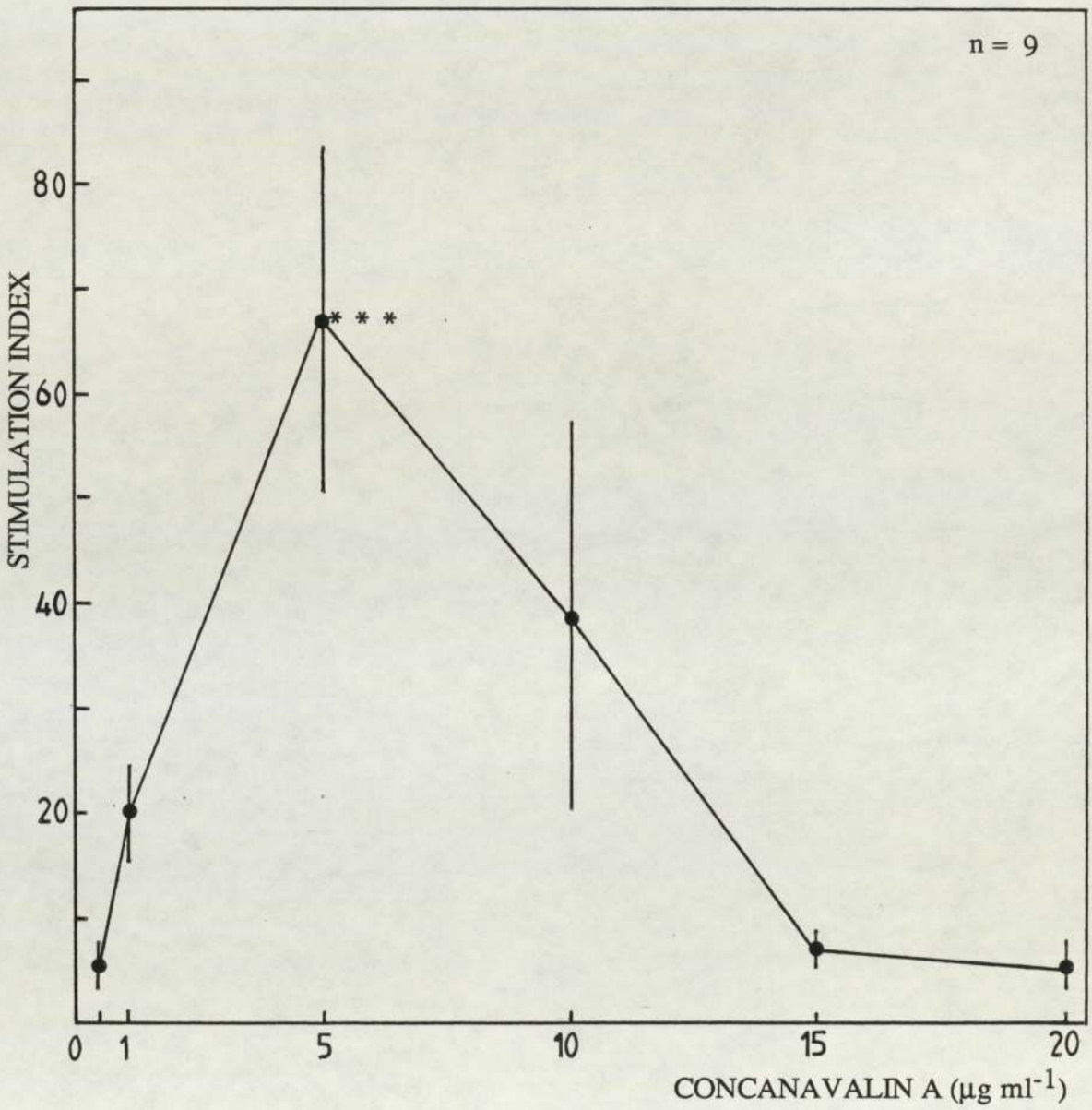


Figure 1: Dose-response curve of the effect of Concanavalin A on thymidine incorporation in isolated TO murine splenocytes

Results expressed as stimulation index (SI) represent the mean (± SEM) of triplicate determinations in nine experiments

$$SI = \frac{\text{Incorporation in the presence of Con A (cpm/well)}}{\text{Incorporation in the unstimulated control (cpm/well)}}$$

n = the number of separate experiments for each treatment values significantly different from those of the other cultures and indicated by asterisks

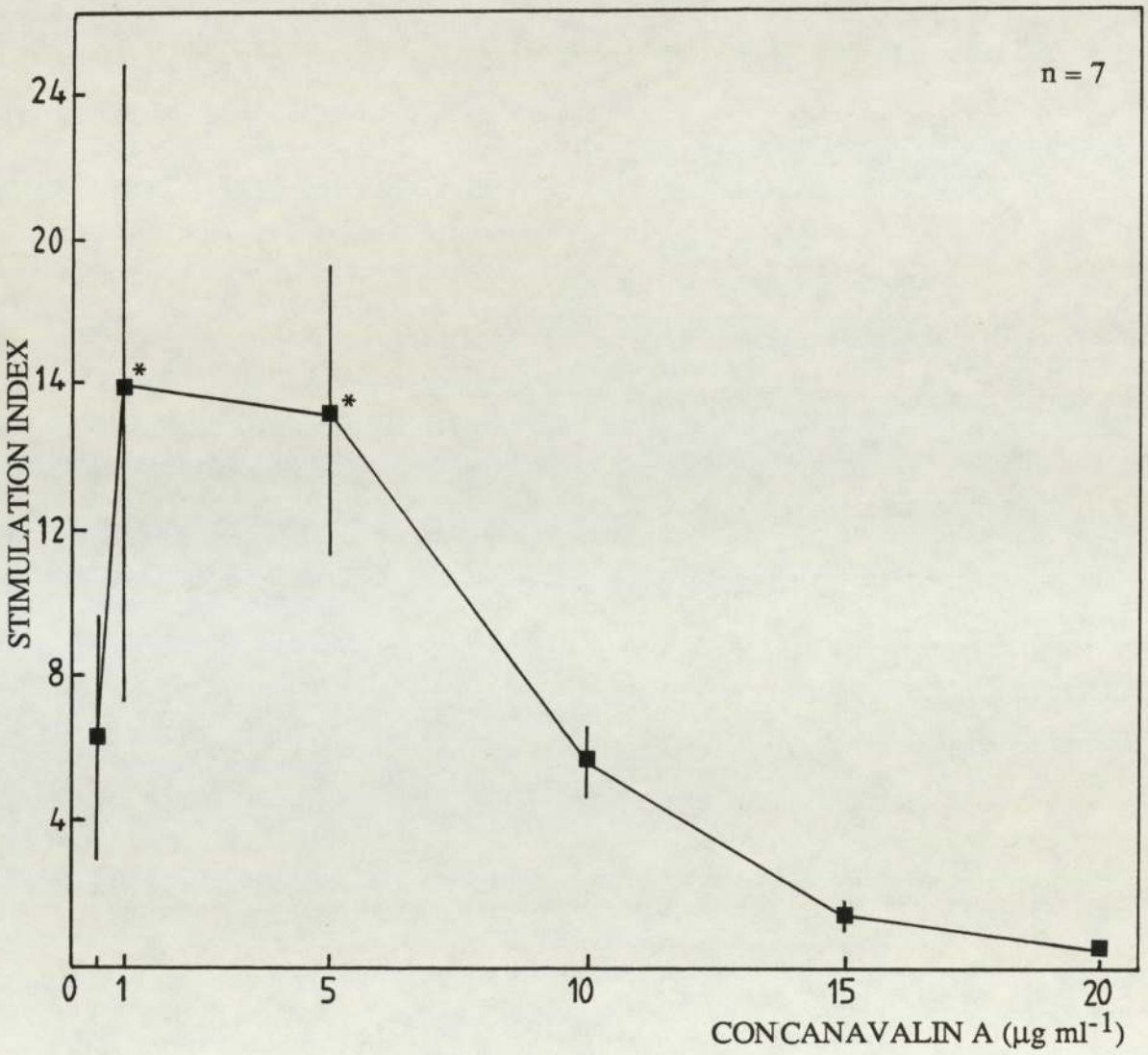


Figure 2: Dose-response curve of the effect of Concanavalin A on thymidine incorporation in isolated C_{57} BL/6 murine splenocytes.

Values significantly different from those of the other cultures are indicated by asterisks. Other details as in Figure 1.

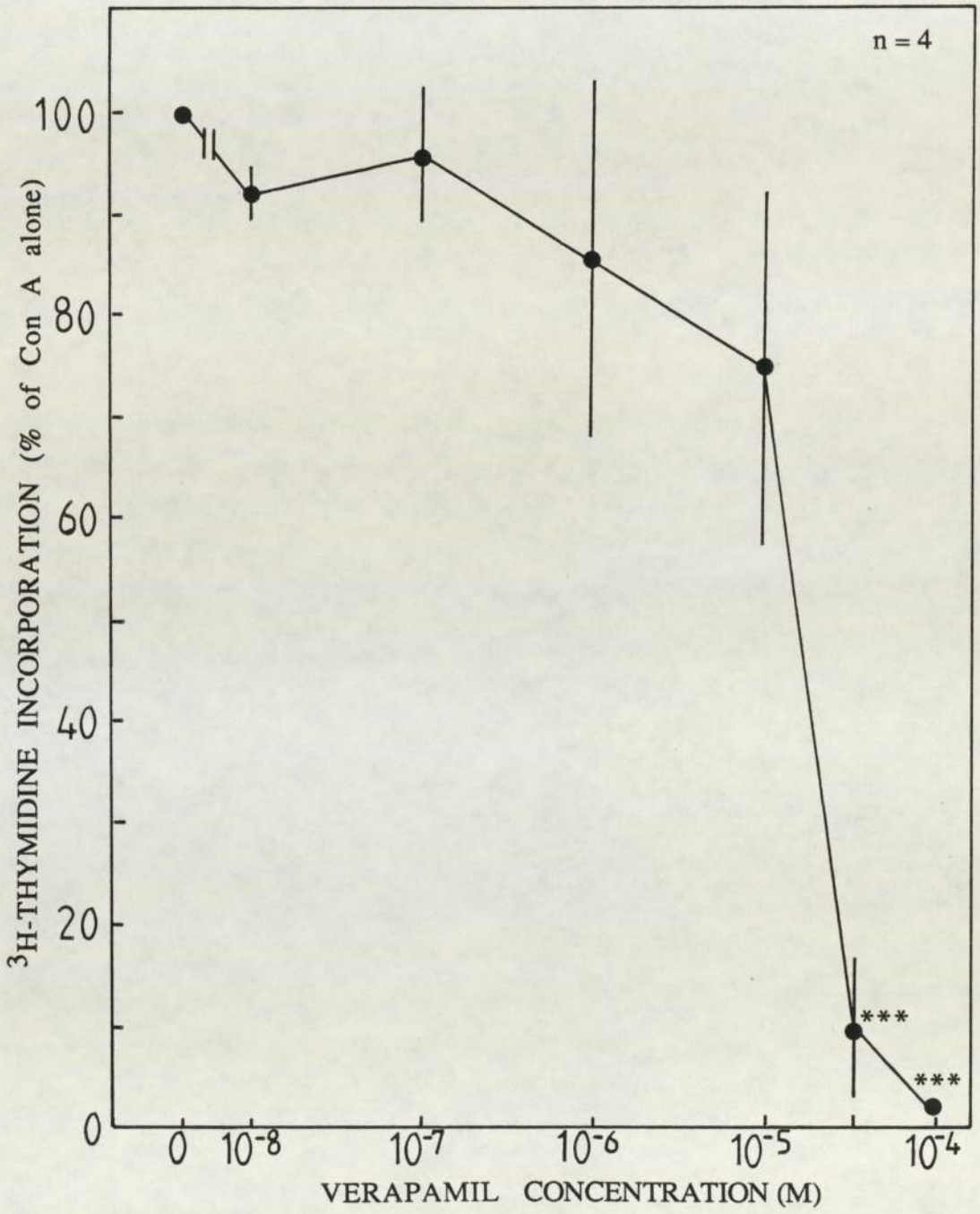


Figure 3: Effect of Verapamil on Con A induced murine splenocyte proliferation.

The level of thymidine incorporation in cultures stimulated by 5 μg ml^{-1} Con A alone is taken as 100% and the incorporation in cultures incubated with 5 μg ml^{-1} Con A plus various concentrations of verapamil expressed as a percentage thereof. Values significantly different from corresponding cultures without the drug are indicated by asterisks.

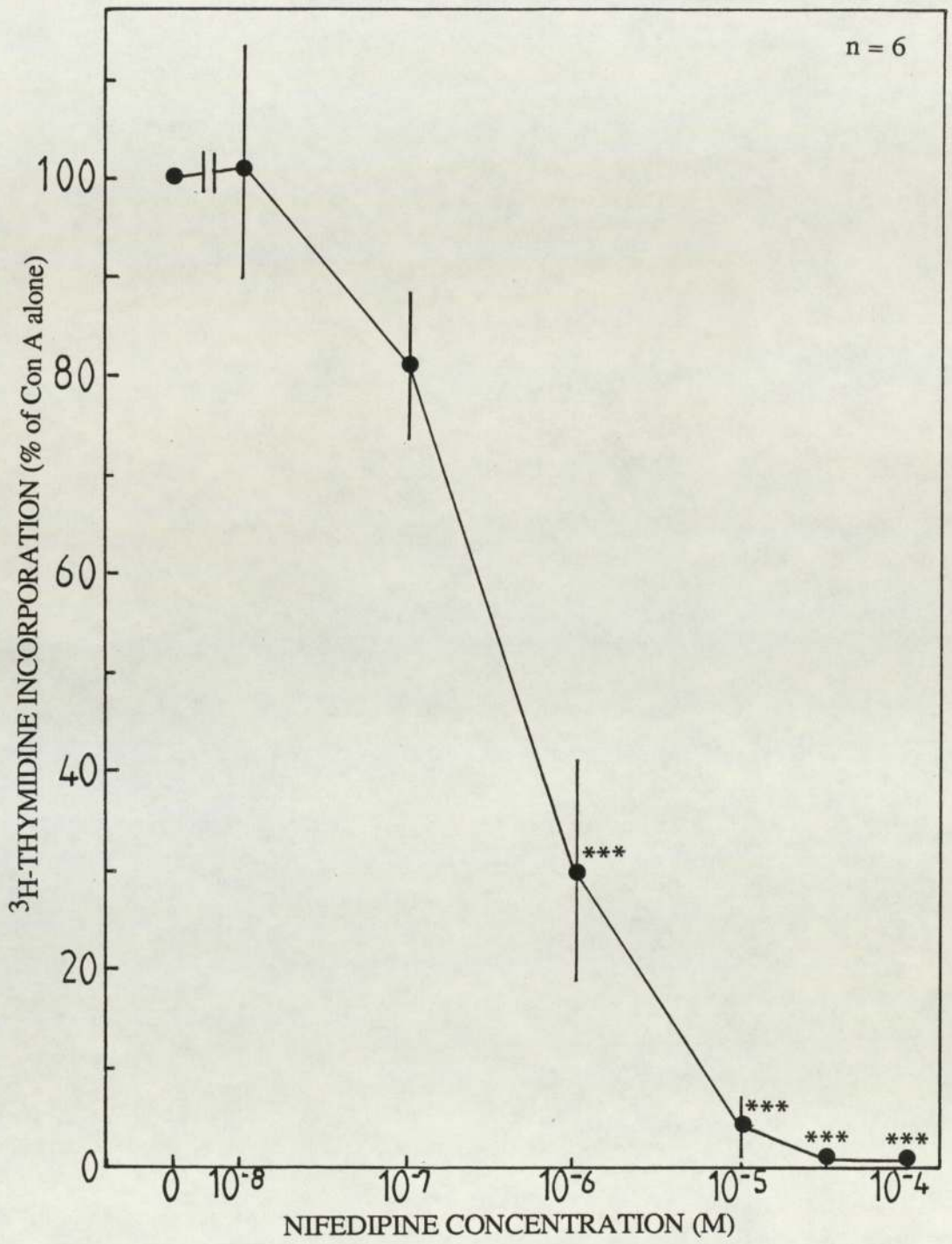


Figure 4: Effect of Nifedipine on Con A (5 μg ml^{-1}) induced murine splenocyte proliferation.

Values significantly different from corresponding cultures without the drug are indicated by asterisks. Other details as in Figure 3.

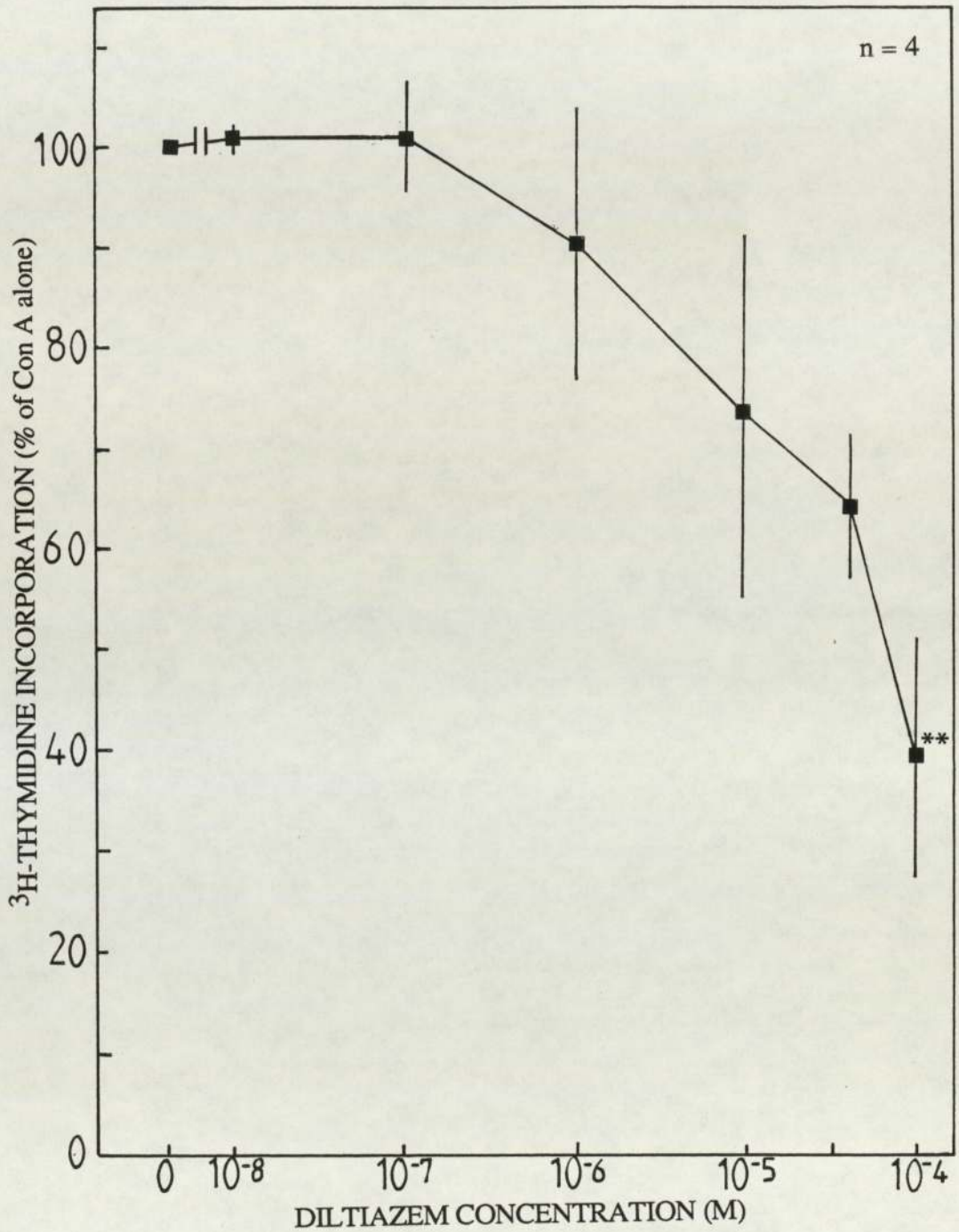


Figure 5: Effect of Diltiazem on Con A (5 μg ml^{-1}) induced murine splenocyte proliferation.

Values significantly different from corresponding cultures without the drug are indicated by asterisks. Other details as in Figure 3.

Con A alone was taken as 100% and incorporation in the presence of drugs expressed as a percentage thereof. All three agents suppressed proliferation in a dose-related manner, a significant inhibition of [^{3}H]-thymidine uptake was found at 5 x 10^{-5} M verapamil, 10^{-6} M nifedipine and 10^{-4}M diltiazem. To exclude the possibility that suppression of T cell proliferation is due to cytotoxicity, cell viability was examined in control cultures and those containing the drugs at the above concentrations. 90-95% of splenocytes were viable at the end of the incubation period as assessed by trypan blue exclusion, under all culture conditions. Thus, Ca^{2+} influx appears to be a pre-requisite for the Con A induced mitotic response in splenocytes. To verify this hypothesis the radioisotope of calcium, $^{45}Ca^{2+}$, was used to monitor calcium influx in splenocytes at rest and during their recruitment from quiescence by Con A. Preliminary studies revealed that the time course of $^{45}Ca^{2+}$ uptake in Con A treated splenocytes was not significantly different from control cells (Figure 6) implying that either Con A induced mitotic stimulation does not involve an enhanced Ca^{2+} influx or that the technique employed (see Section 2.4 for details) fails to monitor an enhanced Ca^{2+} influx. Since cells treated with the calcium ionophore A23187 (10^{-7}M) also failed to demonstrate any increase in $^{45}Ca^{2+}$ uptake over control values during a thirty minute period it appears likely that this microcentrifugation technique is unsuitable for monitoring Ca^{2+} fluxes in murine splenocytes.

Since $^{45}Ca^{2+}$ tracer studies were unable to define the role of Ca^{2+} fluxes in Con A induced proliferation, it seemed appropriate to monitor intracellular calcium levels following lectin stimulation. For this purpose fura-2 (see Section 2.3.1) was employed. The calcium ionophore A23187 was used to demonstrate the effectiveness of the procedure. An increase in cytosolic Ca^{2+} concentration is represented by a figure greater than one, since resting levels are considered equal to unity. A23187 10^{-7}M caused a rapid (within one minute) increase in fluorescence (Table 1) whereas the addition of an equal volume of physiological saline did not influence the $[Ca^{2+}]i$ over a ten minute period (results not shown).

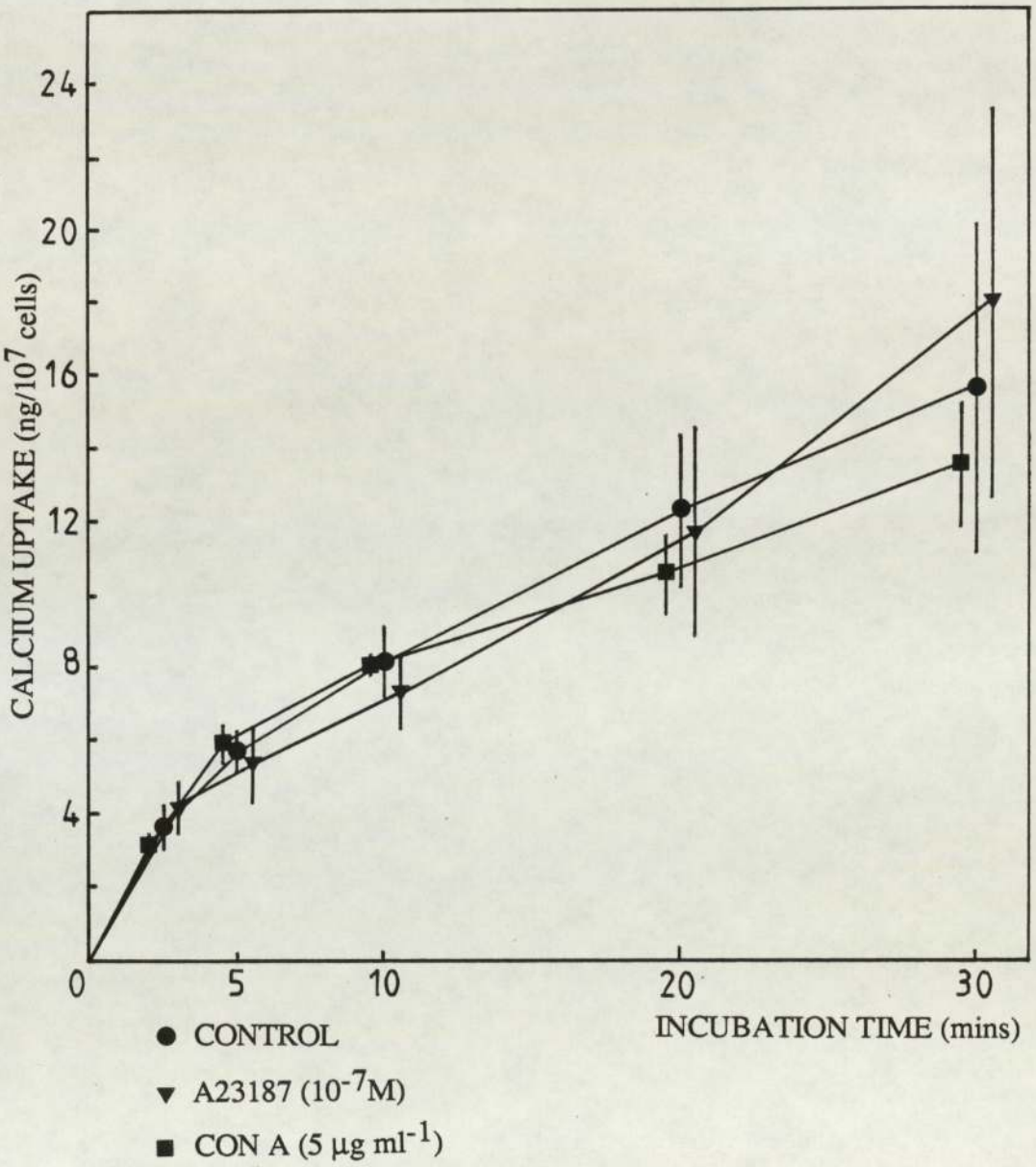


Figure 6: Effect of Con A (5 μg ml^{-1}) and ionophore A23187 (10^{-7}M) on calcium uptake in isolated murine splenocytes

Each value represents the mean (± SEM) of triplicate determinations in three experiments.

Table 1 - Effect of A23187 10^{-7}M on the intracellular calcium concentration of murine splenocytes

Resting $[Ca^{2+}]i$ levels (R) (nM)	Stimulated $[Ca^{2+}]i$ levels (nM) (S)	Increase (S/R)	Mean increase ± SEM
189	560	2.96	
145	251	1.73	2.23 ± 0.37
137	273	1.99	

Medium cation concentration was 0.6 mM Ca^{2+}, 1.0 mM Mg^{2+} in all fura-2 studies unless otherwise stated.

Splenocytes retained their ability to respond to calcium ionophore even after two hours incubation at 37°C although some fura-2 leakage was apparent (see Section 2.3.1); cell viability was also maintained at greater than 90% throughout this period. When intracellular calcium was monitored following Con A treatment a rapid increase in $[Ca^{2+}]i$ was observed reaching a maximum by 1 1/2 minutes (see Diagram 4 for a typical recording). The rise in $[Ca^{2+}]i$ in response to Con A in murine splenocytes demonstrates an increase of 1.45 (± 0.16 SEM n = 39) over resting levels. The $[Ca^{2+}]i$ prior to stimulation, ie. the resting level is 186 nM (± 10 SEM n = 39). The Ca^{2+} signal persists for at least one hour following interaction of Con A with its receptor (results not shown) and possibly longer. Attempts to monitor $[Ca^{2+}]i$ following Con A stimulation during the whole of the 48 hour incubation period were unsuccessful; fura-2 loading performed at intervals after Con A application gave variable results such that no trends were distinguishable.

The Ca^{2+} signal generated by Con A treatment could result from a stimulation of Ca^{2+} influx, an inhibition of active Ca^{2+} efflux across the plasma membrane and/or mobilisation of Ca^{2+} from an intracellular store(s) (see Section 1.5.2). To elucidate the nature of the Ca^{2+} signal the effect of the calcium channel blockers was investigated. Verapamil (5 x 10^{-5}M) at a concentration known to suppress Con A induced proliferation (Figure 3) significantly diminished the lectin

stimulated $[Ca^{2+}]i$ increase (Table 2).

Table 2: The effect of Verapamil on the Con A induced increase in intracellular calcium concentration $[Ca^{2+}]i$ of murine splenocytes

	Treatment	
	Con A (5 μg ml^{-1})	Con A (5 μg ml^{-1}) Verapamil (5 x 10^{-5}M)
Increase in $[Ca^{2+}]i$ above basal levels (considered equal to unity)	1.59	1.35
	1.50	1.40
	1.25	1.18
	1.27	1.16

Whilst investigating the effects of various agents on the Con A induced Ca^{2+} signal, experiments involving the potential antagonists were conducted alongside control 'runs' of Con A alone wherever possible and the subsequent results analysed by the 'paired T test'. Verapamil (5 x 10^{-5}M) has a significant ($P < 0.05$) effect on the Con A induced increase in $[Ca^{2+}]i$ in murine splenocytes. In contrast, the concentration of diltiazem (10^{-4} M) found to inhibit proliferation (Figure 5) was without effect on the increase in $[Ca^{2+}]i$ promoted by Con A (Table 3).

Table 3: The effect of Diltiazem on the Con A induced increase in $[Ca^{2+}]i$ of murine splenocytes

	Treatment	
	Con A (5 μg ml^{-1})	Con A (5 μg ml^{-1}) + Diltiazem (5 x 10^{-4}M)
Increase in $[Ca^{2+}]i$ over basal levels (considered equal to unity)	1.40	1.34
	1.48	1.54
	1.40	1.37

Attempts to monitor the effect of nifedipine, on the Con A stimulated rise in $[Ca^{2+}]i$ were unsuccessful due to its ability to interfere with the fluorescence signal, possibly by filtering out excitation and emission energies.

The equivocal nature of these results may reflect the differing abilities of the calcium antagonists to block a calcium influx component of the early Ca^{2+} signal. The effects of the Ca^{2+}channel blockers on the Con A induced Ca^{2+} signal were only monitored for approximately five minutes. However, it must be borne in mind that in a 48 hour incubation period they may cause other alterations in the intracellular calcium concentrations. Additional interpretations include the possibility that this early Ca^{2+} signal may be irrelevant in the signal transduction pathway following Con A stimulation; or that some feature of the early Ca^{2+} signal may be important or finally, that these calcium antagonists exert effects on proliferation unrelated to calcium.

The Ca^{2+} signal in response to Con A was partially diminished when Ca^{2+} was absent from the extracellular medium (achieved by washing twice and resuspending in Ca^{2+} free medium), when Ca^{2+} was subsequently added to the medium a further increase in $[Ca^{2+}]i$ was monitored (Figure 7, Table 4) suggesting that the Con A induced early Ca^{2+} signal depends on both a mobilisation of intracellular Ca^{2+} and an influx of Ca^{2+} ions across the plasma membrane.

Table 4: The effect of extracellular Ca^{2+} ions on the Con A (5 $\mu g\ ml^{-1}$) induced increase in $[Ca^{2+}]i$ in murine splenocytes

Resting $[Ca^{2+}]i$ levels (nM) in Ca^{2+} free media (R)	Con A (5 $\mu g\ ml^{-1}$) addition: stimulated $[Ca^{2+}]i$ levels (nM) (S)	Increase in $[Ca^{2+}]i$ (S/R)	Ca^{2+} addition (0.6 mM): stimulated $[Ca^{2+}]i$ levels (nM) (S')	Increase in $[Ca^{2+}]i$ (S'/R)
200	230	1.15	297	1.49
155	192	1.24	232	1.50
137	190	1.39	266	1.94
164	208	1.27	247	1.51
136	178	1.31	216	1.59
Mean + SEM				
158.4±11.69	199.6±8.98	1.27±0.04	251±14.04	1.61±0.09

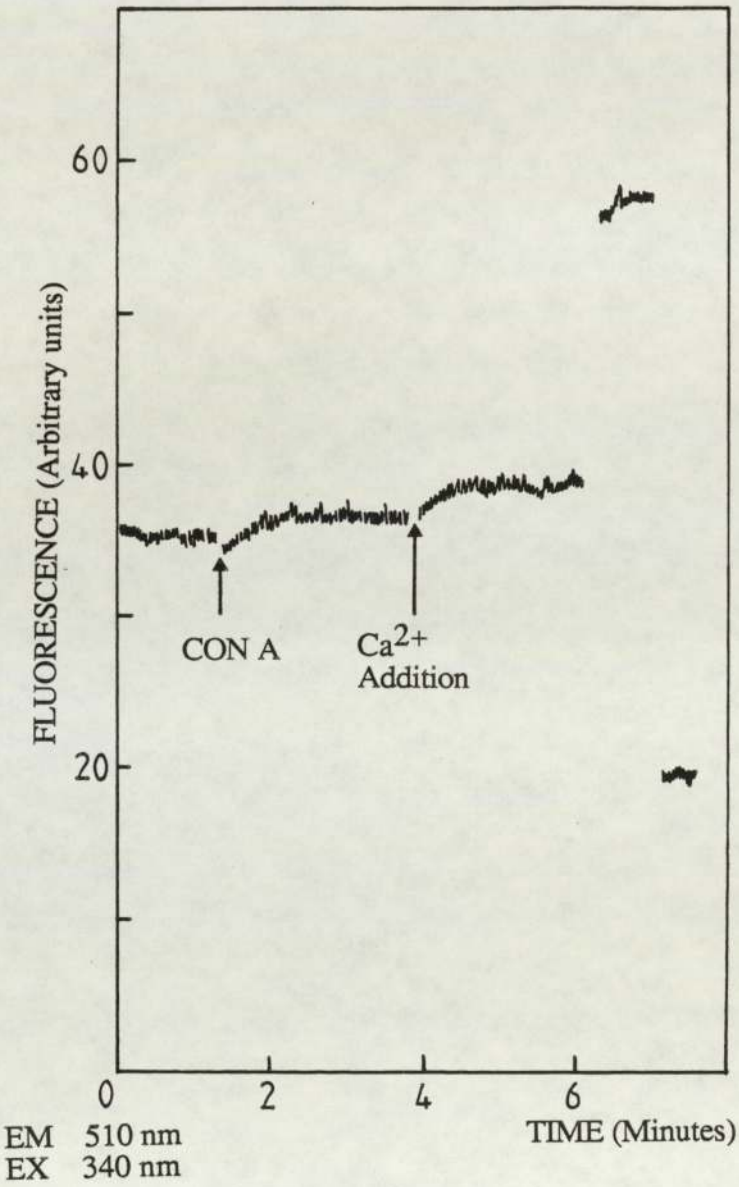


Figure 7: The effect of Con A (5 μg ml^{-1}) on the $[Ca^{2+}]i$ of fura-2 loaded murine splenocytes incubated in Ca^{2+} free medium followed by subsequent addition of Ca^{2+} ions (0.6 mM) to the extracellular environment.

The fluorescence values correspond to resting calcium levels of 155 nM in Ca^{2+} free medium which rise to 192 nM following Con A stimulation, a further increase to a $[Ca^{2+}]i$ of 232 nM occurs upon addition of Ca^{2+} ions to the medium.

Removal of Ca^{2+} from the medium for short time periods did not appear to alter the resting $[Ca^{2+}]i$ levels from cells incubated in medium with the cation present (compare resting levels in Tables 1 and 4). The fluorescence signal of fura-2 in splenocytes incubated for up to 10 minutes in Ca^{2+}-free physiological saline was not increased over 5-10 minutes after addition of external calcium (results not shown). This suggests that addition of Ca^{2+} ions to cells in Ca^{2+} free saline has no effect *per se* on the $[Ca^{2+}]i$ in murine splenocytes.

Although the viability of cells appeared to be unaffected during brief periods in Ca^{2+}-free medium (results not shown) Ca^{2+} depletion may have had other effects on the cells in addition to the effects on the Ca^{2+} signal. Nevertheless these studies suggest that the early Ca^{2+} signal generated by Con A stimulation in murine splenocytes is dependent on Ca^{2+} in the medium and mobilisation of Ca^{2+} from an intracellular pool.

A justifiable supposition is that the increase in intracellular calcium ion concentration probably activates the calcium regulatory protein, calmodulin (see Section 1.5.2). Consequently, the effects of two calmodulin antagonists, trifluoperazine (TFP) and M&B,13,573 (May & Baker Ltd) on the Con A induced mitotic response were investigated. As predicted both agents suppressed proliferation in a dose-dependent fashion, with approximately 50% inhibition being achieved at 10^{-7}M in both cases (Figure 8); at these concentrations cell viability was unaffected (results not shown) confirming that cytotoxicity was not responsible for the diminished [^{3}H]-thymidine incorporation. Higher concentrations of TFP ~ 50 μM are thought to exhibit non-specific effects on protein kinase C and reduce ATP levels (see Discussion, Chapter 4 for further details).

Thus, whilst these preliminary studies demonstrate that a considerably lower TFP concentration inhibits splenocyte proliferation it is evident that the effects of calmodulin inhibitors must be interpreted cautiously. Nevertheless, the inhibition of Con A induced proliferation by calmodulin antagonists corroborates the evidence gained from the proliferative studies employing Ca^{2+} channel blockers.

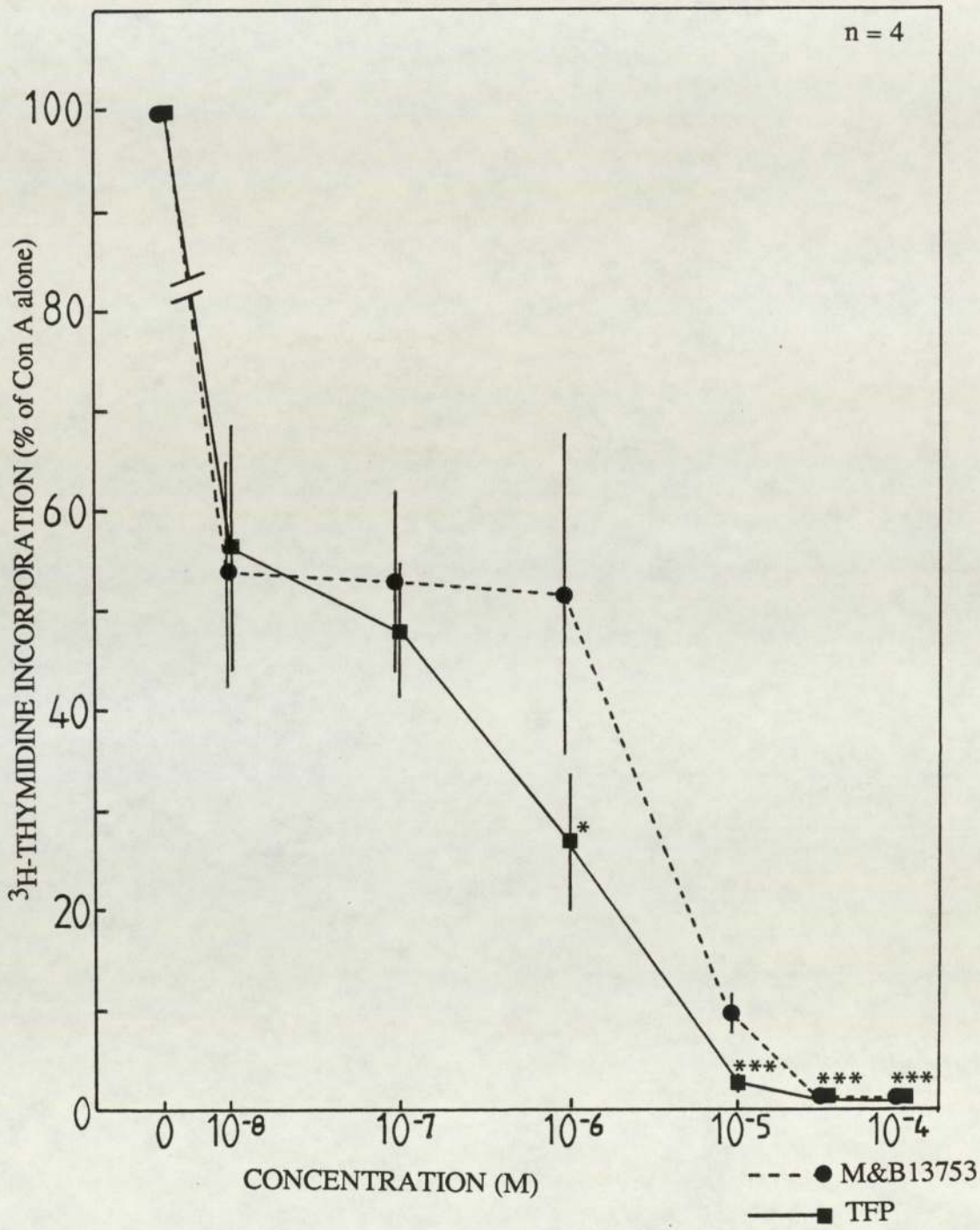


Figure 8: Effect of calmodulin inhibitors on Con A (5 μg ml^{-1}) induced murine splenocyte proliferation.

Values significantly different from those of cultures without drugs are indicated by asterisks.

Thus, Ca^{2+} appears to be involved in the signal transduction pathway following Con A stimulation. However, to establish whether the early Ca^{2+} signal is obligatory (rather than a passive accompaniment to some other unknown signal) for subsequent DNA synthesis to occur it is essential to find an agent which not only blocks the early Ca^{2+} signal demonstrable in fura-2 studies but which also inhibits [^{3}H]-thymidine incorporation following 48 hours incubation with Con A. Agents that elevate intracellular cAMP or membrane-permeant cyclic AMP analogues such as 8-bromo cyclic AMP (8-Br cAMP) and dibutyryl cyclic AMP also block the early Ca^{2+} signal and inhibit subsequent DNA synthesis in response to Con A in murine thymocytes (Hesketh et al, 1985; Metcalfe et al, 1985). Neither of these membrane permeant cyclic AMP analogues impaired the Con A induced early Ca^{2+} signal at any concentration tested (Tables 5 & 6) following a 3 minute preincubation period and only a rather unphysiological concentration of dibutyryl cAMP (10^{-4}M) caused a significant reduction in DNA synthesis (Figure 9).

Table 5: The effect of preincubation with 8-Br cAMP, on the Con A induced increase in intracellular calcium concentration of murine splenocytes

	Con A (5 μg ml^{-1})	Treatment Concentration (M)	Con A (5 μg ml^{-1}) + 8-Br cAMP
Increase in $[Ca^{2+}]i$ above basal levels (considered equal to unity)	1.43	10^{-5}	1.55
	1.46	10^{-6}	1.40
	1.61	10^{-7}	1.57

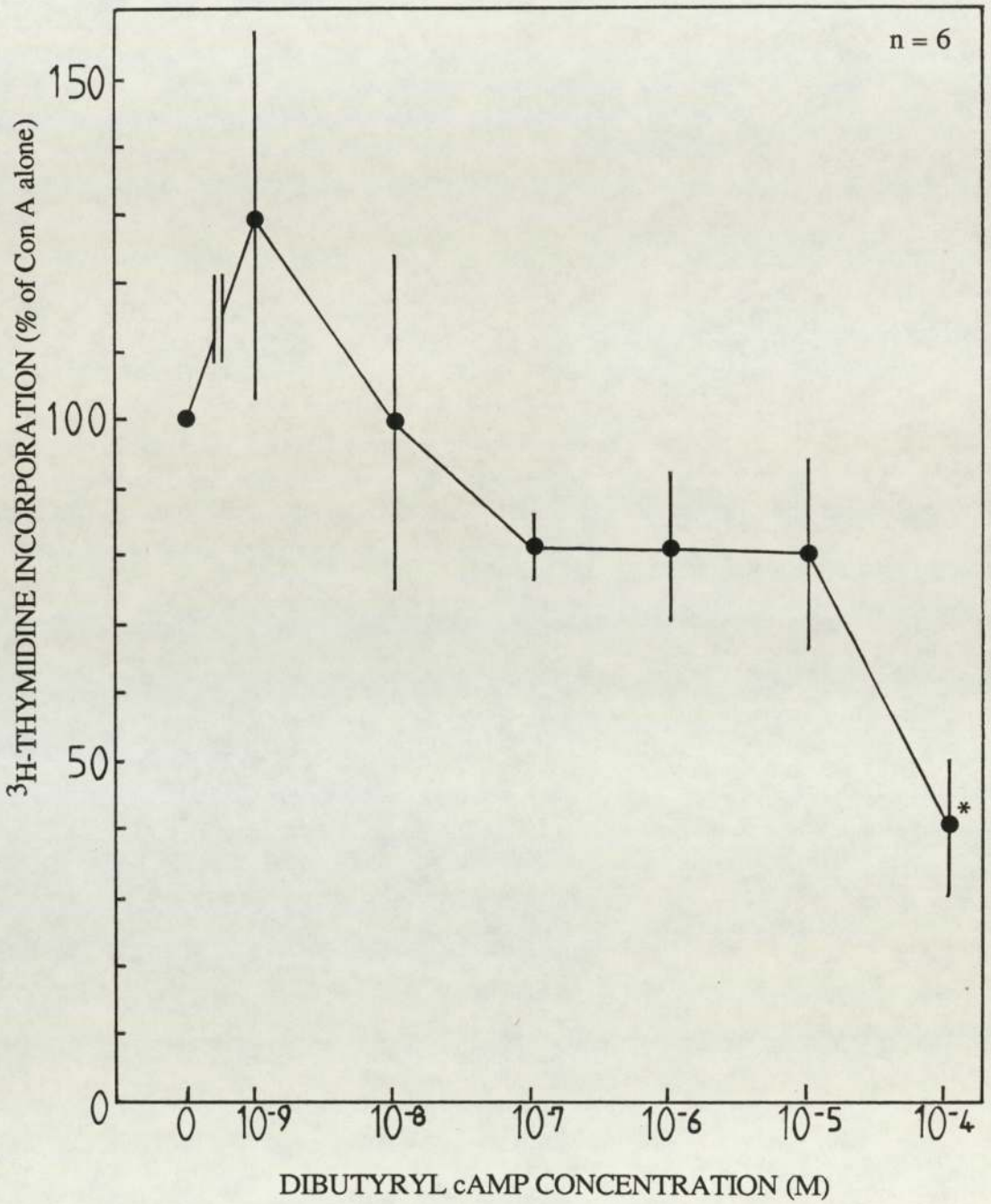


Figure 9: Effect of Dibutyryl cyclic AMP on Con A (5 $\mu g\ ml^{-1}$) induced murine splenocyte proliferation.

Values significantly different from those of cultures with Con A alone are indicated by an asterisk.

Table 6: The effect of preincubation with dibutyryl cAMP on the Con A induced increase in intracellular calcium concentration of murine splenocytes

	Treatment		
	Con A (5 μg ml^{-1})	Con A (5 μg ml^{-1}) + dibutyryl cAMP Concentration (M)	
Increase in $[Ca^{2+}]i$ above basal levels (considered equal to unity)	1.61	10^{-5}	1.57
	1.46	10^{-6}	1.39
	1.61	10^{-7}	1.58

Since these membrane permeant cyclic AMP analogues were unable to block either the early Ca^{2+} signal or DNA synthesis except at high concentrations it seemed appropriate to investigate the effects of forskolin an agent which elevates intracellular cAMP, on Con A induced DNA synthesis. It is apparent that an increase in endogenous cAMP does indeed inhibit [^{3}H]-thymidine incorporation in a dose-related manner (Figure 10) unless forskolin has effects on DNA synthesis unrelated to cAMP production. Forskolin was initially dissolved in DMSO but the final concentrations of DMSO had no effect on [^{3}H]-thymidine incorporation. (Results not shown). Since fura-2 studies were not undertaken to investigate the effects of forskolin on the early Ca^{2+} signal it has not yet been established how forskolin mediates its inhibitory effects. Forskolin alone had no effect on basal [^{3}H]-thymidine incorporation in murine splenocytes (results not shown), however, this does not imply that cAMP is not involved in stimulus-mitosis coupling in murine splenocytes. Thus, the timing of a cAMP signal may be critical in recruiting quiescent cells to enter the cell cycle (see Section 1.5.1).

Attempts to directly correlate the early Ca^{2+} signal with the subsequent DNA synthesis measured 48 hours after Con A stimulation have proved difficult. Thus, no agent has been found which abolishes the early Ca^{2+} signal in addition to inhibiting [^{3}H]-thymidine incorporation 48 hours later. Nevertheless, the studies with verapamil demonstrate that the early Ca^{2+} signal does indeed have some relevance for

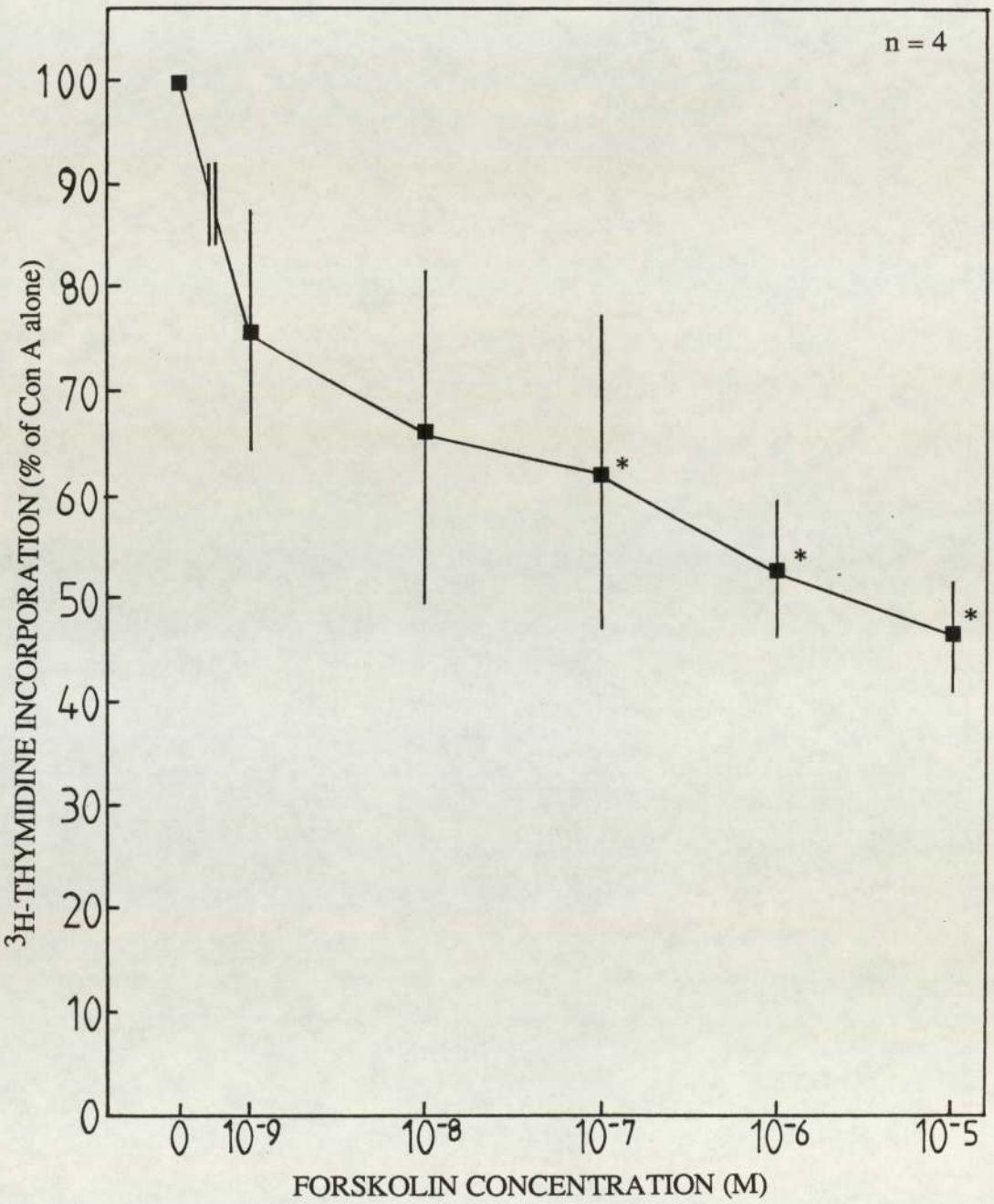


Figure 10: Effect of Forskolin on Con A (5 μg ml^{-1}) induced murine splenocyte proliferation.

Values significantly different from those of cultures without forskolin are indicated by an asterisk.

the initiation of DNA synthesis. An attempt was made to establish the chronology of the Ca^{2+} requirement during Con A stimulation. If extracellular Ca^{2+} was chelated throughout the incubation period then DNA synthesis was obliterated (Figure 11). But if Ca^{2+} presence was allowed to persist for the first four hours no inhibition occurred. If Ca^{2+} ions were restored to Ca^{2+} free medium at four hours following Con A stimulation, DNA synthesis was still severely reduced (Figure 11); these results indicate that the Ca^{2+} requirement comes early in the incubation period. To determine the crucial period of time which elapses between Con A application and commitment to division (the 'activation' period) experiments were conducted where the mitogenic effect of Con A was systematically inhibited by the addition of α methyl mannoside to the culture medium. At a concentration previously shown to maximally inhibit Con A induced DNA synthesis (Figure 12), α methyl mannoside (50 mM) suppressed $[^3H]$-thymidine incorporation in murine splenocytes when applied during the first 6 hours of incubation after which time it had no effect (Figure 13). Thus, receptor occupancy is necessary for the first 4-6 hours of Con A stimulation whilst extracellular calcium is only required for the first 4 hours of the 48 hours incubation period implying that in addition to the rise in $[Ca^{2+}]i$ following stimulation - the putative mitotic signal, Con A occupancy also serves some additional purpose(s).

Many mitogens that use Ca^{2+} as a second messenger induce hydrolysis of membrane phosphoinositides resulting in a transducing mechanism that initiates a signal cascade comprising mobilisation of Ca^{2+} and the activation of protein kinase C (see Section 1.5.2). Con A is known to stimulate the breakdown of phosphatidylinositol (4,5)-bisphosphate ($PtdInsP_2$) to inositol (1,4,5)-trisphosphate ($InsP_3$) in murine thymocytes (Moore et al, 1984; Metcalfe et al, 1985). The two limbs of this signal pathway can be mimicked by the calcium ionophore A23187 and the phorbol ester TPA (12-0-tetradecanoyl phorbol 13-acetate) which is assumed to activate specifically protein kinase C (Metcalfe et al, 1985) (see Section 1.5.2).

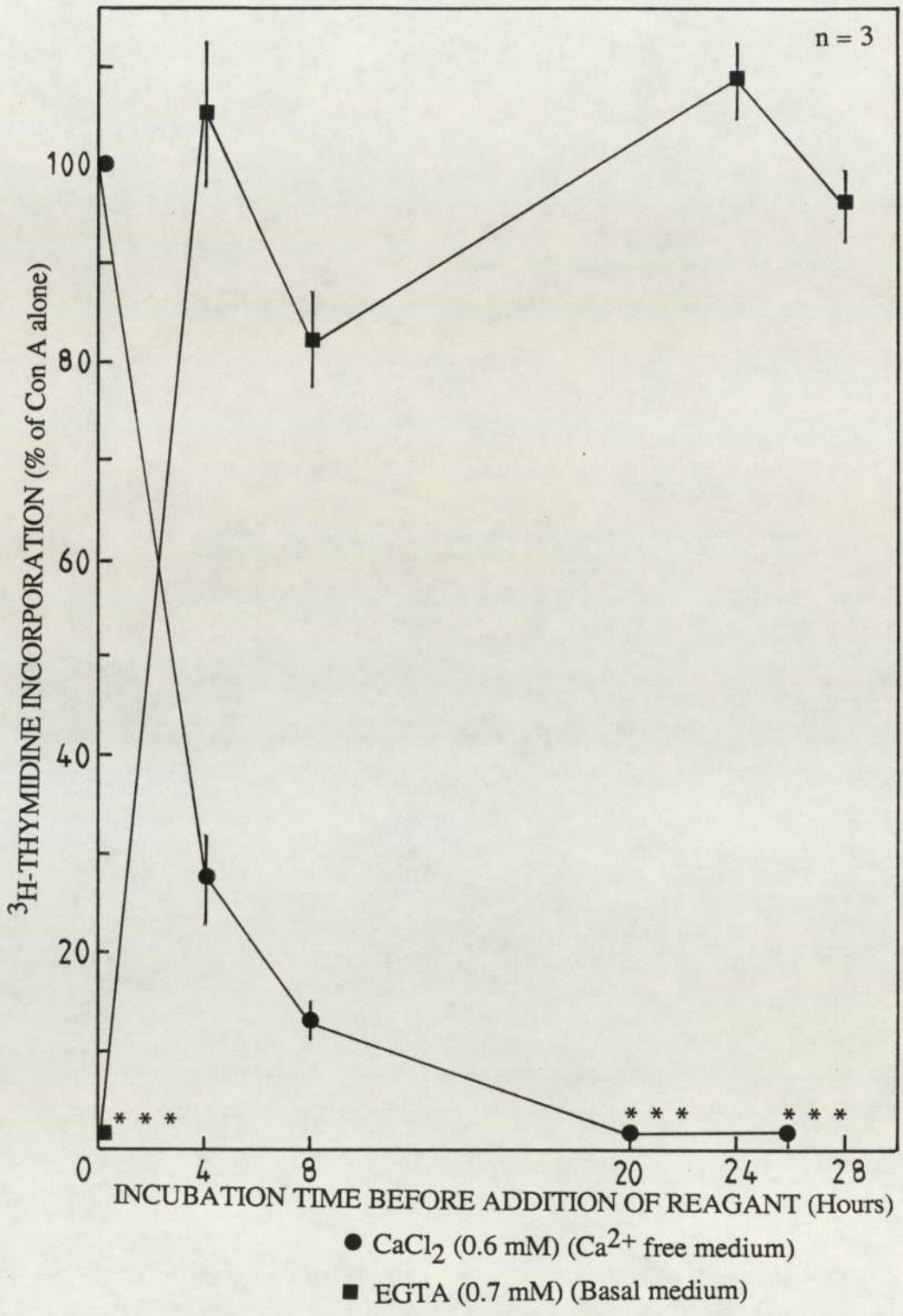


Figure 11: Effect of sequential additions of EGTA and $CaCl_2$ during the 48 hour incubation on Con A (5 $\mu g\ ml^{-1}$) induced murine splenocyte proliferation.

Values significantly different from those of cultures with Con A and basal medium are indicated by asterisks. Basal medium contains 0.6 mM Ca^{2+} sequential additions of $CaCl_2$ (0.6 mM) are made to cultures in medium rendered Ca^{2+} free by the addition of EGTA (0.7 mM).

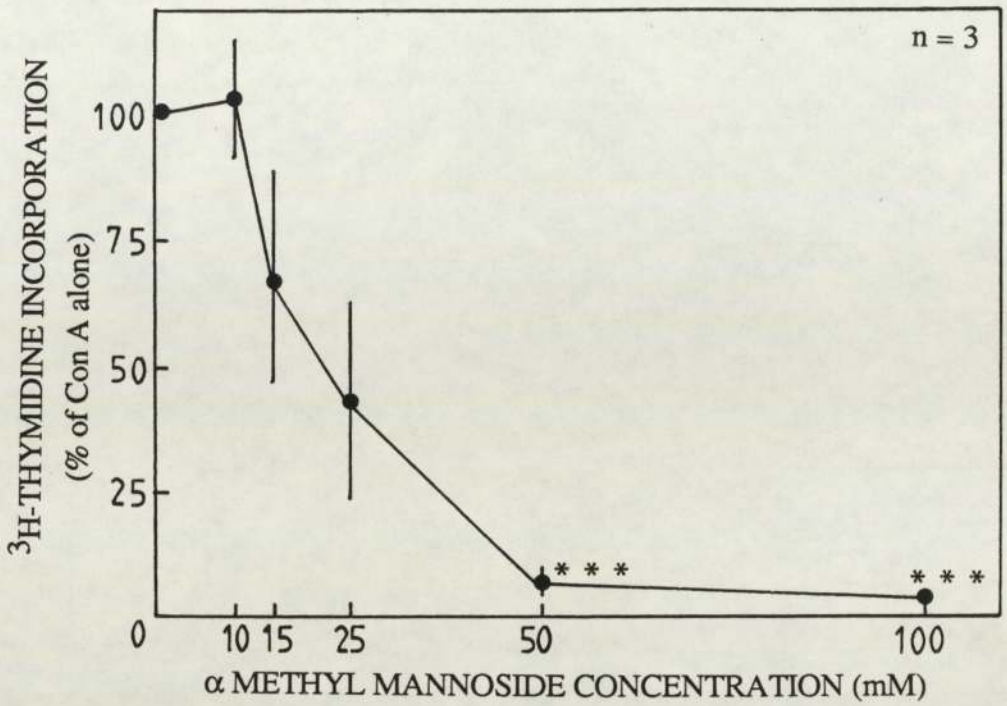


Figure 12: Dose-response curve of the effect of α methyl-mannoside on Con A (5 μg ml^{-1}) induced murine splenocyte proliferation

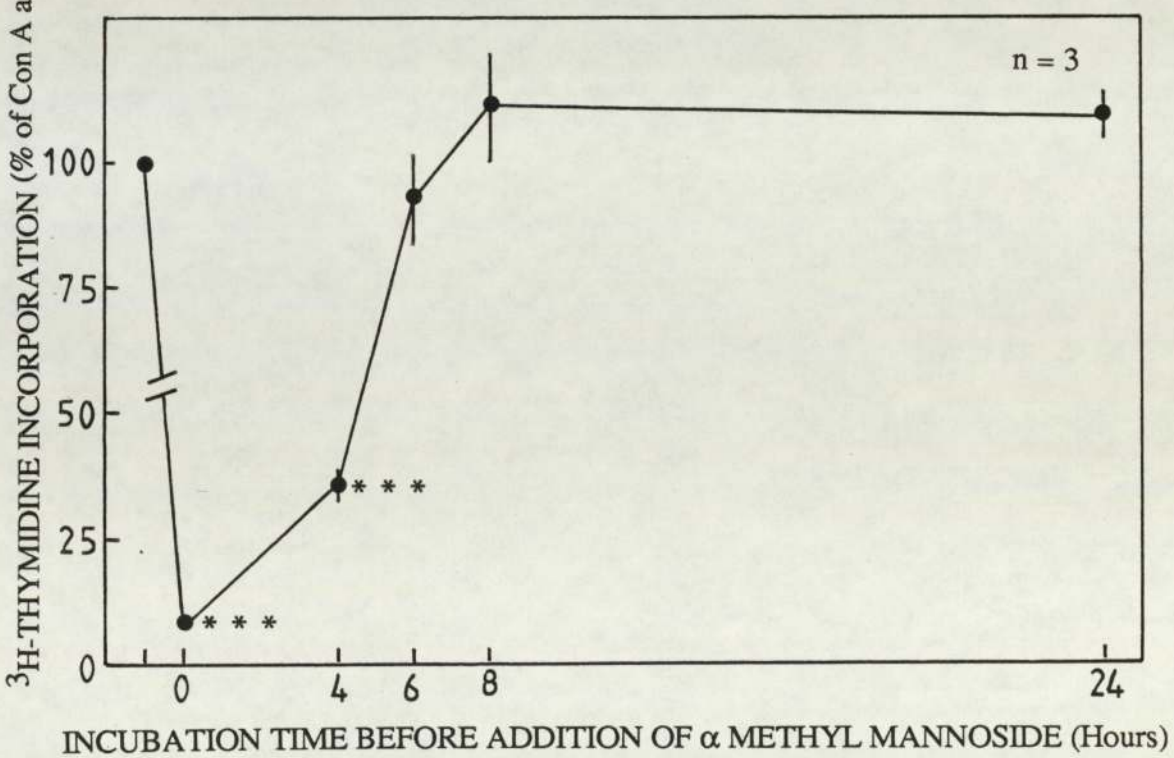


Figure 13: Effect of sequential additions of α methyl-mannoside (50 mM) during the incubation period on Con A (5 μg ml^{-1}) induced murine splenocyte proliferation.

Values significantly different from corresponding cultures without the drug are indicated by asterisks.

Therefore proliferative studies were undertaken to determine if this transducing mechanism is involved in Con A stimulated murine splenocyte proliferation. These studies demonstrated that A23187 alone did not stimulate DNA synthesis at any concentration tested (Figure 14) whilst TPA alone caused a very modest (in comparison to Con A) increase in [^{3}H]-thymidine incorporation (Figure 14). However, when varying concentrations of A23187 in combination with TPA (10^{-9}M) were investigated for their mitotic potential, a synergistic interaction emerged with A23187 (10^{-7}M) and TPA (10^{-9}M) (Figure 14) showing a stimulation index equivalent to about 50% of the Con A (5 μg ml^{-1}) stimulation index under identical conditions (results not shown). To elucidate whether the Ca^{2+} signal and protein kinase C activation were able to compensate for one another, lower concentrations of A23187, (10^{-8}M and 10^{-9}M) in conjunction with varying concentrations of TPA were tested for their proliferative actions. Neither concentration of ionophore produced a significant increase in [^{3}H]-thymidine incorporation with any concentration of TPA (Figures 15 and 16) suggesting that the magnitude of the Ca^{2+} signal generated by ionophore and protein kinase C activation by TPA are critical in stimulating DNA synthesis.

The opportunistic comitogens, TPA (10^{-9}M) and A23187 (10^{-7}M) presumably bypass the physiological pathway acting through $PtdInsP_3$ breakdown, by increasing [Ca^{2+}]i and activating PKC directly. Indeed, TPA does seem to be exerting its affects by enhancing PKC activity rather than by a non-specific membrane perturbation since the synergism could not be repeated using 4 α-phorbol-12,13-didecanoate, a phorbol ester which has no PKC enhancing activity (Castagna et al, 1982) (Figure 17). These studies indicate that the phosphoinositide transducing mechanism may be important in murine splenocyte proliferation and that PKC activation is necessary in addition to a Ca^{2+} signal and it is predicted that they may be compulsory secondary responses (or signals) for mitogenic stimulation by Con A.

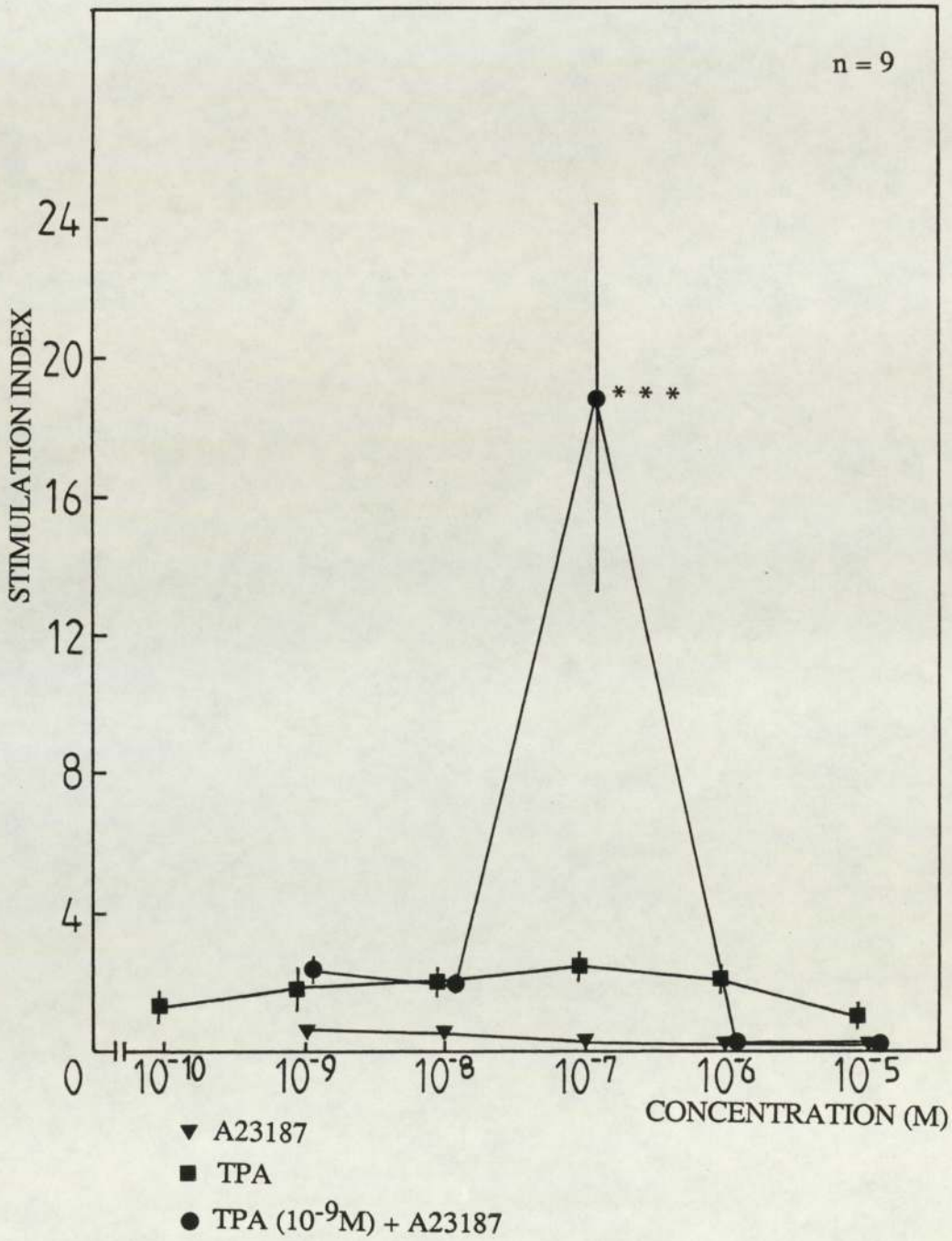


Figure 14: Dose response curves of the effects of A23187 and TPA alone and in combination on thymidine incorporation in isolated murine splenocytes.

Values significantly different from those of the other cultures are indicated by asterisks.

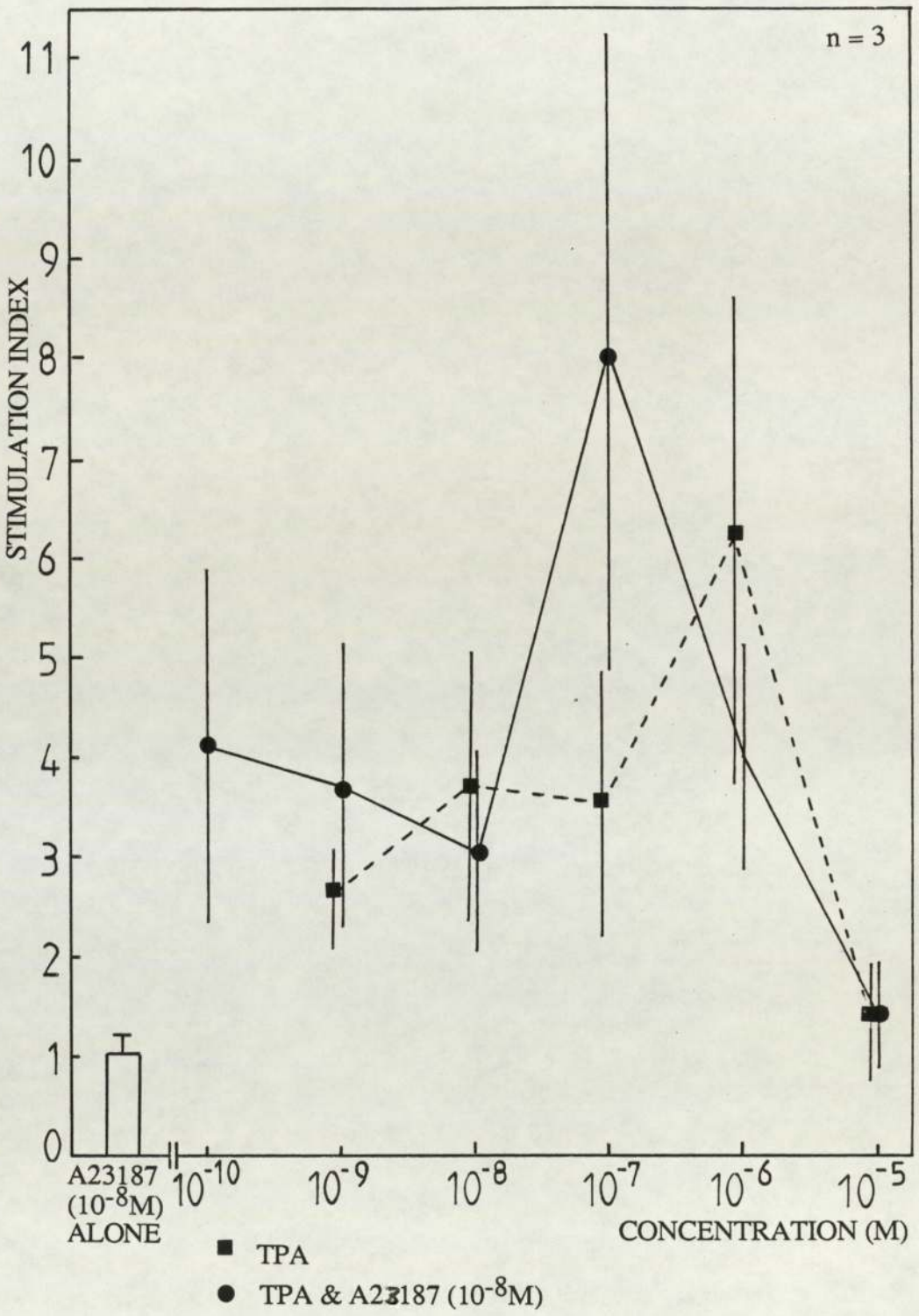


Figure 15: Effects of TPA with and without A23187 (10^{-8} M) on thymidine incorporation in isolated murine splenocytes.

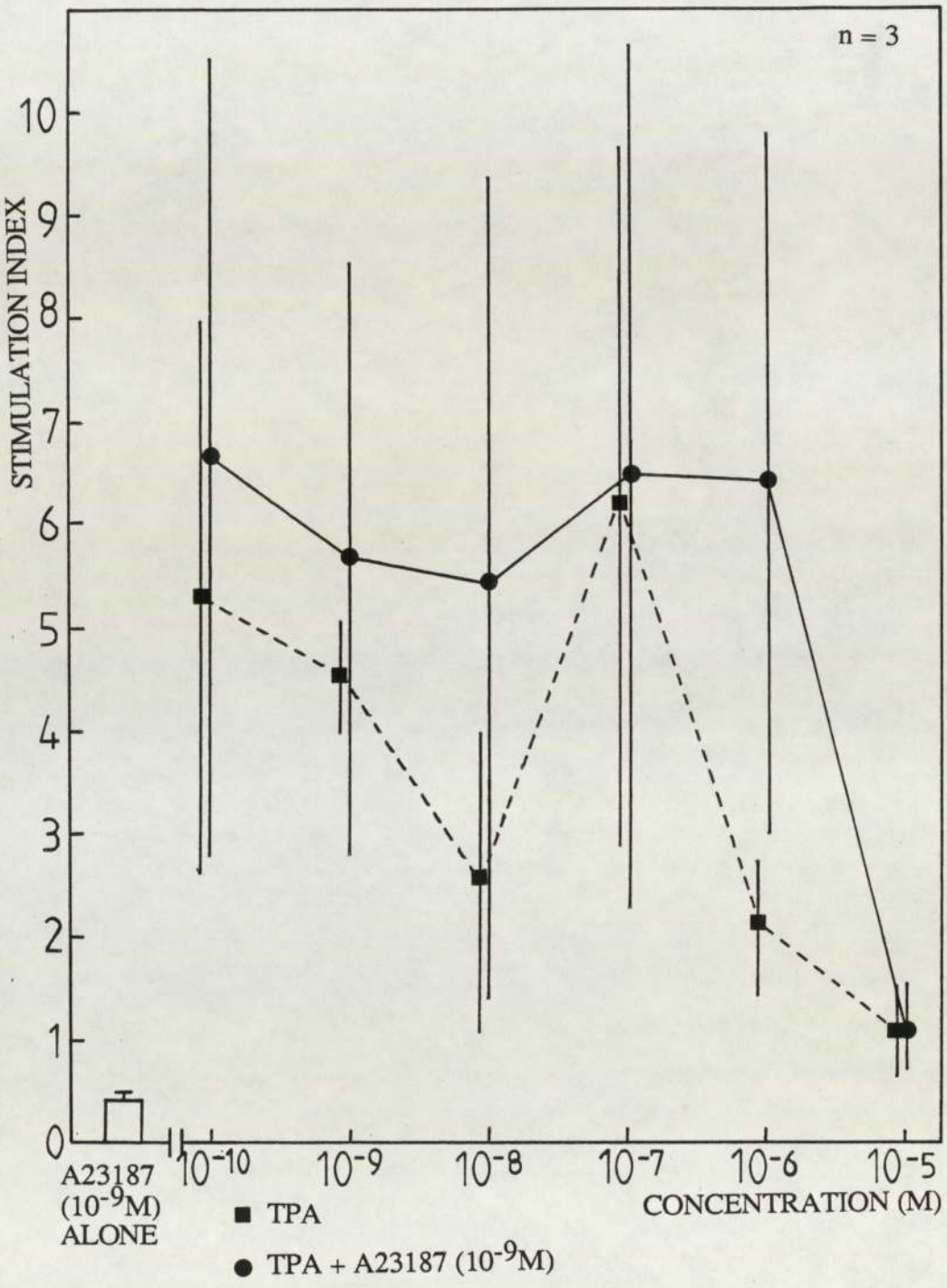


Figure 16: Effects of TPA with A23187 (10^{-9}M) and without ionophore on thymidine incorporation in isolated murine splenocytes

Fura-2 studies substantiate the hypothesis that a Ca^{2+} signal alone is insufficient to trigger mitosis since A23187 10^{-7}M caused an early Ca^{2+} signal of greater magnitude than Con A (Table 1) although it was not mitogenic by itself (Figure 14). The early Ca^{2+} signal was unaffected by the simultaneous addition of TPA (10^{-9}M) (Table 1 & 7) although the two acted synergistically to increase DNA synthesis (Figure 14). Since A23187 (10^{-7}M) and 4 α,12,13-phorbol didecanoate (10^{-9}M) together produced a Ca^{2+} signal of the same proportion (Table 7) but do not act as co-mitogens (Figure 17) it appears that in addition to the early Ca^{2+} signal there must be another signal presumably PKC activation. Neither phorbol ester had any effect on resting $[Ca^{2+}]i$ levels at these concentrations (results not shown).

Table 7: The effect of simultaneous administration of calcium ionophore A23187 and phorbol esters on intracellular calcium concentrations of murine splenocytes

	A23187 (10^{-7}M) + TPA (10^{-9}M)	Treatment A23187 (10^{-7}M + 4 α,12,13 phorbol didecanoate (10^{-9}M)
Increase in $[Ca^{2+}]i$ above basal levels (considered equal to unity)	1.64 2.29 1.76	2.46 3.48 2.45

Preliminary studies revealed that when the Ca^{2+} signal and the putative PKC activation generated by Con A were mimicked by appropriate combinations of A23187 and TPA the subsequent DNA synthesis was reduced by approximately 50% compared with the responses to Con A (results not shown). This implies that the lectin promoted a further unidentified signal in addition to the Ca^{2+} signal and any PKC activation. Further studies demonstrated surprisingly, that no concentration of Con A tested, further enhanced DNA synthesis stimulated by the co-mitogens TPA

(10^{-9}M) and A23187 (10^{-7}M (Figure 18). Moreover the stimulation indices of the optimal concentration of Con A (5 μg ml^{-1}) and the combination of A23187 and TPA are virtually identical (Figure 18). These results are in stark contrast to preliminary studies which suggested that Con A (5 μg ml^{-1}) produced a much greater signal, and possibly relate to fluctuations between different batches of TPA since in later studies the concentration of TPA required to synergise with A23187 was rather variable (results not shown). Unfortunately, these ambiguous results are unable to confirm or deny the necessity of a third signal in addition to the Ca^{2+} mobilisation and PKC activation.

There are indications in the literature that TPA at higher concentrations can interfere with receptor dependent increases in cytosolic Ca^{2+} (Naccache et al, 1985; Staddon & Hansford, 1986; Muldoon et al, 1987). Indeed, if murine splenocytes were preincubated with varying concentrations of TPA for 3 minutes the Ca^{2+} signal measured by fura-2 fluorescence in response to Con A was diminished by doses of 10^{-8}M TPA and above (Table 8). Cells preincubated with the co-mitogenic concentration of TPA (10^{-9}M) did not demonstrate a diminution in the Ca^{2+} signal (Table 8).

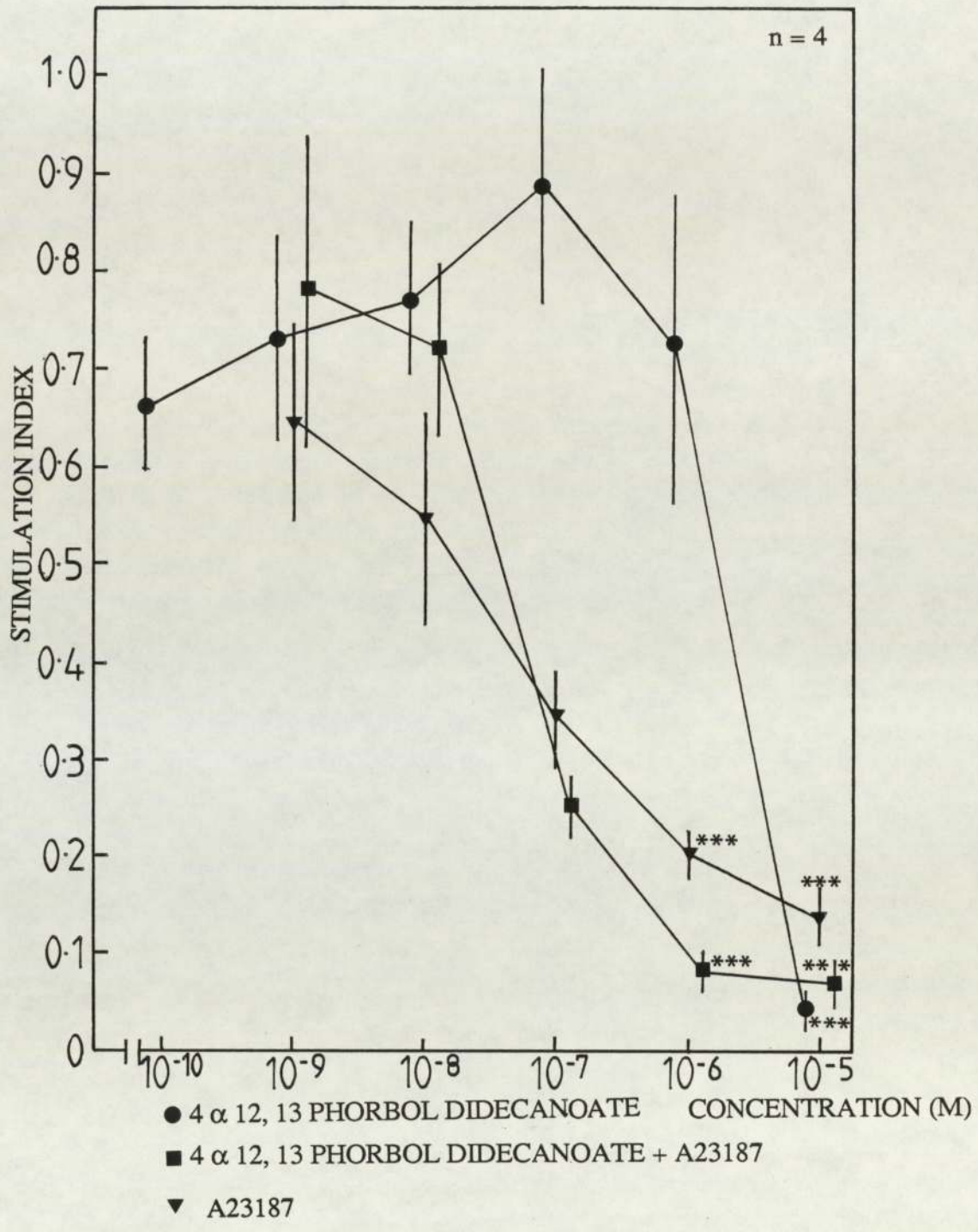


Figure 17: Effects of 4α, 12,13 phorbol didecanoate with and without A23187 (10^{-7}M) on thymidine incorporation in isolated murine splenocytes

Values significantly different from other cultures are indicated by asterisks.

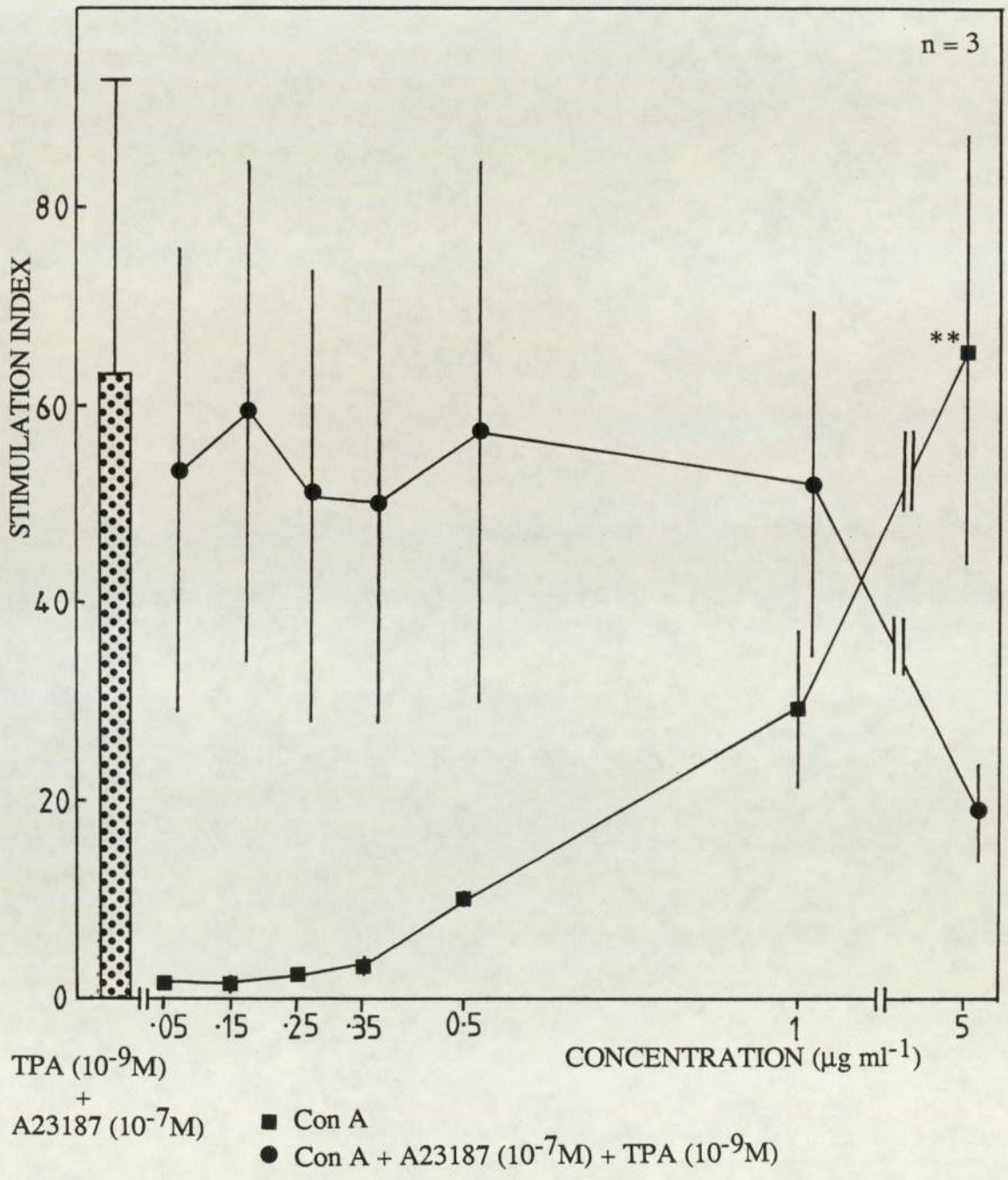


Figure 18: Effects of the co-mitogens A23187 (10^{-7}M) and TPA (10^{-9} M) with varying concentrations of Con A.

Values significantly different from other cultures are indicated by an asterisk.

Table 8: The effect of preincubating (3 mins) murine splenocytes with varying concentrations of TPA on the Con A induced increase in intracellular calcium concentration

	Treatment		
	Con A (5 $\mu g\ ml^{-1}$)	Concentration (M)	Con A (5 $\mu g\ ml^{-1}$) + TPA
	1.61	10^{-9}	1.58
	1.94	10^{-8}	1.25
	1.62		1.63
	1.61		1.47
Increase in $[Ca^{2+}]i$ above basal levels (considered equal to unity)	1.35	10^{-7}	1.19
	1.46		1.15
	1.43		1.23
	1.82		1.25
	1.76		1.25
	1.61		1.22

TPA (10^{-7}M) has a highly significant ($P < 0.01$) effect on the Con A induced increase in $[Ca^{2+}]i$.

4 α 12,13 phorbol-didecanoate which lacks tumorigenicity and the ability to activate PKC had no effect on the Con A induced increase in $[Ca^{2+}]i$ at a similar concentration (Table 9), implying that the inhibition of the Ca^{2+} signal by TPA is mediated by protein kinase C.

Table 9: The effect of preincubating (3 mins) murine splenocytes with 4 α 12,13 phorbol didecanoate on the Con A induced increase in intracellular calcium concentration

	Treatment	
	Con A (5 μg ml^{-1})	Con A (5 μg ml^{-1}) + 4 α 12,13 phorbol didecanoate (10^{-7}M)
Increase in $[Ca^{2+}]i$ above basal levels (considered equal to unity)	1.46 1.53 1.59 1.76 1.62 1.61	1.65 1.69 1.49 1.75 1.67 1.47

Even when the preincubation time was increased to ten minutes this inactive phorbol ester had no effect on the Con A induced intracellular Ca^{2+} increase (Table 10) whilst TPA (10^{-7}M) had a marked effect, although the Ca^{2+} signal was never completely obliterated (Table 10), (Figure 19).

Table 10: The effect of preincubating (10 minutes) murine splenocytes with TPA (10^{-7}M) and 4 α 12,13 phorbol didecanoate (10^{-7}M) on the Con A induced increase in intracellular calcium concentration

	Con A (5 μg ml^{-1})	Treatment	Con A (5 μg ml^{-1}) + phorbol ester (10^{-7}M)
Increase in $[Ca^{2+}]i$ above basal levels (considered equal to unity)	1.42 1.33 1.22 1.29 1.63	TPA	1.10 1.11 1.10 1.07 1.25
	1.42 1.29 1.65	4α12,13 phorbol didecanoate	1.40 1.27 1.65

TPA (10^{-7}M) has a highly significant ($P < 0.01$) effect on the Con A induced increase in $[Ca^{2+}]i$

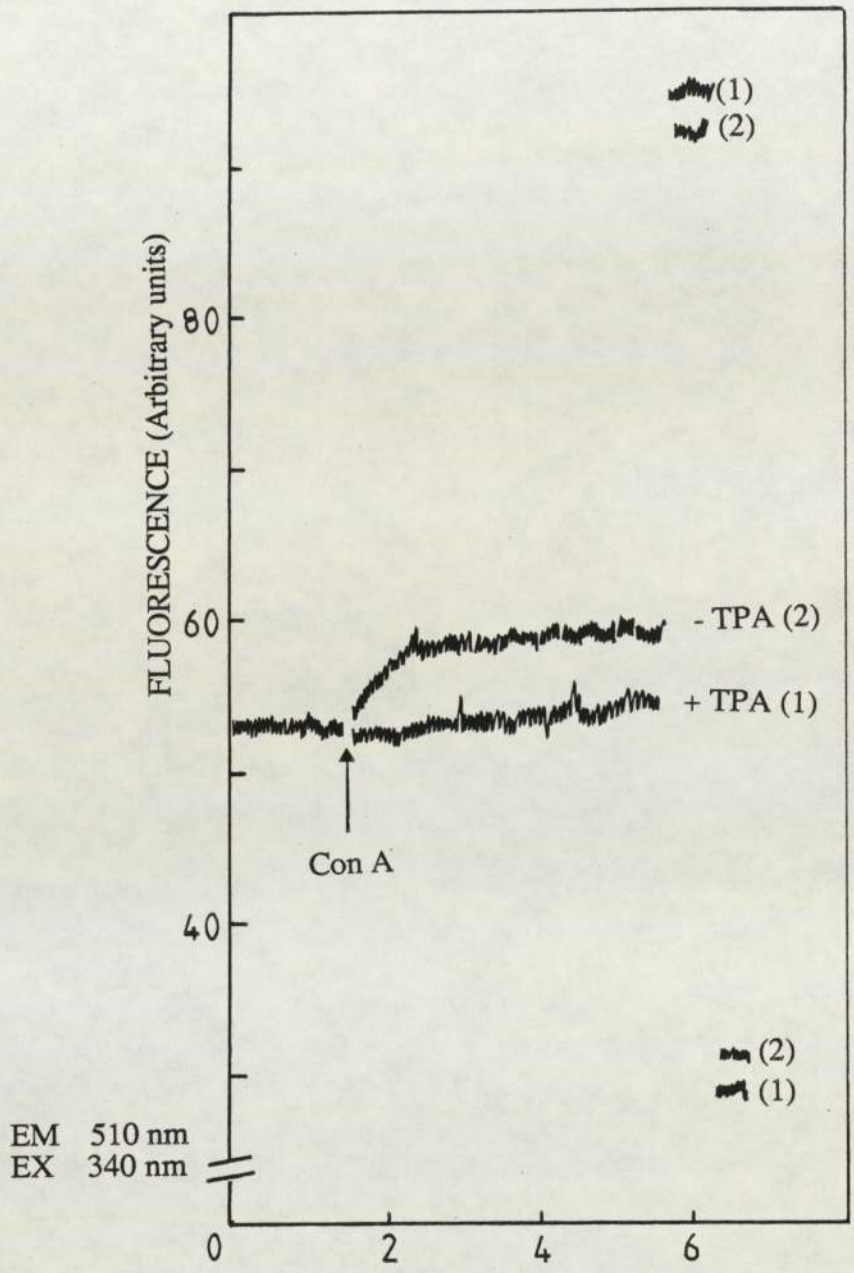


Figure 19: Comparison of the effects of Con A (5 μg ml^{-1}) on the $[Ca^{2+}]i$ of fura-2 loaded murine splenocytes preincubated in normal medium or medium treated with TPA (10^{-7}M) for 10 minutes.

The fluorescence values correspond to resting calcium levels of 124 nM (1) and 134 nM (2) rising to 137 nM and 190 nM respectively following Con A stimulation.

To elucidate the effects of the diminished Ca^{2+} signal on subsequent DNA synthesis, murine splenocytes were preincubated with TPA (10^{-7}M) and other concentrations of TPA for 10 minutes prior to administration of Con A 5 $\mu g\ ml^{-1}$. After 48 hours incubation DNA synthesis was increased 2 fold when compared to control cells (Con A alone) (Figure 20) following TPA treatment. Under identical conditions, the inactive phorbol ester, 4α 12,13 phorbol didecanoate had no effect on the Con A induced stimulation (Figure 20). This is further confirmation of the hypothesis that the Ca^{2+} signal alone is insufficient to commit cells to division.

One of the consequences of PKC activation is the stimulation of Na^+/H^+ exchange which leads to an increase in intracellular pH. To define a role for PKC activation in signal transduction following the interaction of lectin with its receptor, the effect of amiloride an agent used to inhibit the Na^+/H^+ antiport (Besterman et al, 1985) on Con A stimulated DNA synthesis was investigated. Amiloride significantly inhibited [^{3}H]-thymidine incorporation into murine splenocytes at concentrations of 10^{-6}M and higher (Figure 21). These results suggest that the Na^+/H^+ antiport is indeed involved in T-lymphocyte proliferation possibly as a result of PKC activation. Na^+/H^+ exchange is in principle detectable as a cytoplasmic alkalinization, thus investigations were conducted using the pHi sensitive fluorescent probe Quene 1 (Rogers et al, 1983). No pHi increases were detected following Con A (5 $\mu g\ ml^{-1}$) stimulation or as a result of application of the 'co-mitogen' TPA (10^{-9}M) a direct activator of protein kinase C (results not shown). Although this fluorescent probe detected pHi increases following treatment with NH_4Cl 5mM (see Diagram 5) no significant cytoplasmic alkanization was monitored following administration of either ligand, thus either this model system is insensitive to such pHi changes that do occur or these mitogens do not signal T cells to divide via Na^+/H^+ exchange. It seemed appropriate therefore to repeat the experiments utilising a further pHi sensitive fluorescent probe BCECF, (bis (carboxyethyl)-carboxyfluorescein. In these studies Con A (5 $\mu g\ ml^{-1}$) induced a pHi increase which develops more slowly than the Ca^{2+}

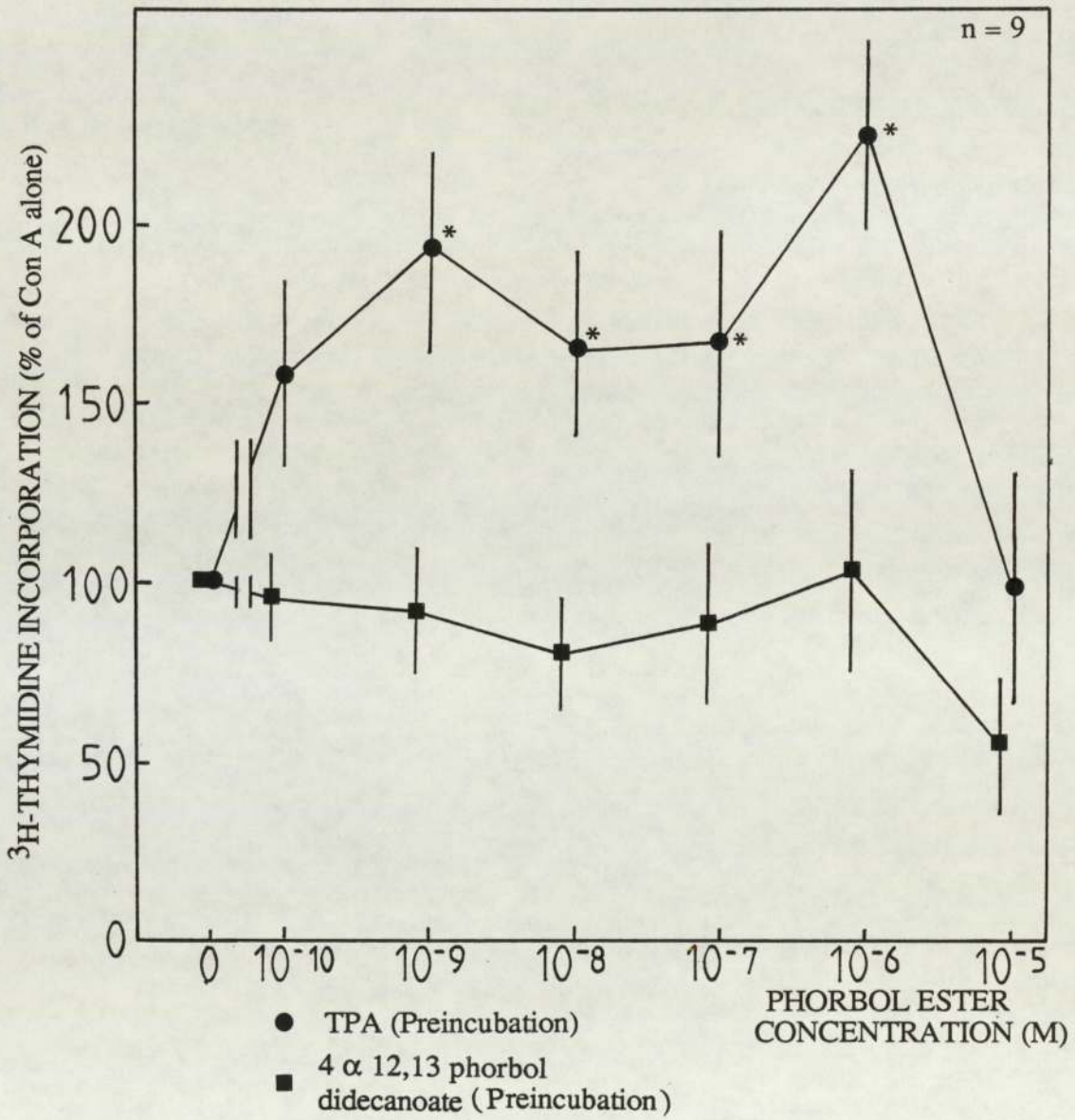


Figure 20: Effects of preincubation with phorbol ester (10 minutes) on the Con A (5 $\mu g\ ml^{-1}$) induced murine splenocyte proliferation

Values significantly different from those of corresponding cultures without phorbol esters are indicated by an asterisk.

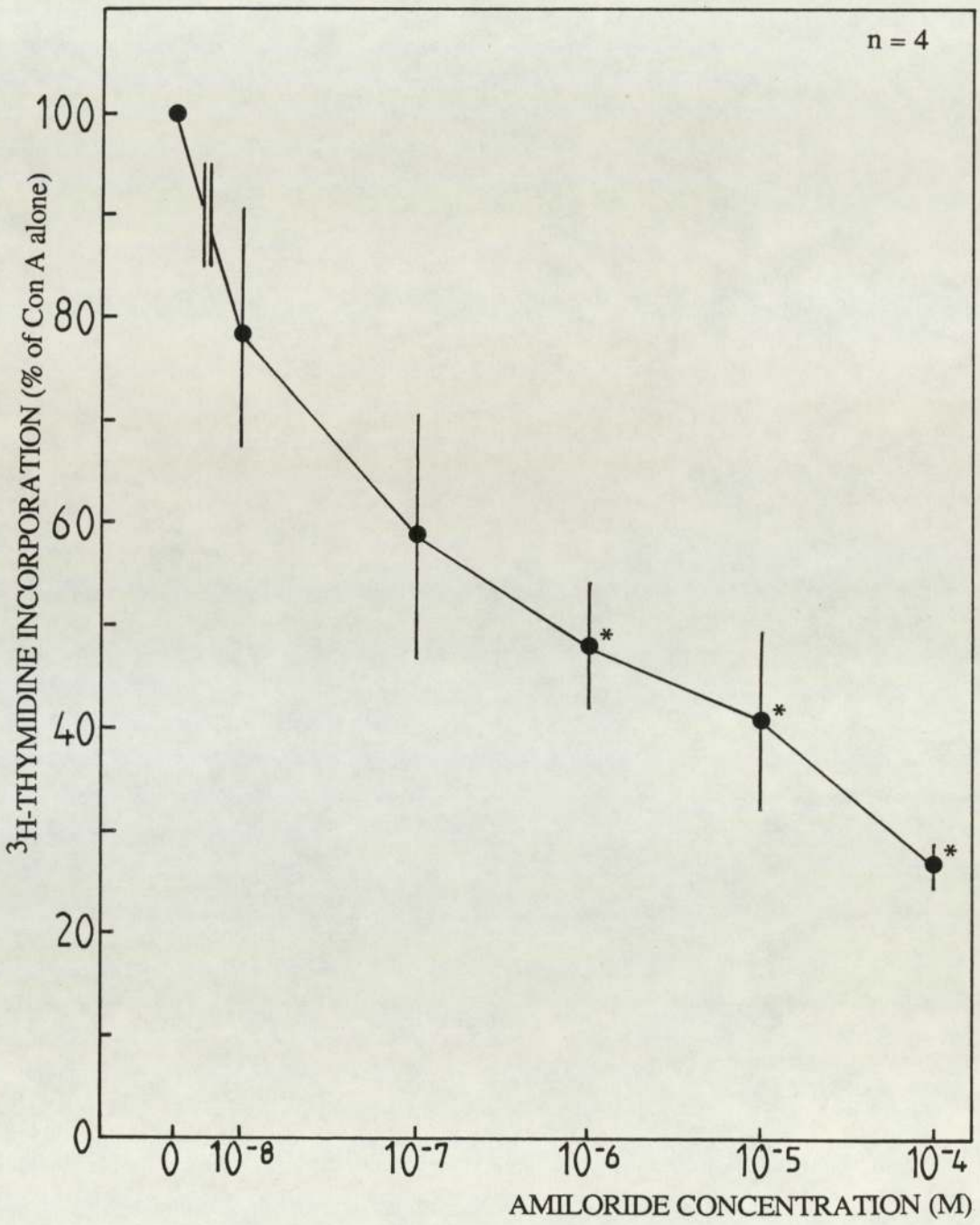


Figure 21: Effect of Amiloride on Con A (5 μg ml^{-1}) induced murine splenocyte proliferation.

Values significantly different from corresponding cultures without amiloride are indicated by an asterisk.

signal and only reaches a maximum after ~ 10 minutes (compare Diagram 4 and Figure 22). The co-mitogenic concentration of TPA (10^{-9}M) also generated a pHi increase (Figure 22) whilst physiological saline over the same period had no effect (results not shown). When cells from the same mouse were loaded with either quene 1 or BCECF, a pHi increase following Con A (5 μg ml^{-1}) was demonstrable only in BCECF loaded splenocytes suggesting that quene 1 is an unsatisfactory fluorescent probe in this particular system. This is in agreement with earlier quene 1 studies in thymocytes (Rogers et al, 1983) and perhaps this indicator should now be considered obsolete.

The $[Ca^{2+}]i$ and pHi responses induced by Con A stimulated T lymphocytes are presumably generated by the breakdown of $PtdInsP_2$ releasing $InsP_3$ and diacylglycerol. Protein kinase C activation is then probably responsible for the increased Na^+/H^+ exchange leading to cytoplasmic alkalinization (see Section 1.5.2).

Many putative second messengers have been linked to T-lymphocyte activation (see Section 1.7) of these, a Ca^{2+} signal and pHi increase are implicated in Con A stimulation of murine splenocytes. Another, second messenger, ornithine decarboxylase is thought to be involved not only in T lymphocyte proliferation but in the generation of Ca^{2+} signals in other cell types (see Section 1.5.5). Investigations were therefore performed to determine the effects of an irreversible inhibitor of ornithine decarboxylase, DFMO (α(Difluoromethyl) ornithine hydrochloride monohydrate) (a gift from Dr Tom Brown, May & Baker Ltd) on the Con A induced Ca^{2+} signal in murine splenocytes. DFMO (5 mM), (a concentration known to irreversibly inhibit ornithine decarboxylase (Mamont et al, 1978)) was without significant effect on the Con A induced Ca^{2+} signal (Table 11).

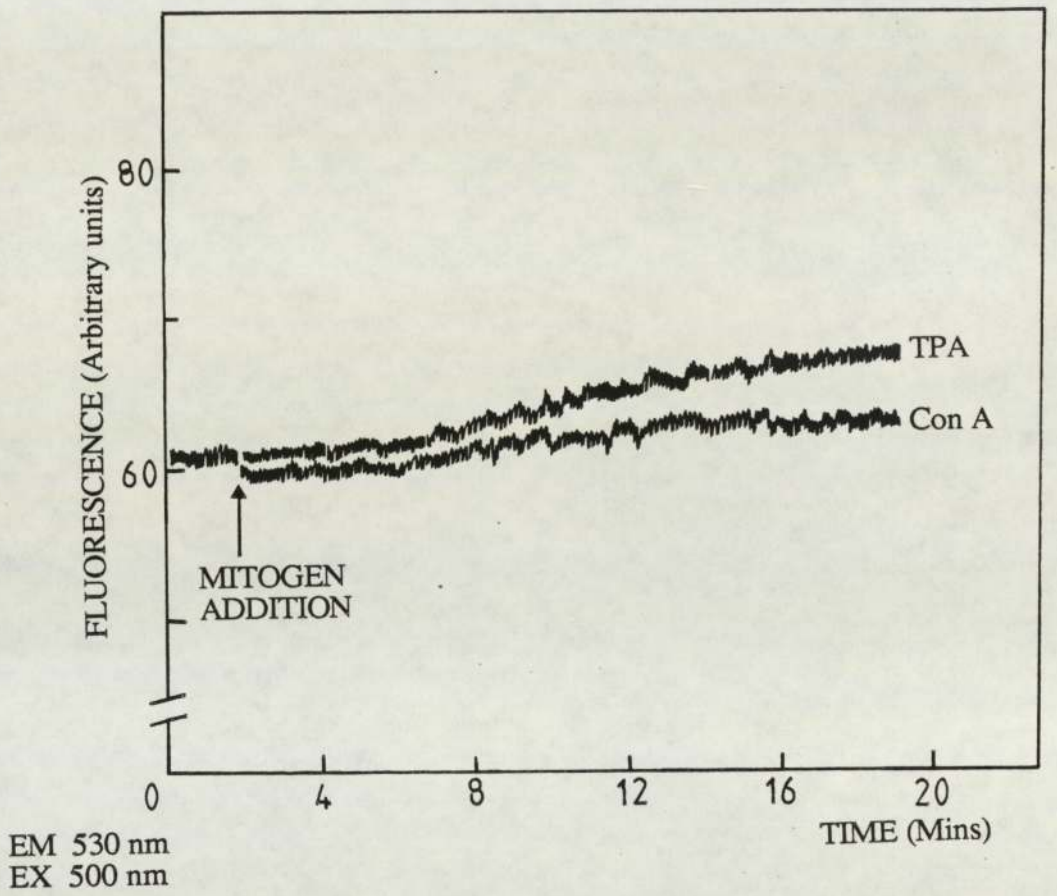


Figure 22: Comparison of the effects of Con A (5 $\mu g\ ml^{-1}$) and TPA ($10^{-9}M$) addition upon the pHi levels of BCECF loaded murine splenocytes.

Fluorescence recordings demonstrate qualitative changes in the pHi of murine splenocytes stimulated with both mitogens. No attempt has been made to calibrate these values (see Chapter 2 for details).

Table 11: The effect of DFMO (5 mM) on the Con A induced increase in cytosolic Ca^{2+} concentration of murine splenocytes

	Treatment	
	Con A (5 μg ml^{-1})	Con A (5 μg ml^{-1}) DFMO (5 mM)
Increase in $[Ca^{2+}]i$ above basal levels (considered equal to unity)	1.27 1.27 1.31	1.30 1.10 1.20

DFMO has no significant effect on the Con A induced increase in $[Ca^{2+}]i$

Furthermore, no concentration of DFMO tested inhibited [^{3}H]-thymidine incorporation into Con A stimulated murine splenocytes (Table 12).

Table 12: The influence of DFMO on Con A induced DNA synthesis in murine splenocytes n = 4

	Treatment
DFMO Concentration (M)	% of Con A (5 μg ml^{-1}) alone induced [^{3}H]-thymidine incorporation
10^{-6}	82.83 ± 7.25
10^{-5}	111.55 ± 23.68
10^{-4}	123.28 ± 21.66
10^{-3}	85.65 ± 16.19
5×10^{-3}	75.32 ± 12.69
10^{-2}	96.23 ± 16.76

Since normal cellular growth requires polyamines and the only route to putrescine is via the enzyme ornithine decarboxylase (Pegg & McCann, 1982) it must be assumed that the inhibitor DFMO was lacking in activity and therefore the fura-2 studies should be viewed with scepticism.

Having outlined as far as possible the mechanism of Con A induced murine splenocyte proliferation, the effects of known physiological modulators of immunological proliferation were investigated. An objective of this study was to discern the mechanism(s) by which sex steroids inhibit lymphocyte transformation and to determine whether it has any relevance to the modulation of the immune system *in vivo* by gonadal steroids (see Section 1.7). Thus, the ability of testosterone and certain metabolites to inhibit the stimulation induced by 5 $\mu g\ ml^{-1}$ Con A in splenocytes was investigated. Testosterone is indeed an inhibitor of DNA synthesis but only at a pharmacological concentration of $10^{-4}M$ (Figure 23). 5 α dihydro-testosterone (5 α DHT) an active metabolite more potent than testosterone in certain tissues was also inhibitory but failed to inhibit DNA synthesis at a lower concentration than testosterone itself (Figure 23); whereas testosterone glucuronide, a conjugated metabolite was without effect (Figure 23). α-oestradiol and ß-oestradiol were equally effective in blocking proliferation but only at pharmacological doses of $10^{-4}M$ (Figure 24). The effects of cholesterol, a C27 compound from which steroids are derived, failed to have an inhibitory effect on Con A stimulation at any concentration tested (Figure 25). These results clearly show that sex steroids are able to inhibit mitogen induced blast transformation albeit at high concentrations. To exclude the possibility that inhibition of DNA synthesis was due to cytotoxicity cell viability was examined in control cultures and those containing steroids at the inhibitory concentrations. 90-95% of splenocytes were viable at the end of the incubation period as assessed by trypan blue exclusion, under all culture conditions. The steroids were initially dissolved in ethanol, but the final concentrations of ethanol had no effect on $[^3H]$-thymidine incorporation (results not shown). In order to define the mechanism of the immuno-suppressive action of sex steroids, the effect of testosterone on the Con A induced increase in $[Ca^{2+}]i$ measured by the Ca^{2+} sensitive fluorescent dye fura-2 was investigated. Testosterone ($10^{-4}M$) had no effect on the Ca^{2+} signal (Table 13)

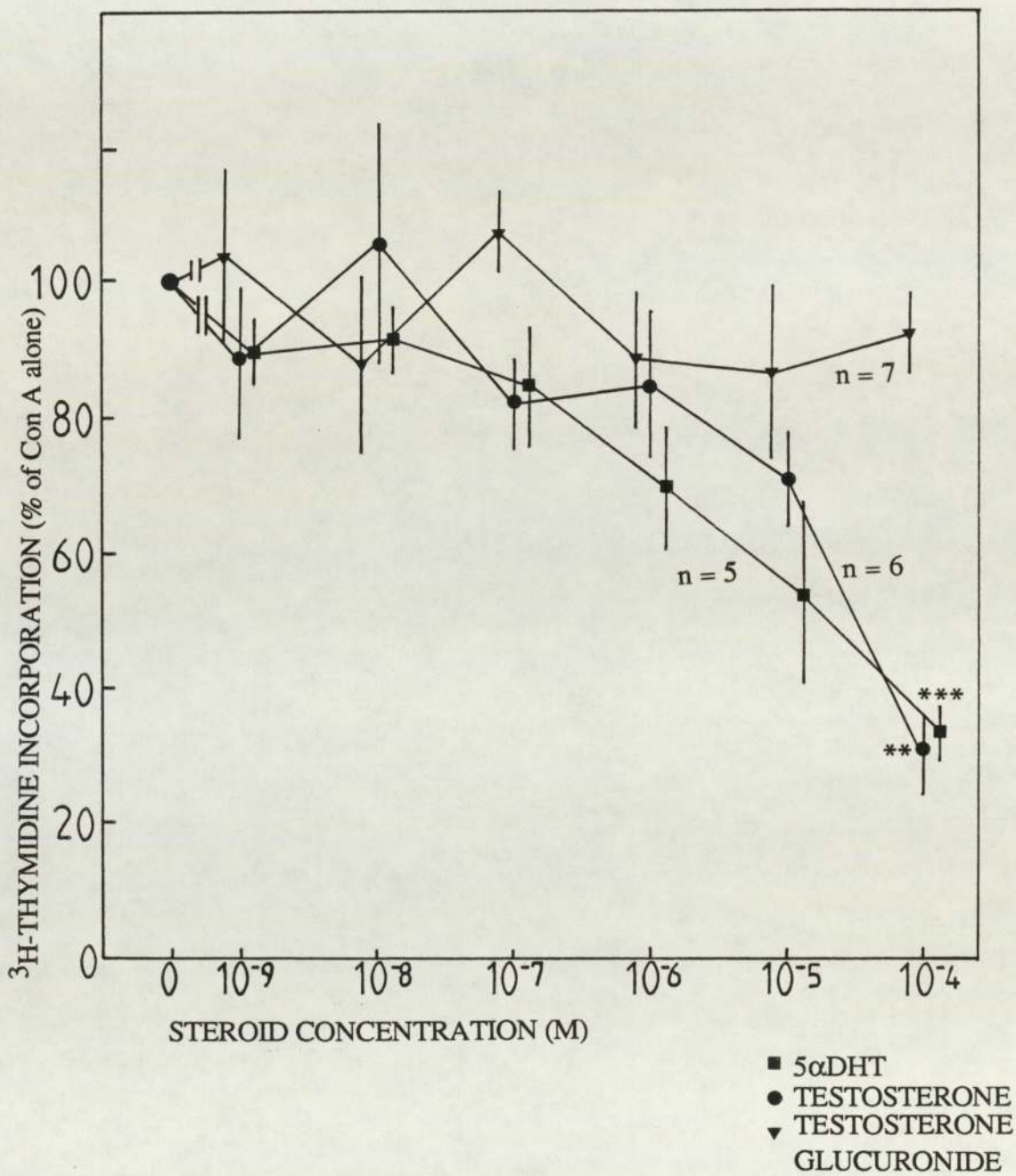


Figure 23: Effects of Testosterone, 5αDihydrotesterone (5αDHT) and Testosterone Glucuronide on Con A (5 μg ml^{-1}) induced murine splenocyte proliferation

Values significantly different from corresponding cultures without steroids are indicated by asterisks

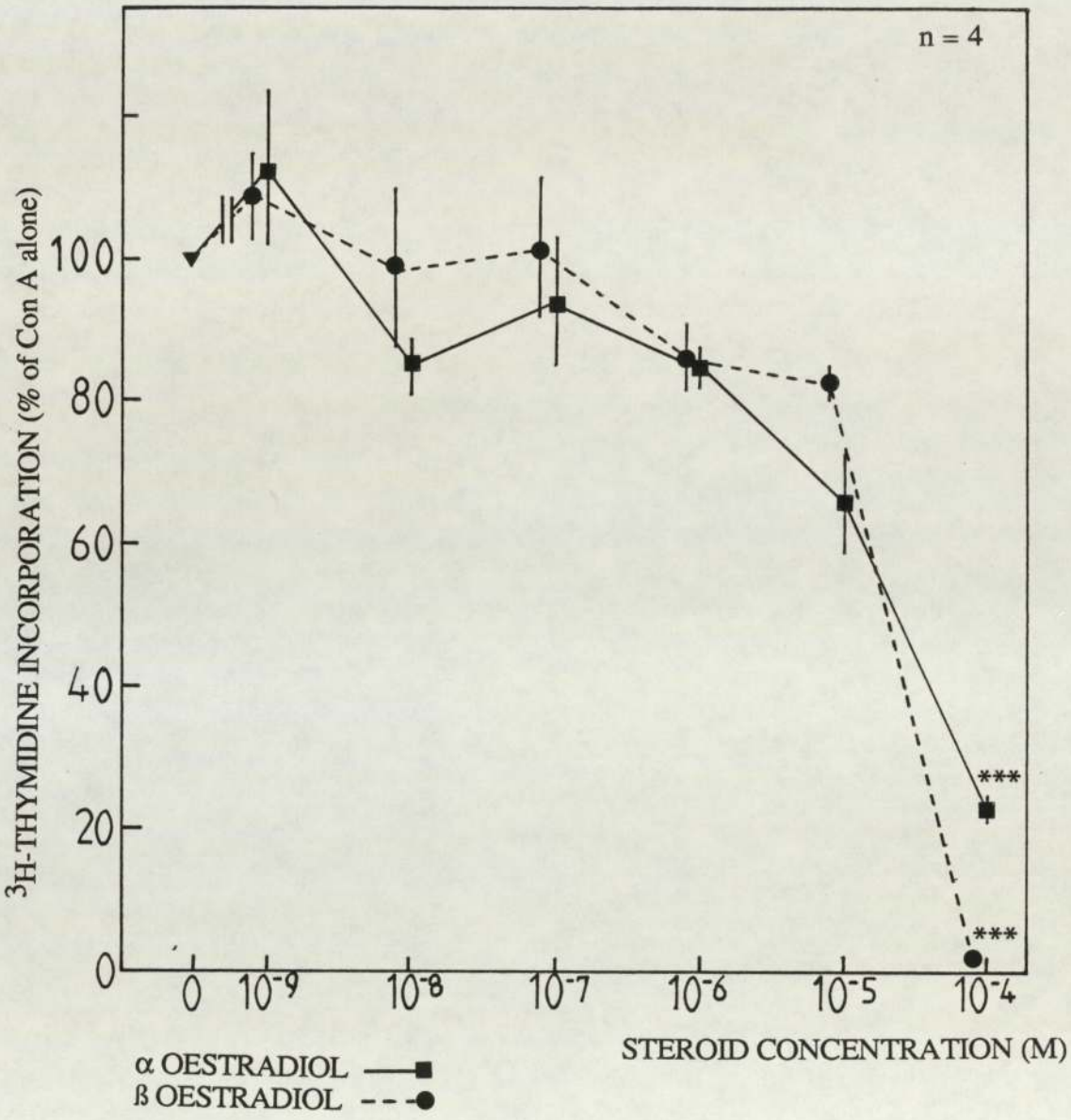


Figure 24: Effects of α and ß Oestradiol on Con A (5 μg ml^{-1}) induced murine splenocyte proliferation.

Values significantly different from corresponding cultures without steroids are indicated by asterisks.

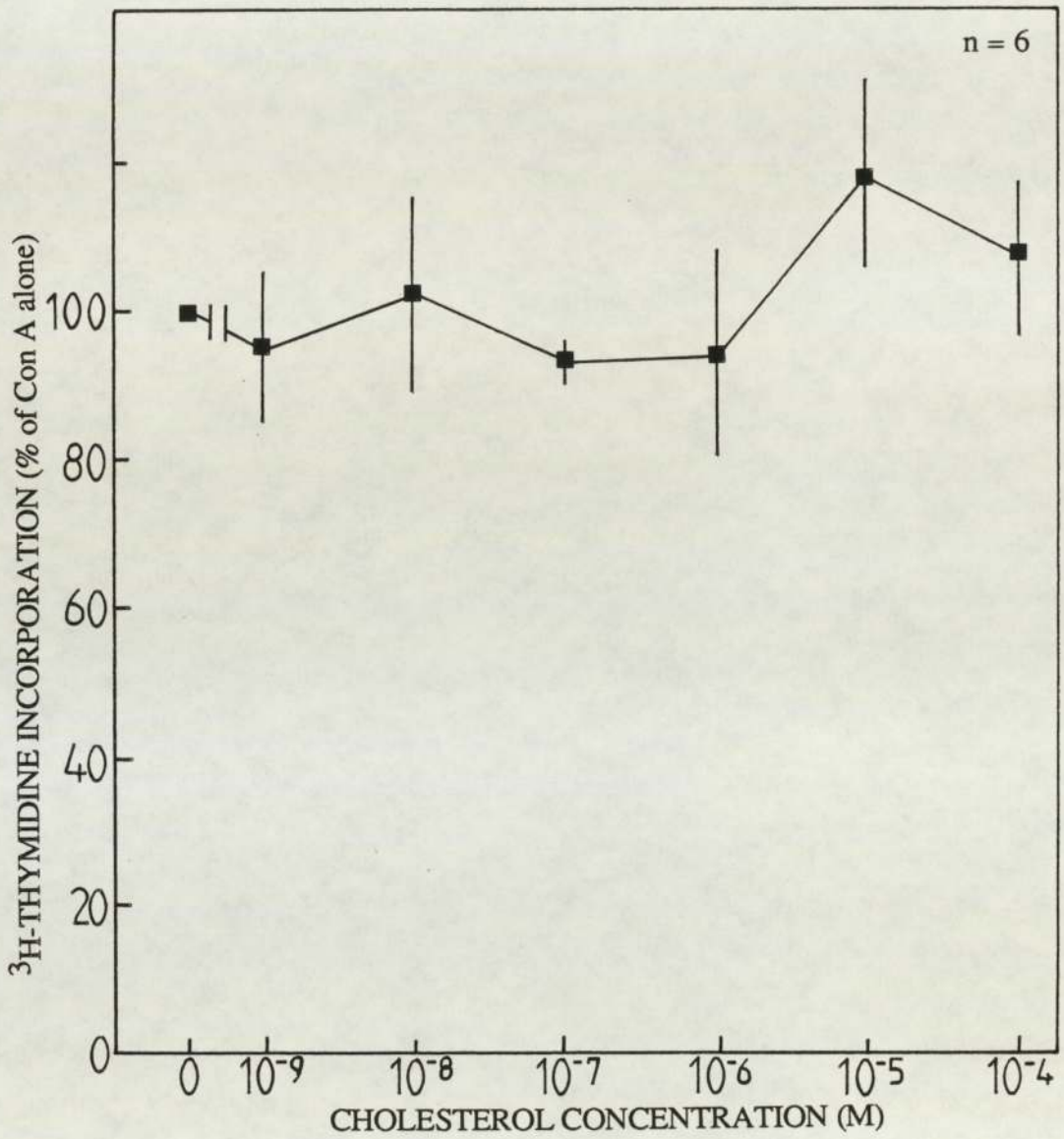


Figure 25: Effect of Cholesterol on Con A (5 μg ml^{-1}) induced murine splenocyte proliferation.

implying that its inhibitory action must be mediated elsewhere.

Table 13: The effect of testosterone (10^{-4}M) on the Con A induced increase in cytosolic calcium in murine splenocytes

	Treatment	
	Con A (5 μg ml^{-1})	Con A (5 μg ml^{-1}) + Testosterone 10^{-4}M
Increase in $[Ca^{2+}]i$ above basal levels (considered equal to unity)	1.41 1.29 1.51 1.72 1.46 1.59	1.30 1.30 1.47 1.64 1.33 1.61

Investigations were undertaken to elucidate the time during the 48 hour culture period at which testosterone (10^{-4}M) exerts its abrogation of the Con A induced stimulation of murine splenocytes. Surprisingly, testosterone (10^{-4}M) caused a significant inhibition of DNA synthesis even when administered after 44 hours of incubation with Con A (5 μg ml^{-1}) (Figure 26). In view of the high concentrations of sex steroids necessary to inhibit lymphocyte stimulation *in vitro*, it is probable that other factors contribute to the observations of sex steroid inhibited lymphocyte function in the intact animal (see Section 1.7).

Studies were therefore undertaken to investigate the effects of gonadectomy on the lymphoid tissue of mice. The spleens and thymus glands of post-pubertal TO mice were excised at four, six and nine weeks following surgery. Surgery *per se* had little effect since controls never differed significantly from sham-operated animals. In female mice no significant increases were found in the spleen index (spleen to body weight ratio) at any post-operative time (Figure 27), whilst the thymic index of ovariectomised mice was larger than control and sham-operated mice at 9 weeks after surgery (Figure 27).

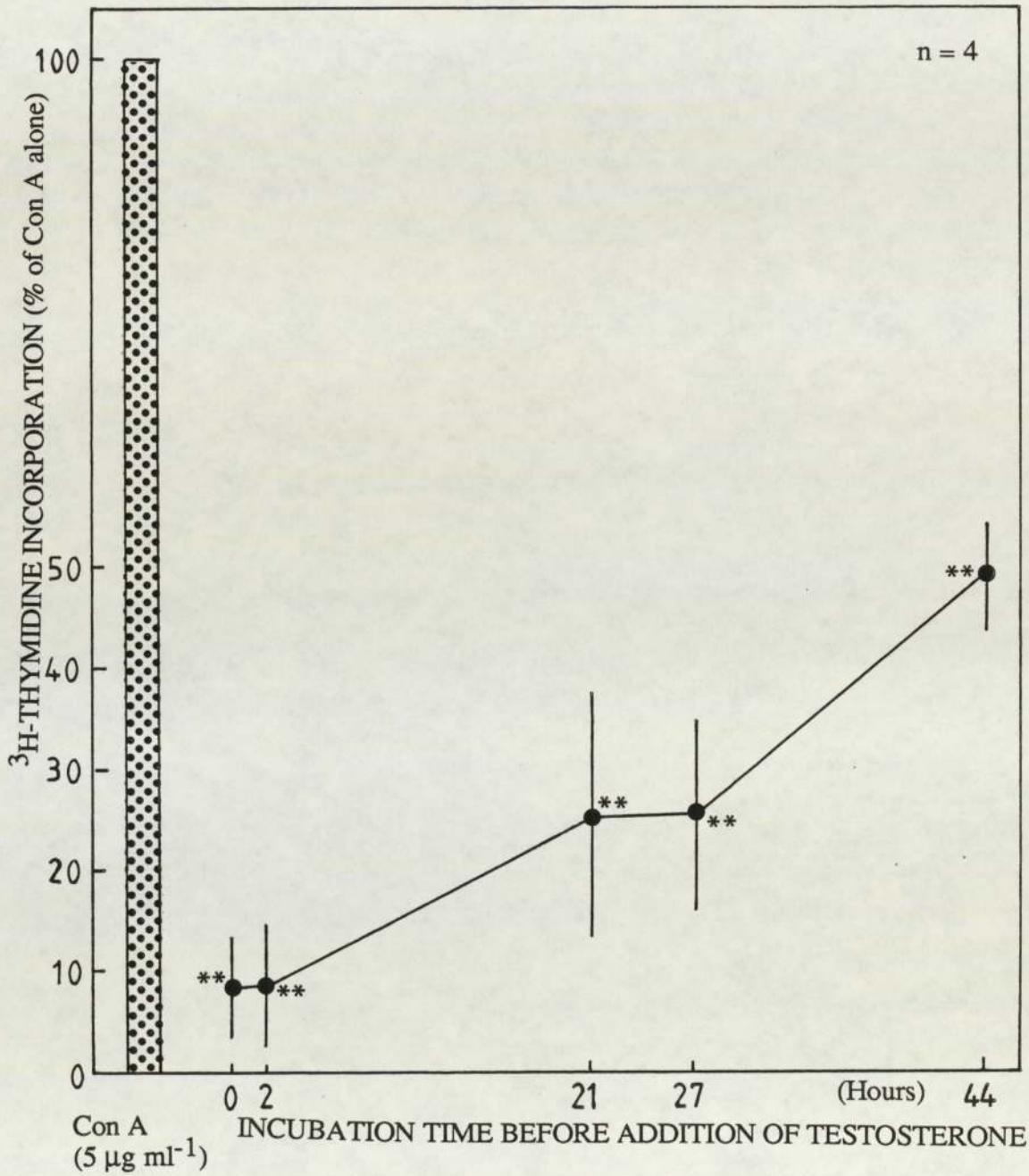


Figure 26: Effect of sequential additions of Testosterone (10^{-4}M) during the incubation period on Con A (5 μg ml^{-1}) induced murine splenocyte proliferation.

Values significantly different from those of cultures incubated with Con A alone are indicated by asterisks.

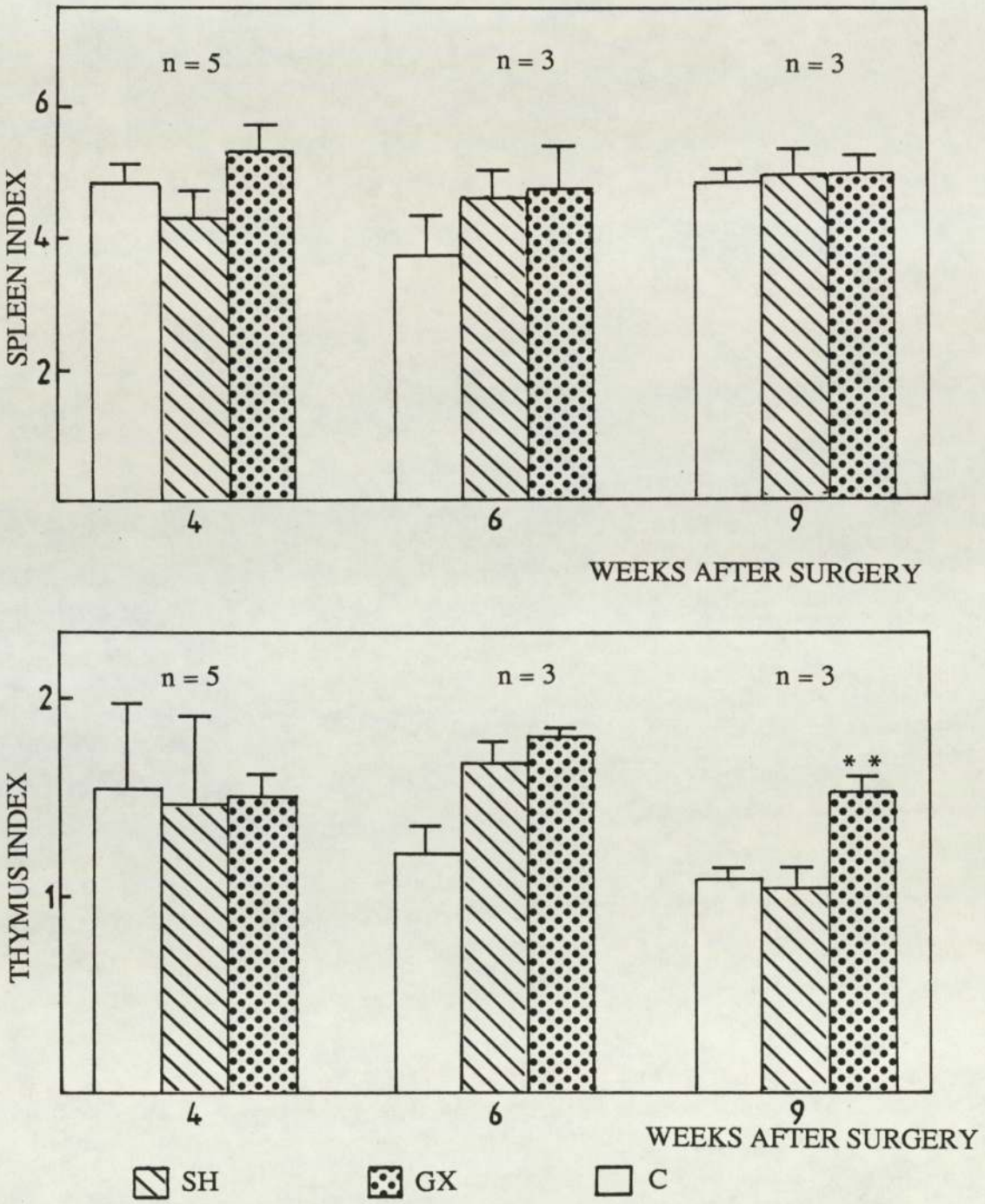


Figure 27: Comparison of thymus and spleen weights of sham operated (SH), gonadectomised (GX) and control (C) female TO mice at 4, 6 and 9 weeks after surgery.

Results are expressed as spleen index (Spleen mg/g body weight) and thymus index (thymus weight mg/g body weight). Values significantly different from those of organ indices of control animals are indicated by asterisks.

In contrast, post-pubertal orchidectomy resulted in a significant increase in the spleen index at 4 weeks post-operation which had disappeared by six weeks (Figure 28). The thymic index of orchidectomised mice was significantly greater than control and sham-operated mice on all occasions following surgery (Figure 28). Thus, although the sex steroids, oestradiol and testosterone had similar effects on proliferation *in vitro*, their effects on lymphoid tissue *in vivo* are apparently quite different.

Investigations were conducted to investigate the hypothesis that gonadectomy may potentiate T cell function in mice. Thus, murine splenocytes derived from control, sham-operated and gonadectomised mice, four weeks after surgery were incubated for 48 hours with varying concentrations of the T cell mitogen Con A. Large variations were apparent in the spleen cell mitogen response for mice of both sexes. The spleen cells of castrate mice responded in a similar way as controls to lectin stimulation, 5 $\mu g\ ml^{-1}$ being the concentration inducing maximum [^{3}H]-thymidine incorporation in both cases (Figure 29). In contrast, the spleen cells of ovariectomised mice did not respond as well as spleen cells derived from sham-operated and control mice to Con A 5 $\mu g\ ml^{-1}$ (Figure 30). Furthermore, spleen cells from control and sham-operated mice displayed increased [^{3}H]-thymidine incorporation at a lower concentration of Con A (1 $\mu g\ ml^{-1}$) than spleen cells from ovariectomised mice (Figure 30). Briefly then, both oestradiol and testosterone (at high concentrations) inhibited T cell proliferation *in vitro*; orchidectomy led to significant increases in lymphoid tissue mass particularly that of the thymus, whereas ovariectomy had relatively little effect on the thymic index and no effect at all on the spleen to body weight ratio post-surgery. Orchidectomy did not appear to potentiate T cell function since splenocytes derived from orchidectomised animals responded to mitotic stimulation by Con A in an identical fashion as cells from control animals. In contrast, these preliminary studies demonstrate that ovariectomy impaired the spleen cell mitogen response implying that oestrogen potentiates T cell function *in vivo*. These findings contradict reports in the literature which suggest that both sex steroids depress the cell mediated immune response (see Section 1.7).

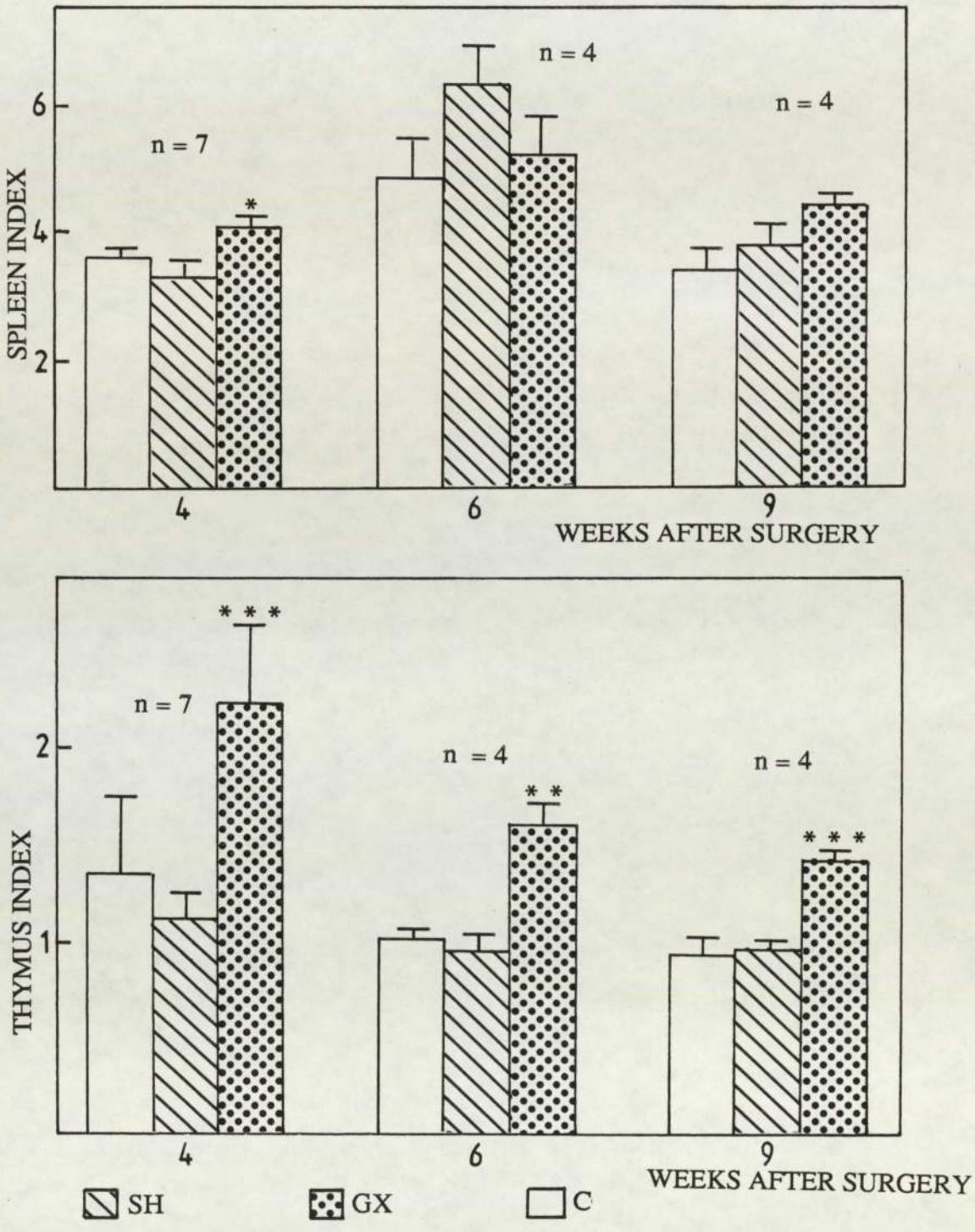


Figure 28: Comparison of Thymus and Spleen weights of Sham operated (SH), gonadectomised (GX) and control (C) male TO mice at 4, 6 and 9 weeks after surgery.

Values significantly different from those of organ indices of control animals are indicated by asterisks. Other details as in Figure 27.

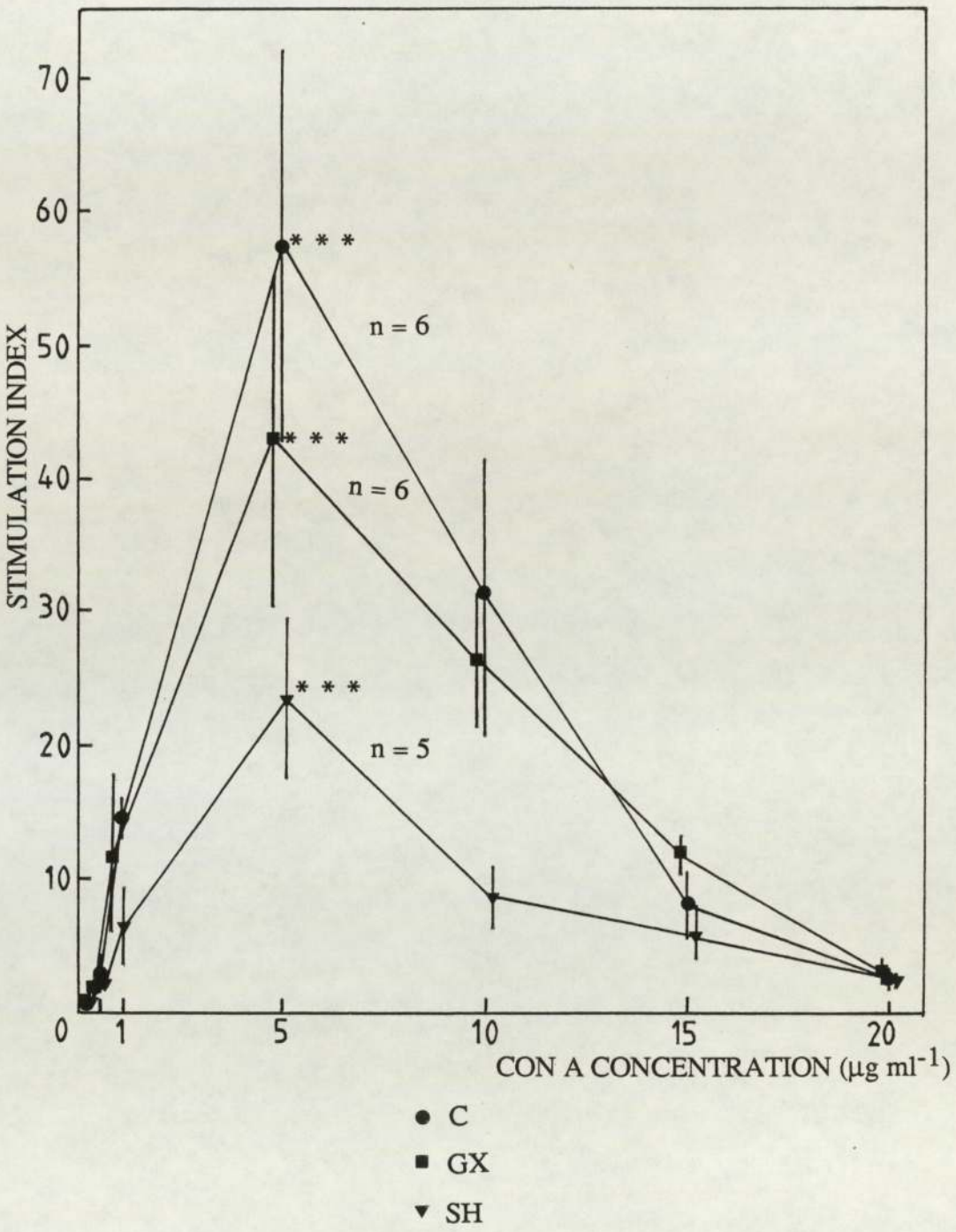


Figure 29: Comparison of dose-response curves of the effect of Con A on thymidine incorporation in splenocytes isolated from post-pubertal sham operated (SH) gonadectomized (GX) and control (C) male TO mice four weeks after surgery.

Values significantly different from those of other cultures within the same treatment group (SH, GX or C) are indicated by asterisks.

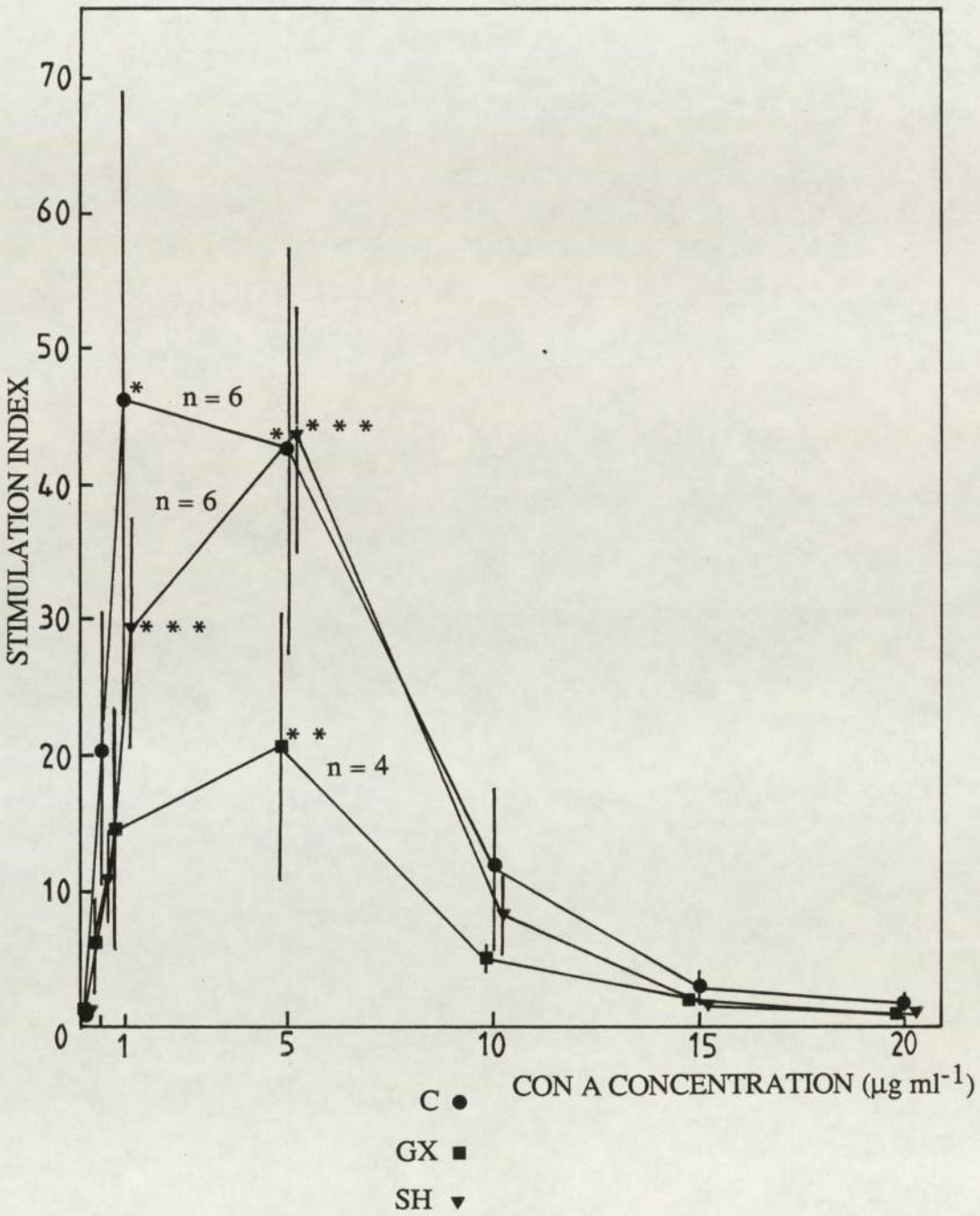


Figure 30: Comparison of dose-response curves of the effect of Con A on thymidine incorporation in splenocytes isolated from post-pubertal sham operated (SH), gonadectomised (GX) and control (C) female TO mice four weeks after surgery.

Values significantly different from those of other cultures within the same treatment group (SH, GX or C) are indicated by asterisks.

There are indications that gonadal steroids may act indirectly in the modulation of the immune system in rats thus, oestradiol is thought to depress the cell mediated immune response through the generation of thymic serum factors (Grossman et al, 1982; Grossman & Roselle, 1983; Grossman, 1985; Myers et al, 1986). To determine whether serum factors were involved in the gonadal steroid modulation of the immune system in mice, splenocytes were incubated *in vitro* for 48 hours in the presence of Con A (2.5 $\mu g\ ml^{-1}$ or 5 $\mu g\ ml^{-1}$), tissue culture media and 20% specific mouse serum fractions and pulse labelled with tritated thymidine. Mouse serum fractions were prepared from control, gonadectomised and sham-operated animals. Remarkably, splenocytes incubated with mouse sera from all sources failed to respond to the mitogenic concentration of Con A in the accustomed way. Thus, splenocytes derived from the same animal cultured in 20% mouse serum demonstrated a stimulation index of <2 in response to Con A 5 $\mu g\ ml^{-1}$ compared with a mean stimulation index of 67.59 (± 38.20 SEM n = 4) when incubated with the same concentration of lectin but with 10% foetal calf serum in the medium (Figure 31). When splenocytes derived from male animals were incubated with the different male serum fractions no significant differences between the serum groups were observed upon addition of Con A, all the stimulation indices were approximately 1 (ie. no stimulation at all). In contrast female control sera significantly enhanced DNA synthesis in response to Con A (2 $\mu g\ ml^{-1}$) in splenocytes derived from female animals (Figure 31), this adds further support to earlier indications that oestrogen potentiates T cell function.

To ascertain whether these surprising findings indicate that mouse serum fails to support lymphocyte proliferation or whether the concentration of 20% mouse serum was too high and potentially toxic, further investigations were conducted. To rule out the possibility that high concentrations of serum might be cytotoxic, splenocytes (derived from male animals) were incubated with Con A (5 $\mu g\ ml^{-1}$) and varying concentrations of foetal calf serum. The combination of 10% foetal calf serum with Con A (5 $\mu g\ ml^{-1}$) stimulated the greatest [^{3}H]-thymidine incorporation (Figure

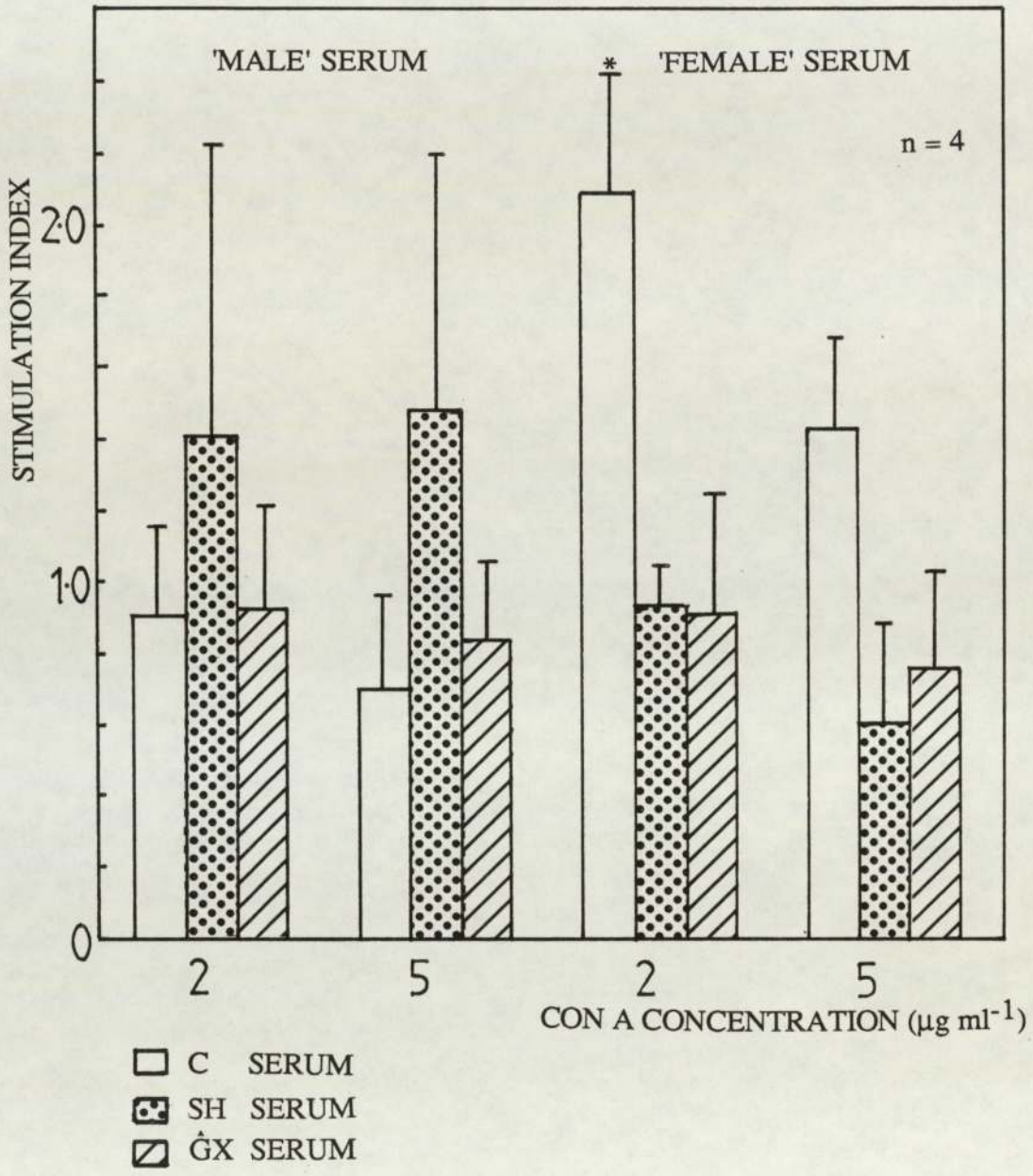


Figure 31: Comparison of the effects of control (C), gonadectomized (GX) and sham operated (SH) male and female mouse serum on normal splenocytes (isolated from male mice) incubated with Con A (2 μg ml^{-1} or 5 μg ml^{-1})

Sera were prepared from the various groups of mice by collecting the blood by cardiac puncture, allowing it to clot at room temperature. It was centrifuged, decanted and stored at -20°C. Before use, the sera was thawed and added to medium at a concentration of 20% and sterilised by filtration with 0.45 micron filter. Values significantly different from those of corresponding cultures incubated with the same concentration of Con A are indicated by an asterisk.

32), however splenocytes incubated with a similar concentration of Con A but with 30% foetal calf serum still exhibited a much higher stimulation index than with 20% mouse serum (Figures 31 and 31). Furthermore, no concentration of normal mouse serum tested in combination with Con A (5 $\mu g\ ml^{-1}$) was able to support the enhanced DNA synthesis in murine splenocytes, normally achieved at this lectin concentration (Figure 32). Intriguingly not only does mouse serum not support splenocyte proliferation but it actually inhibits in a dose-dependent manner DNA synthesis in murine splenocytes stimulated with Con A (5 $\mu g\ ml^{-1}$) in the presence of 10% foetal calf serum (Figure 32). Cell viability was examined in control cultures and those containing mouse serum at the above concentrations. 90-95% of splenocytes were viable at the end of the incubation period as assessed by trypan blue exclusion, under all culture conditions. Unfortunately, these studies with mouse serum fractions although interesting, are unable to confirm or deny the presence of thymic serum factors which might mediate the effects of gonadal steroids on the immune system.

Although the proliferation of lymphocytes in response to plant lectins is often taken as a model for the proliferative aspect of immune responses (see Section 1.7), it seemed appropriate to investigate the mechanism of action of a more physiological growth factor, Interleukin 2. The mechanism by which quiescent T cells are activated incorporates the following components, the T cell receptor recognises the antigen MHC encoded protein complex on the antigen presenting macrophage, this bonding stimulates the release of IL1 by the macrophage and that in turn activates the T cell which expresses IL2 receptors. The activated blasts undergo proliferative expansion under the influence of IL2 produced by another activated T cell sub-population (see Section 1.7). Stimulation of murine splenocytes with mitogenic plant lectins induces a subpopulation of lymphocytes to become responsive to IL2. Hence, preparations of lectin activated cells can then be used to investigate the mechanism of IL2 induced proliferation (Gearing et al, 1985). Thus, murine splenocytes which had been activated with Con A (Con A blasts) (see Section 2.2.5 for details) were incubated with varying concentrations of recombinant human IL2.

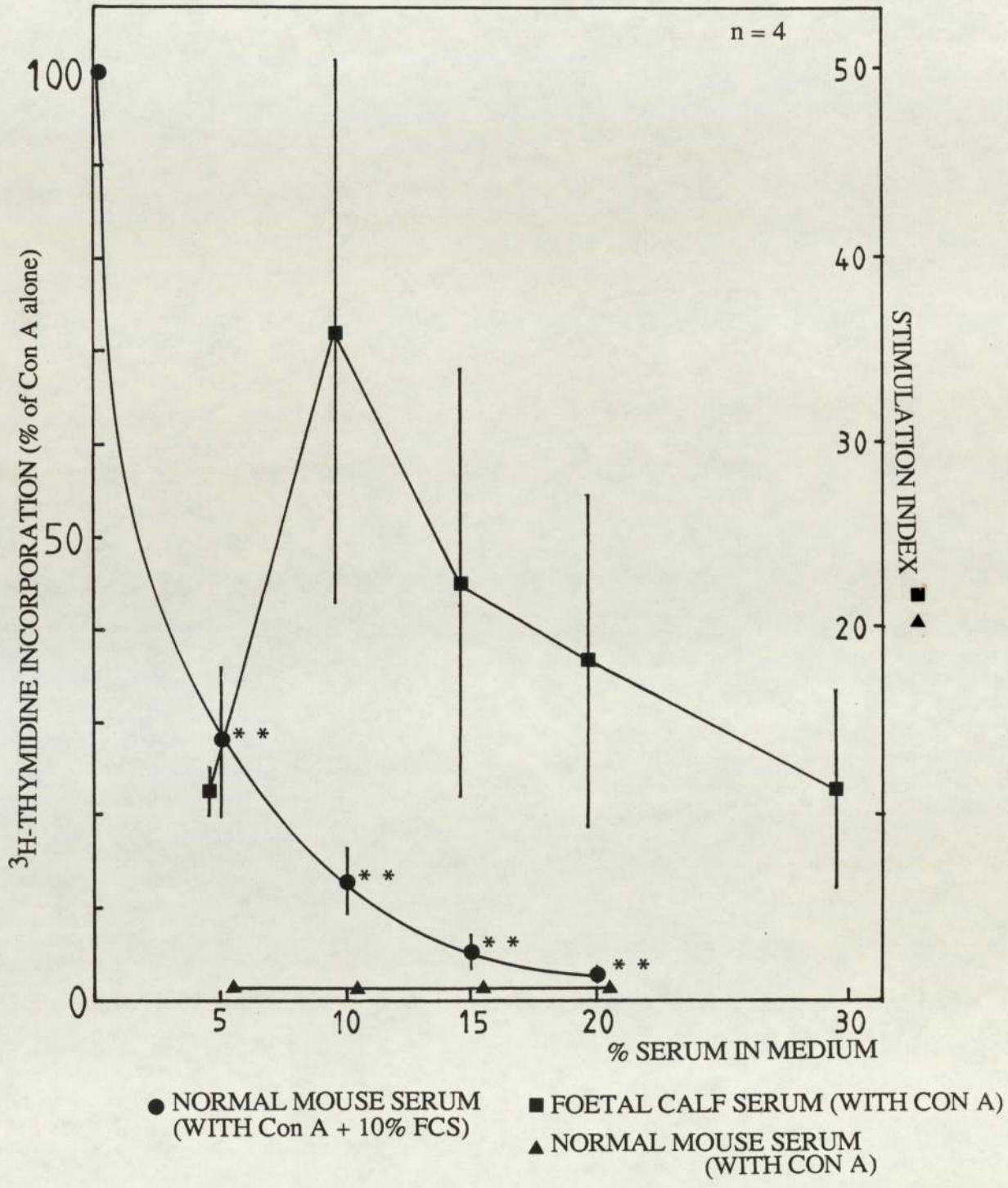


Figure 32: Comparison of the effects of varying concentrations of foetal calf and mouse serum on Con A (5 μg ml^{-1}) induced murine splenocyte proliferation, and the effect of mouse serum on thymidine incorporation in splenocytes incubated with Con A (5 μg ml^{-1}) and 10% foetal calf serum (FCS).

Normal mouse serum was prepared from male mice as described in Figure 30. Values significantly different from those of cultures within the same treatment group are indicated by asterisks.

This lymphokine stimulated DNA synthesis in a dose dependent manner with [^{3}H]-thymidine incorporation into the Con A blasts reaching a plateau at a concentration of 100 U ml^{-1} (Figure 33). In the absence of IL2, incorporation of tritiated thymidine into Con A blasts was negligible. Nifedipine a calcium channel blocker suppressed IL2 (100 U ml^{-1}) induced proliferation at a concentration of 10^{-5}M (Figure 34). Cell viability in control cultures and those containing the drug at this concentration remained at 90-95%. This implies that calcium ions must enter the cytosol from the medium before proliferation can occur, unless this calcium antagonist has effects on proliferation unrelated to calcium.

Use of the calcium sensitive fluorescent probe fura-2 indicated that Con A blasts had an intracellular calcium concentration of 208 nM (± 26 SEM n = 6) compared with quiescent lymphocytes which had basal levels of 186 nM (± 10 SEM n = 39). Following addition of 1L2 (100 U ml^{-1}) the [Ca^{2+}]i appeared to rise (see Figure 35 for a typical recording), however addition of the same volume of physiological saline produced an identical effect (Figure 32). These findings suggest that Con A blasts are susceptible to pronounced and rapid fura-2 leakage and it is therefore impossible to determine whether or not IL2 (100 U ml^{-1}) generates a Ca^{2+} signal.

The effect of a calmodulin antagonist TFP on IL2 induced proliferation of Con A blasts was investigated since there are indications that IL2 might signal cells to divide by generating a Ca^{2+} signal (see Section 1.7). TFP (10^{-5}M) significantly blocked proliferation of murine Con A blasts (Figure 36), this concentration of TFP did not affect cell viability.

The role of PKC activation in IL2 stimulation is controversial (section 1.7) it seemed appropriate therefore to investigate the consequences of treatment with amiloride, an agent used to inhibit the Na^+/H^+ antiport (believed to be activated by PKC). Amiloride (10^{-5}M) significantly inhibited [^{3}H]-thymidine incorporation into Con A blasts stimulated with IL2 (Figure 37).

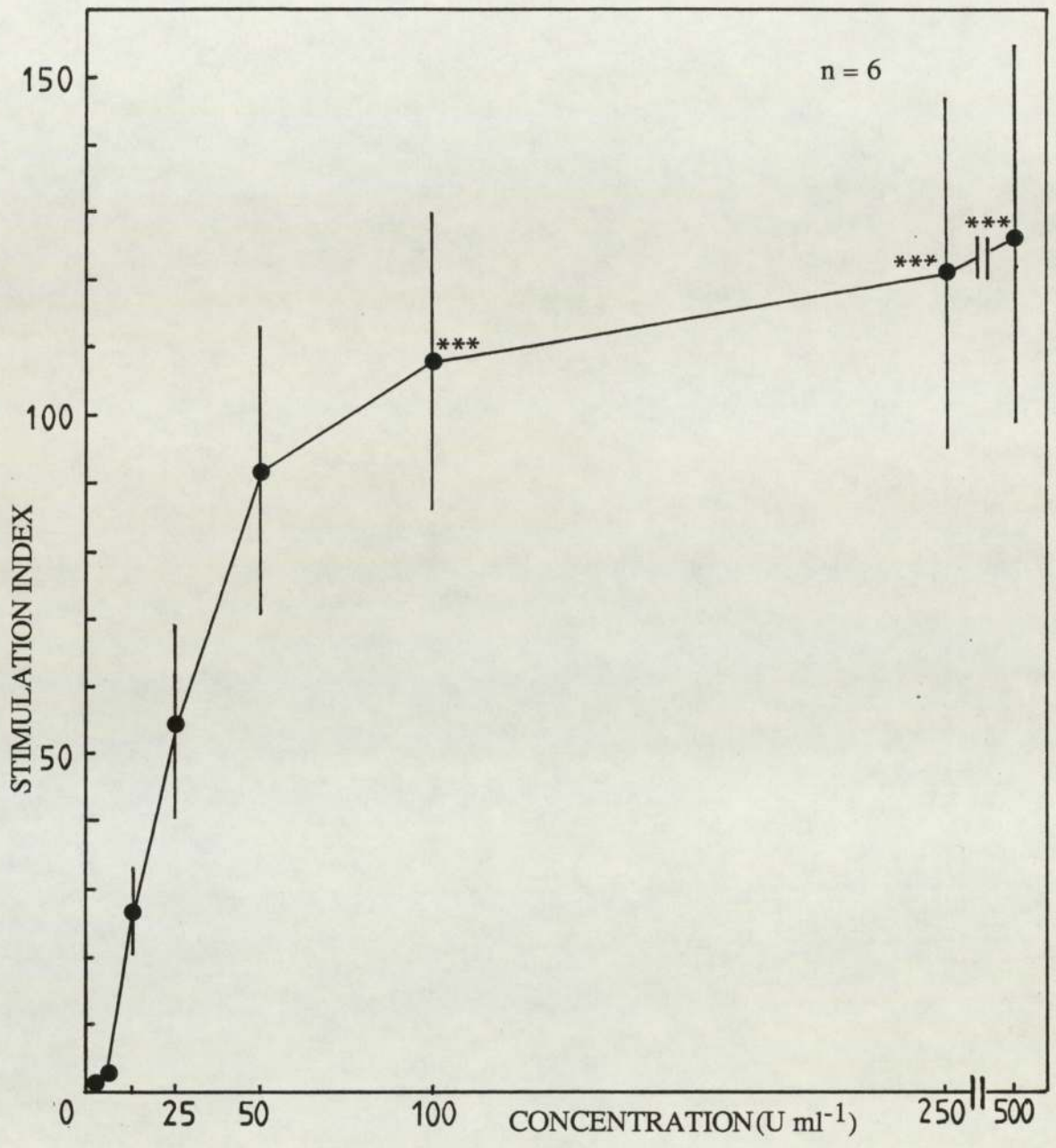


Figure 33: Dose-response curve of the effect of recombinant Human Interleukin 2 on thymidine incorporation in 'Con A blasts'.

Values significantly different from those of other cultures are indicated by asterisks.

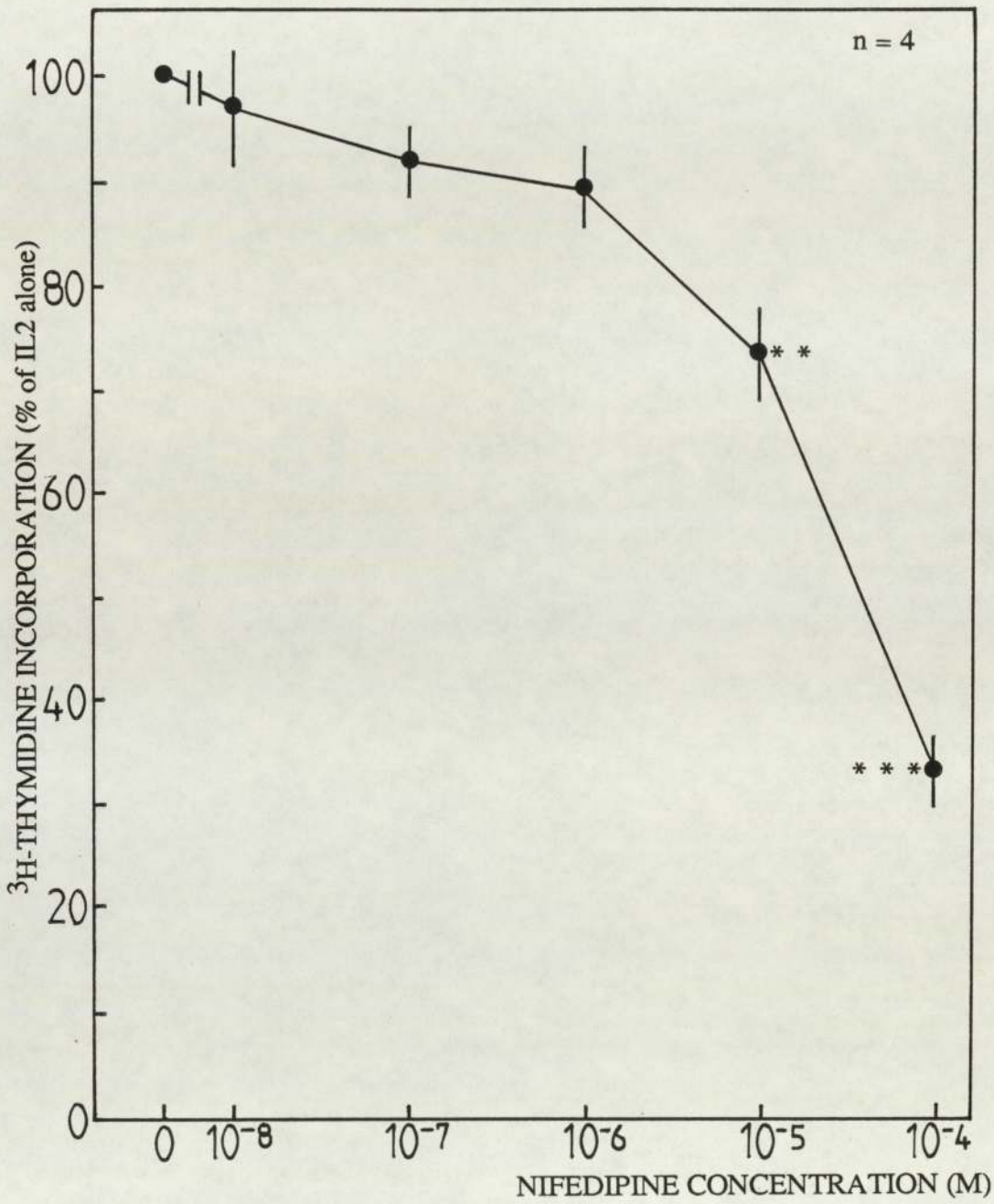


Figure 34: Effect of Nifedipine on the IL2 (100 U ml^{-1}) induced 'Con A blast' proliferation

The level of thymidine incorporation in cultures stimulated by IL2 (100 U ml^{-1}) alone is taken as 100% and the incorporation in cultures incubated with IL2 (100 U ml^{-1}) plus various concentrations of nifedipine expressed as a percentage thereof.

Values significantly different from those of corresponding cultures without the drug are indicated by asterisks.

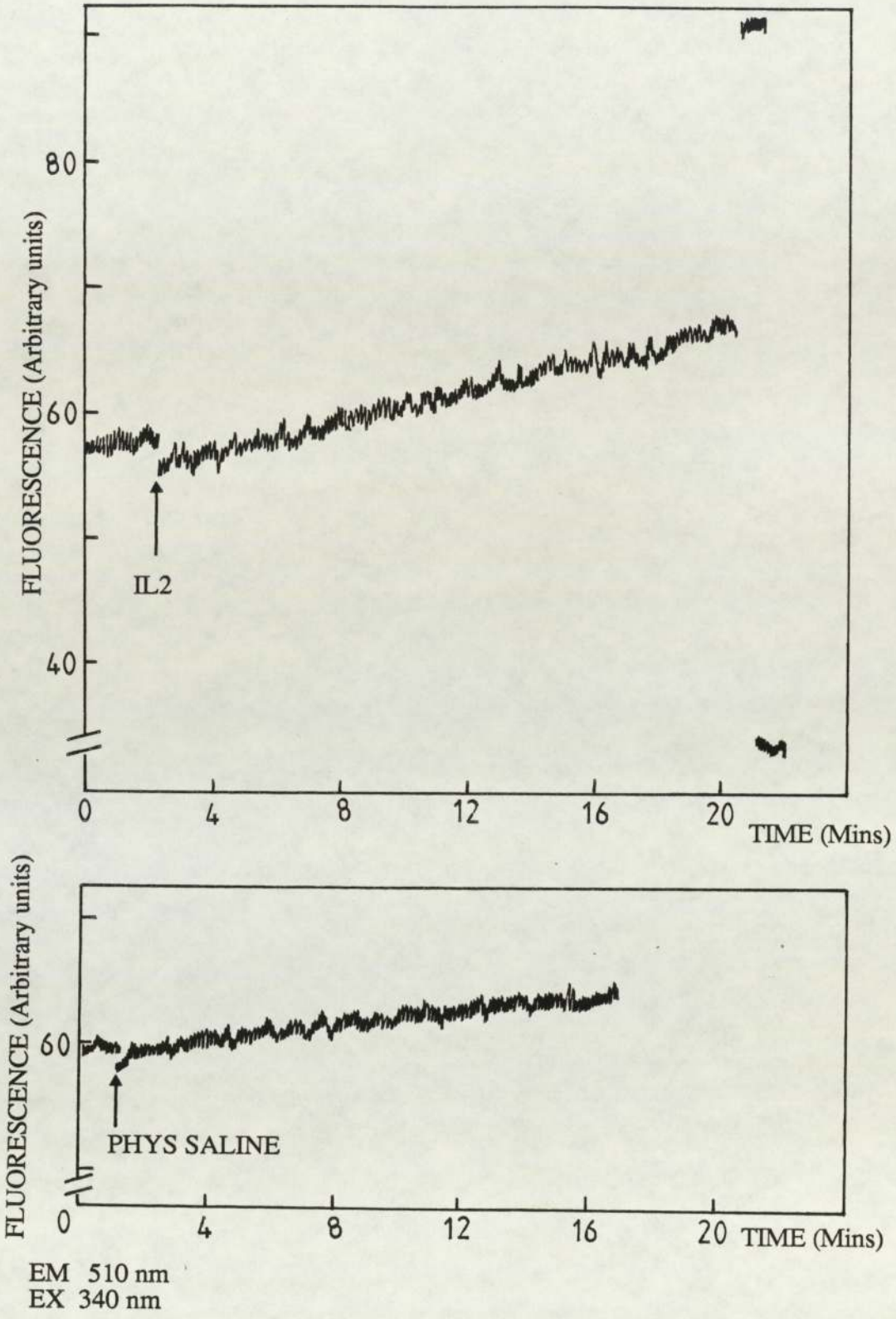


Figure 35: Comparison of the effects of IL2 (100 U ml^{-1}) and physiological saline addition upon the $[Ca^{2+}]i$ of fura-2 loaded Con A blasts.

The fluorescence values correspond to resting calcium levels of 172 nM in the upper trace and 224 nM in the lower trace.

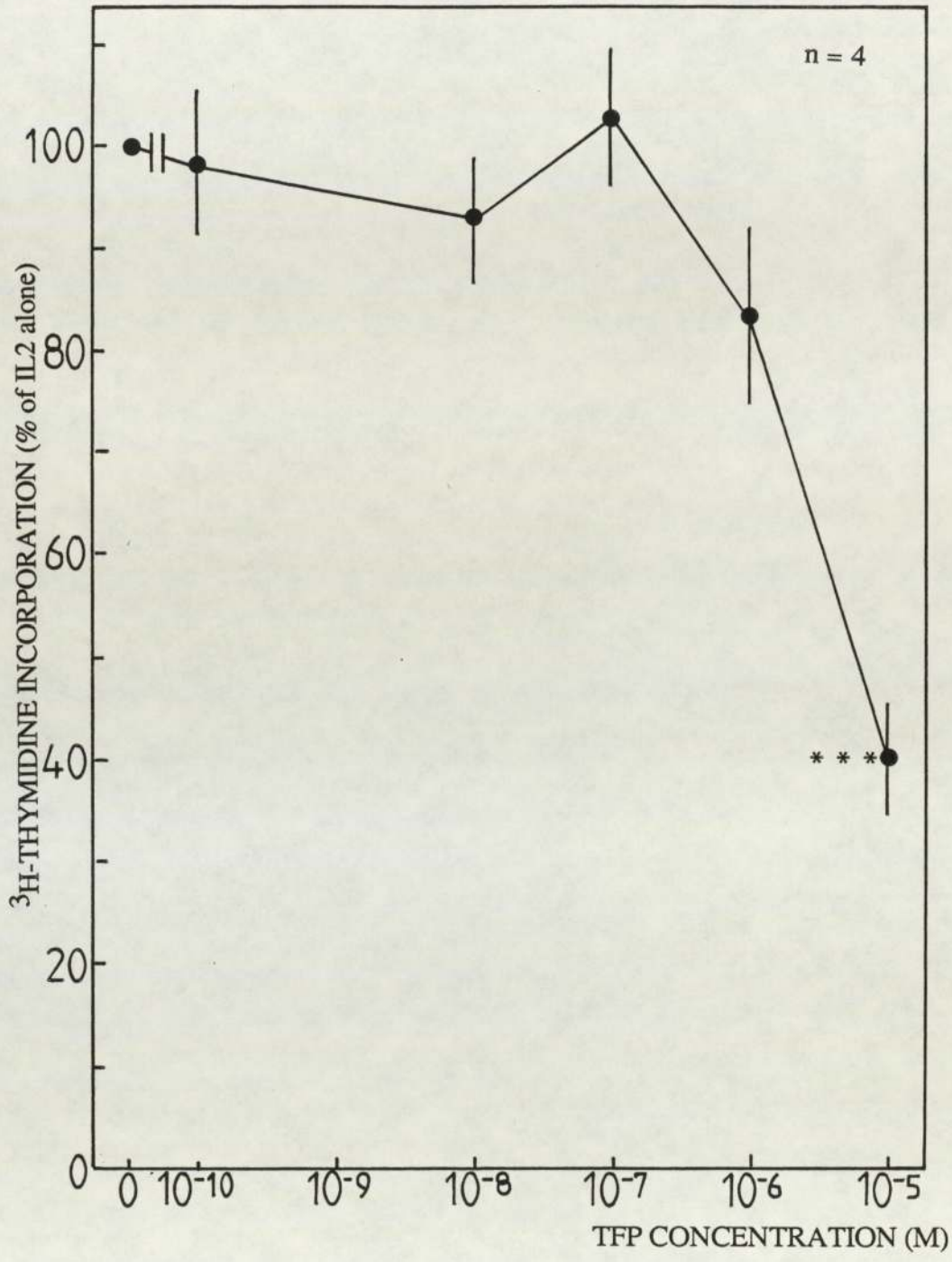


Figure 36: Effect of TFP on the IL2 (100 U ml^{-1}) 'Con A blast' proliferation.

Values significantly different from those of corresponding cultures without the drug are indicated by asterisks.

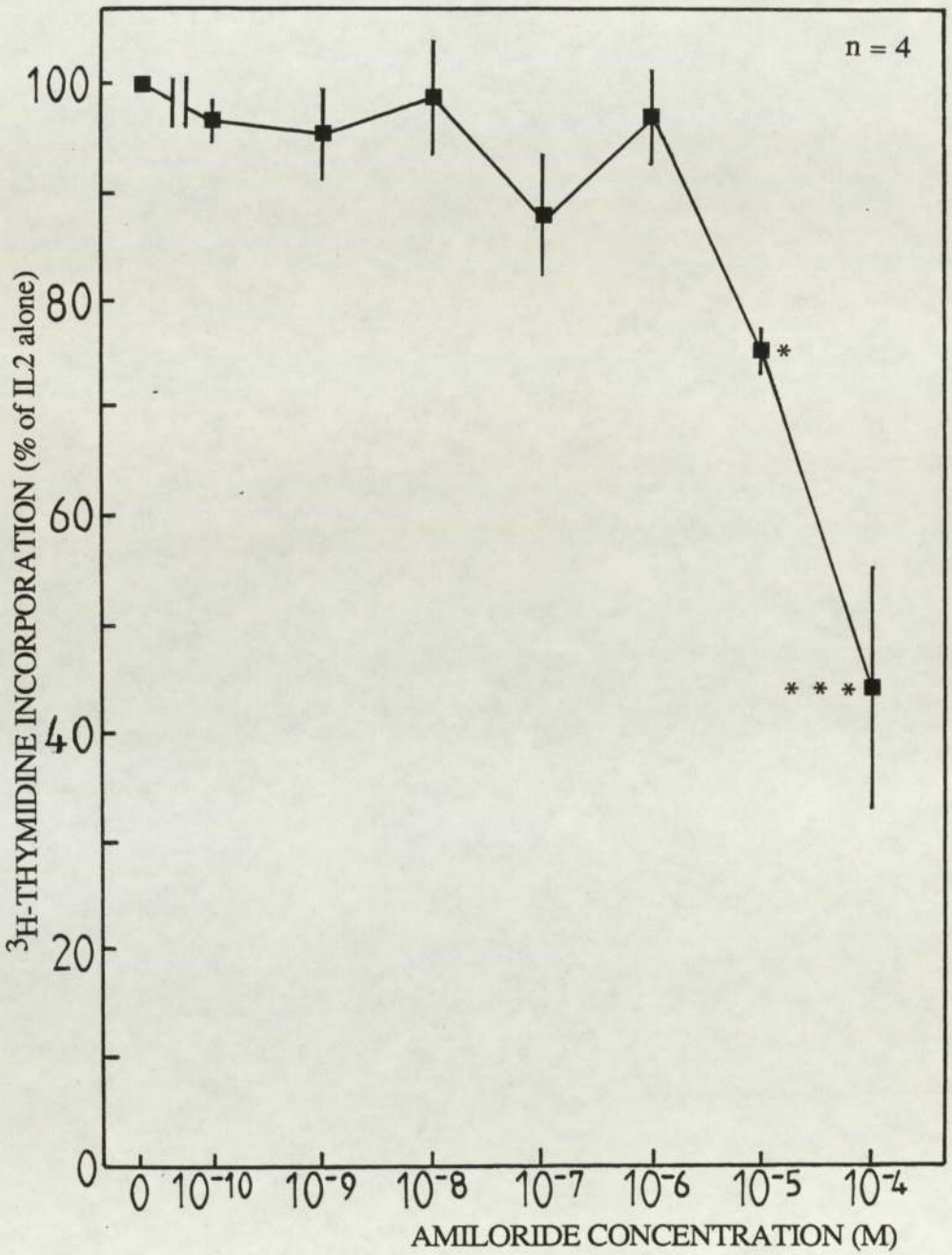


Figure 37: Effect of Amiloride on the IL2 (100 U ml^{-1}) induced proliferation of 'Con A blasts'.

Values significantly different from those of corresponding cultures incubated without the drug are indicated by asterisk(s).

An objective of this study has been to define the mechanism(s) of sex steroid modulation of the immune system. Thus, the effects of sex steroids on the IL2 induced proliferation of Con A blasts were investigated. The hypothesis being that more physiological concentrations of sex steroids might inhibit proliferation induced by this T cell growth factor as opposed to the pharmacological concentrations necessary to block lectin stimulated growth. However, oestradiol and testoesterone still only inhibited DNA synthesis at high concentrations (Figure 38). Nevertheless, this inhibition was not due to cytotoxicity since cell viability was between 90-95% in control cultures and cultures treated with steroids.

Interleukin 1 can stimulate the proliferation of thymocytes in the presence of low amounts of mitogenic lectin (Praetkau et al, 1976). The proliferation is believed to be due to IL2 released by IL1 stimulated T-cells. There are at least two members of the IL1 family, IL1α and IL1ß (March et al, 1985), and both of these lymphokines were investigated for their mitotic potential in murine splenocytes alone and in the presence of Con A (0.35 $\mu g\ ml^{-1}$) for 72 hours. All concentrations of IL1α tested promoted a small increase in DNA synthesis (Figure 39). Administration of varying doses of this lymphokine with Con A (0.35 $\mu g\ ml^{-1}$) had no additive effect on the $[^3H]$-thymidine incorporation stimulated by lectin alone (Figure 39). IL1ß induced a significant, albeit small increment in DNA synthesis at a concentration of $10^{-9} g\ ml^{-1}$ (Figure 40). No concentration of IL1ß significantly enhanced the tritiated thymidine incorporation provoked by Con A (0.35 $\mu g\ ml^{-1}$) alone (Figure 40).

Investigations designed to elucidate the nature of sex steroid modulation of immune function have thus far concentrated on the effects of gonadal steroids on T lymphocyte activity. It seemed appropriate therefore to investigate the actions of sex steroids on B cell function. Lipopolysaccharide, a bacterial endotoxin which specifically stimulates B cells to divide (Greaves and Janossy, 1972), increases DNA synthesis in murine splenocytes with maximum stimulation occurring at a dose of 25 $\mu g\ ml^{-1}$ (Figure 41). Preliminary studies demonstrated that oestradiol inhibits B cell

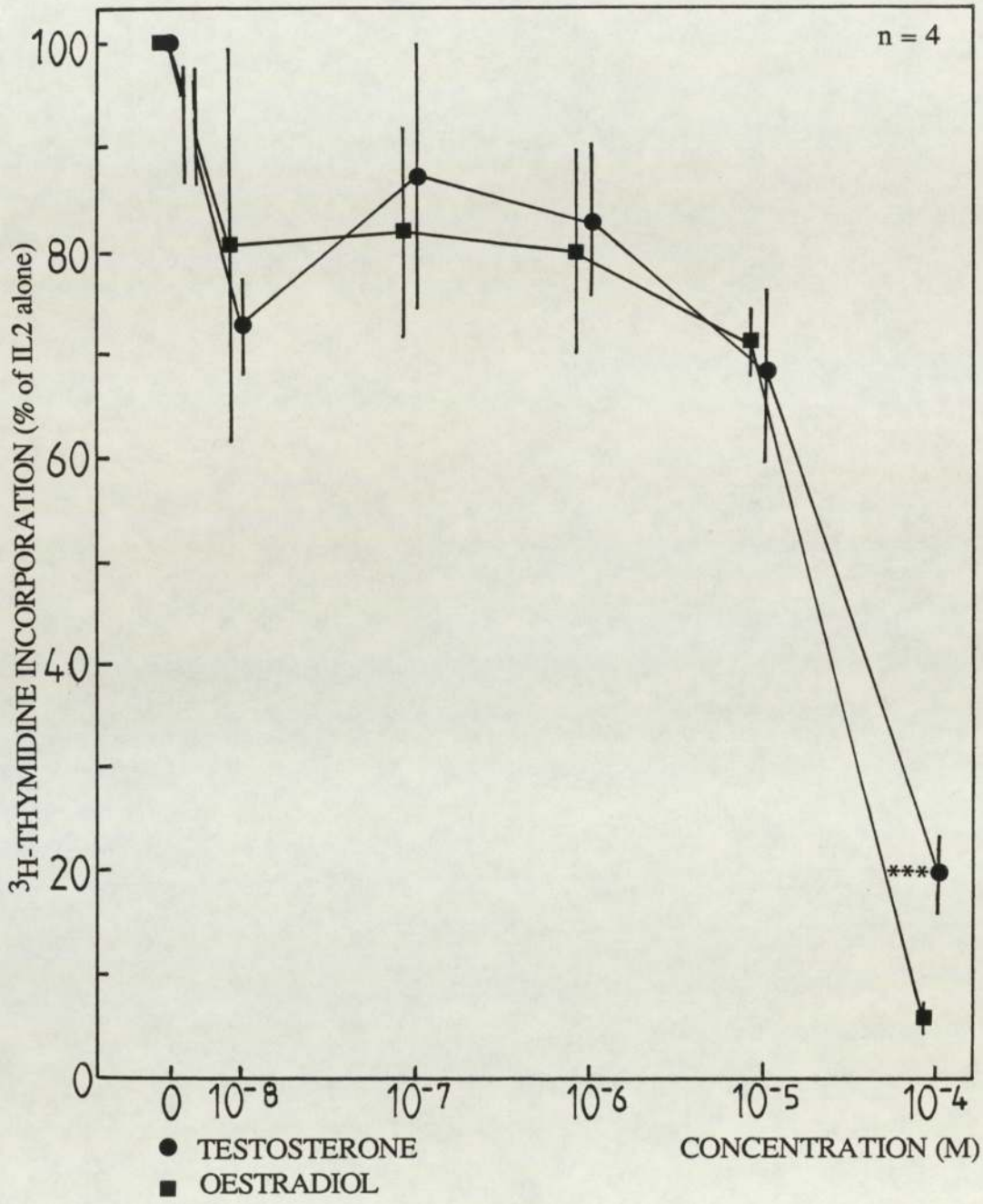


Figure 38: Effect of sex steroids on the IL2 (100 U ml^{-1}) induced proliferation of 'Con A blasts'.

Values significantly different from those of corresponding cultures incubated without steroids are indicated by asterisks.

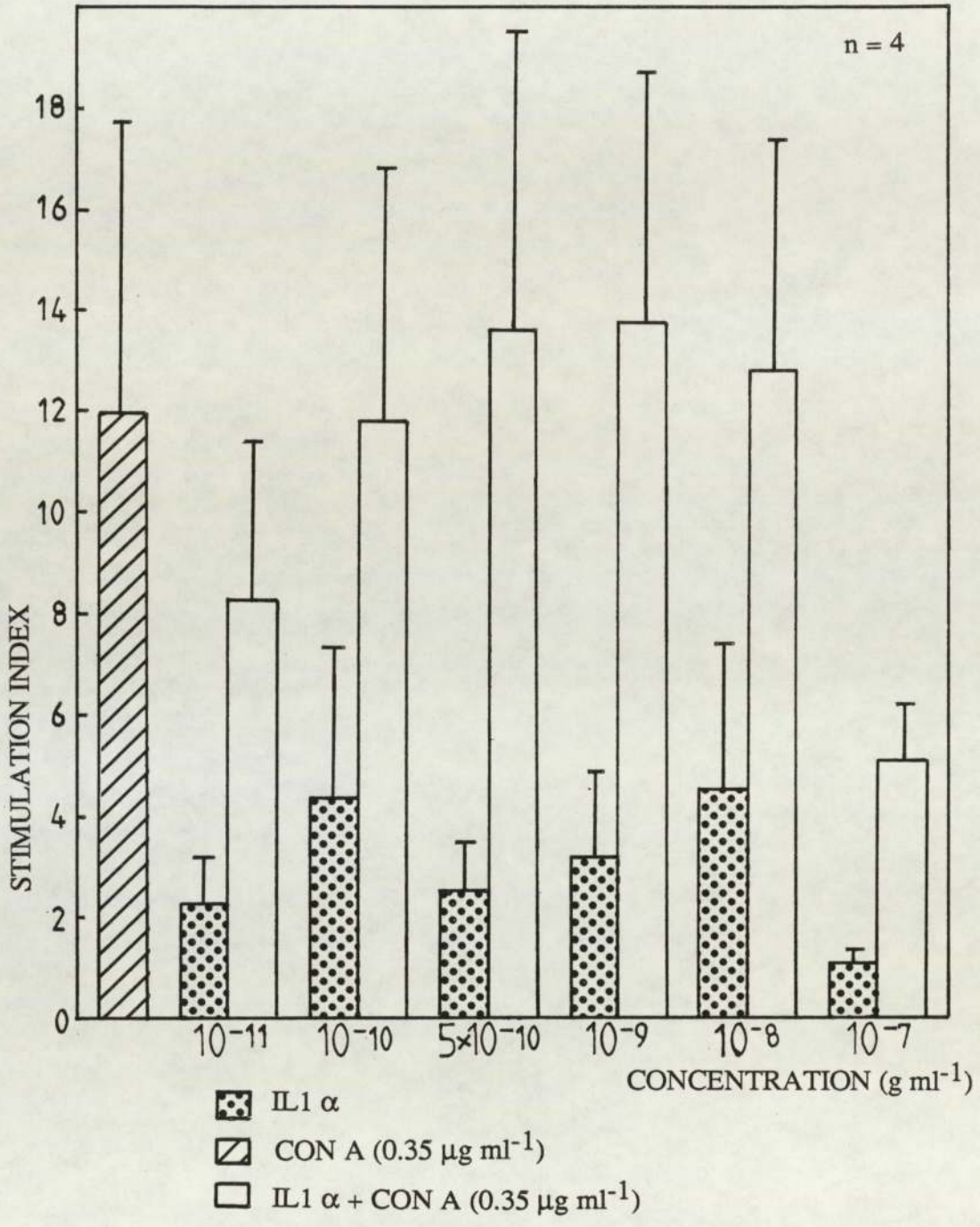


Figure 39: Comparison of the effect of Interleukin 1 α with and without Con A (0.35 µg ml^{-1}) on thymidine incorporation in isolated murine splenocytes.

In this experiment splenocytes were cultured for 72 hours, ^{3}H-thymidine being present during the last 4 hours of incubation.

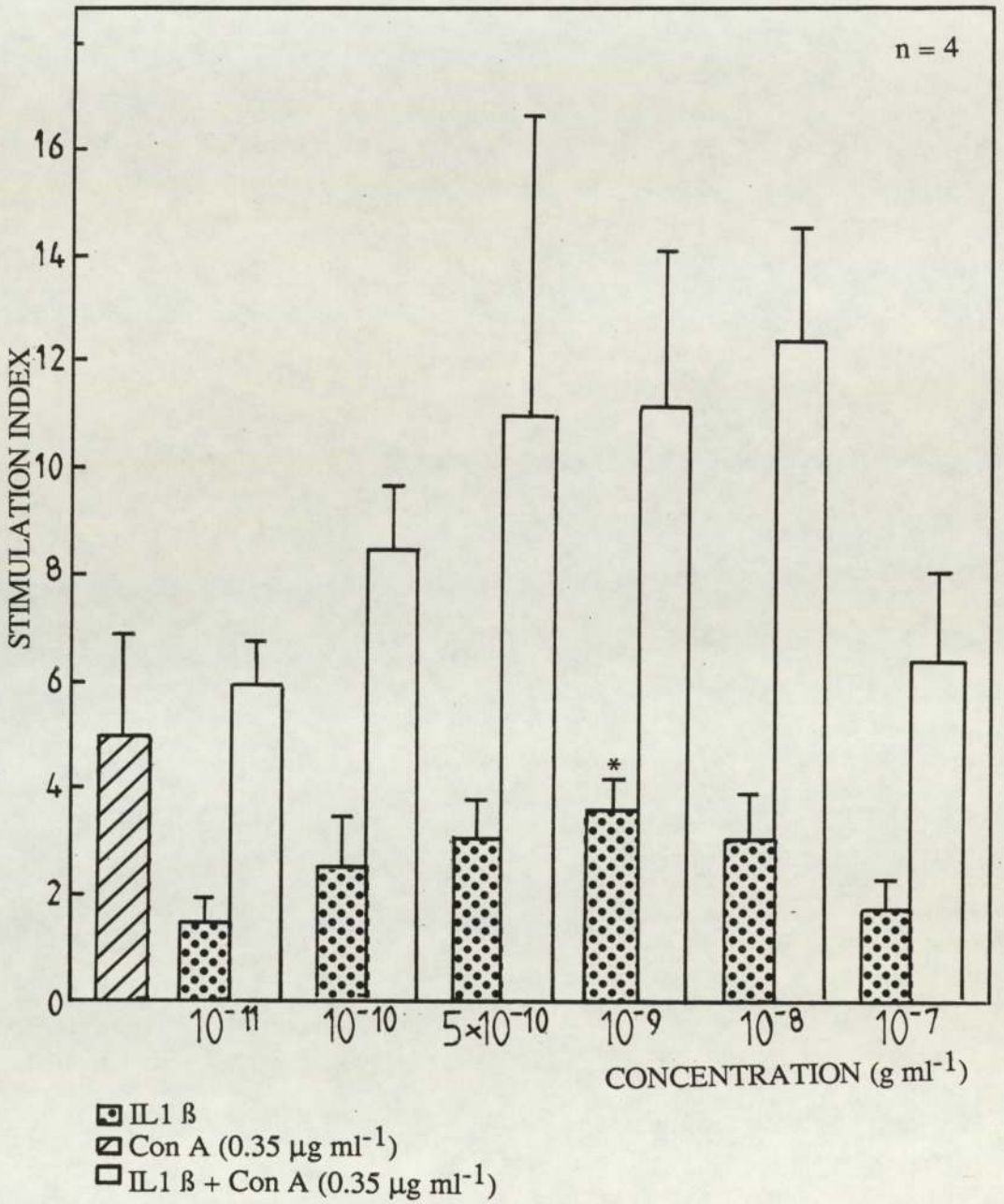


Figure 40: Comparison of the effect of Interleukin 1 ß with and without Con A (0.35 µg ml^{-1}) on thymidine incorporation in isolated murine splenocytes.

Values significantly different from those of other cultures within the same treatment group are indicated by an asterisk. Other details as in Figure 39.

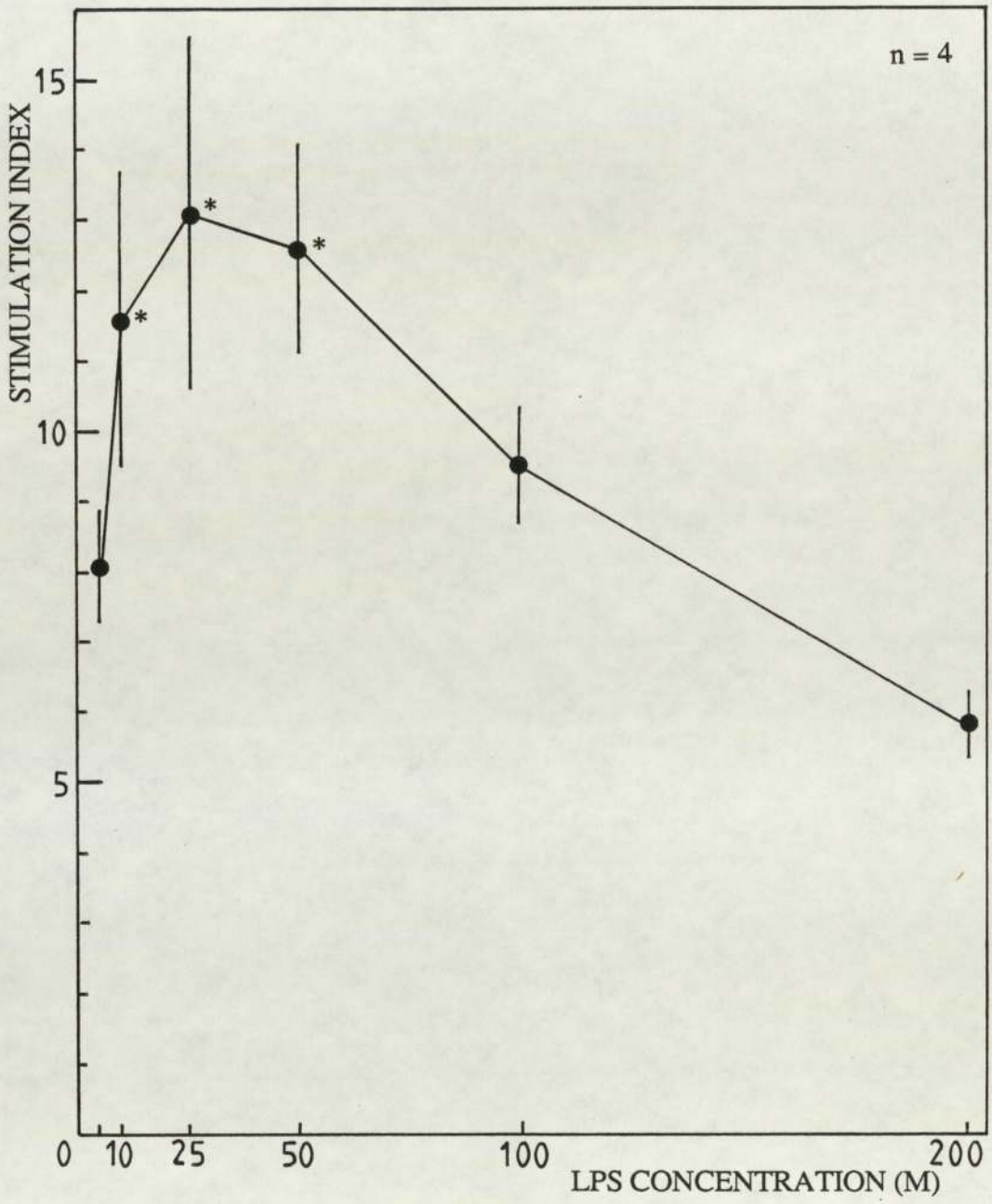


Figure 41: Dose response curve of the effect of Lipopolysaccharide (LPS) on thymidine incorporation in isolated murine splenocytes.

Values significantly different from those of other cultures are indicated by an asterisk.

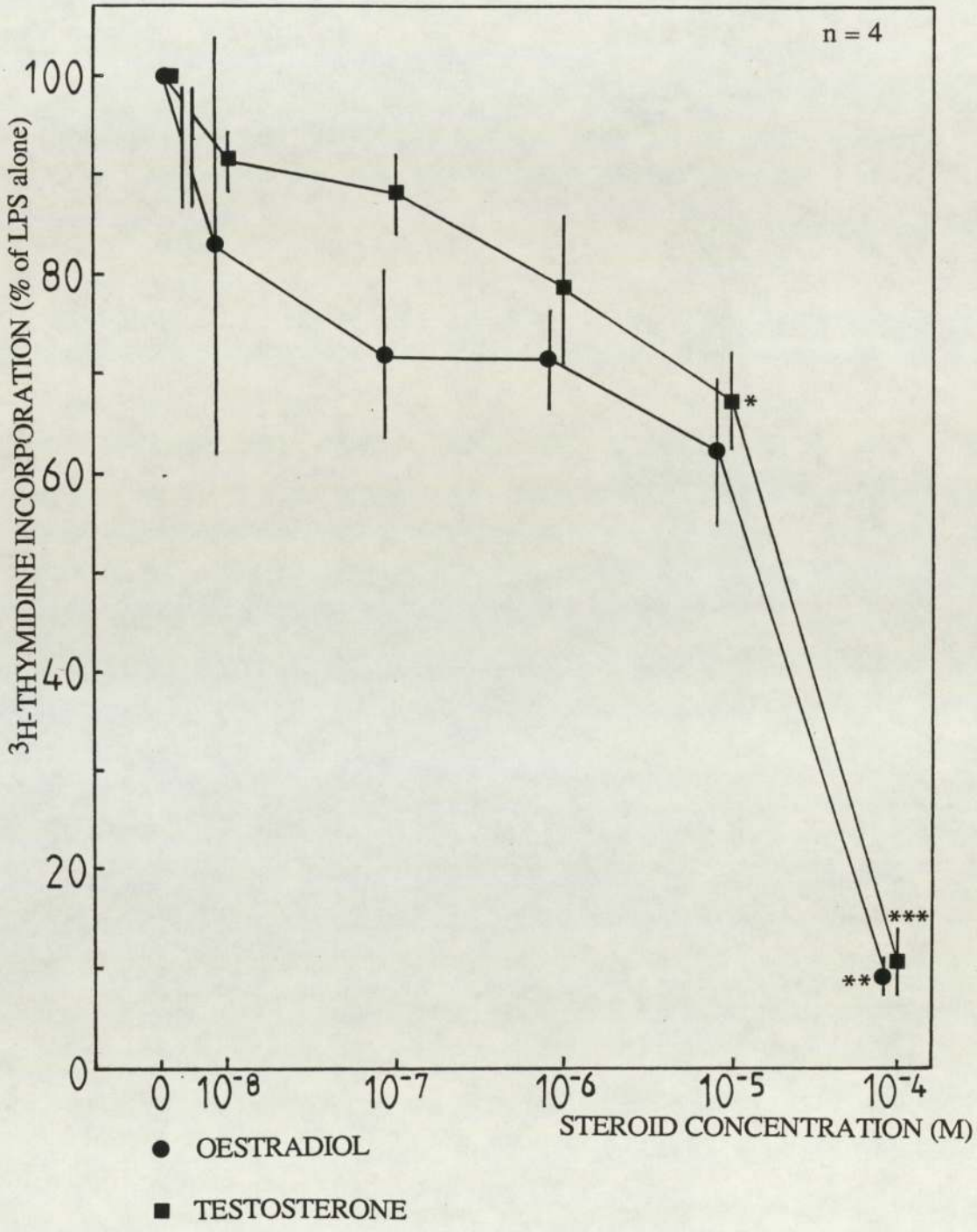


Figure 42: Effect of sex steroids on the LPS (25 μg ml^{-1}) induced proliferation of murine splenocytes.

The level of thymidine incorporation in cultures stimulated by LPS alone is taken as 100% and the incorporation in cultures incubated with LPS plus various concentrations of steroid expressed as a percentage thereof. Values significantly different from those of cultures incubated without steroids are indicated by asterisks.

proliferation at a pharmacological concentration of 10^{-4}M (Figure 42) whilst testosterone (10^{-5}M) significantly inhibits DNA synthesis in murine splenocytes stimulated with LPS (25 $\mu g\ ml^{-1}$) (Figure 42).

The generation and possible interaction of the putative intracellular signals involved in T lymphocyte proliferation stimulated by the plant lectin, Con A and the lymphokine, Interleukin 2 will be discussed in the following section in the light of these experimental findings. The possible mechanism(s) of sex steroid modulation of lymphoid cell function will also be examined with regard to the evidence presented here.

CHAPTER FOUR

DISCUSSION

Cell-mediated immunity provides the main defence against intracellular organisms. The mechanism by which resting T cells are activated in an antigen specific response is believed to incorporate the following steps; T cell receptors interact with antigen in association with Class II products of the major histocompatibility complex on the surface of the antigen-presenting macrophages. The combination of antigen and macrophage-derived interleukin 1 (IL1) activate the T cells which express interleukin 2 (IL2) receptors and subsequently undergo proliferative expansion, under the influence of IL2 secreted by another activated T cell sub-population. The two arms of the cell-mediated response, generation of cytotoxic cells and the release of soluble factors (the lymphokines) are mediated by different T cell subsets, the cytotoxic T cells and helper T cells respectively (see Section 1.7). Preceding activation, T cells are resting in G_0, after interaction with antigen and IL1, intracellular biochemical responses occur that promote entry into the G_1 phase rendering them responsive to an additional signal(s) (IL2) which enables cells to traverse from G_1 into the S phase (DNA synthesis) and progress into mitosis. Despite extensive study, the mechanism(s) by which membrane signals are transduced into the nucleus and thence regulate the cascade of activation and proliferation steps, awaits delineation. Lectin stimulation of T lymphocytes, often used as a model of the proliferative aspect of the immune response has been investigated here in an attempt to gain a better understanding of this complex phenomenon.

In accordance with other studies, Con A, a T cell mitogen enhanced DNA synthesis in murine splenocytes (Figure 1). Mitogen-induced stimulation of T-lymphocytes increased with Con A concentrations up to 5 $\mu g\ ml^{-1}$ and then

decreased with increasing concentrations of Con A. Two hypotheses have been suggested to account for this inhibitory effect of supraoptimal lectin concentration. Thus, rapid capping and internalization of mitogen receptors occurs such that receptors are removed from the cell surface prematurely before commitment to the S phase has occurred (Pozzan et al, 1981). Alternatively or in addition, supra-optimal concentrations are thought to bind to proteins (other than mitogen receptors) on the membrane, thereby blocking essential surface-modulating activity controlled by the cytoskeleton (McClain & Edelman, 1976).

T lymphocyte proliferation induced by the optimal mitogenic concentration of Con A, 5 $\mu g\ ml^{-1}$ was significantly inhibited by three specific calcium antagonists, verapamil ($5 \times 10^{-5}M$), nifedipine ($10^{-6}M$) and diltiazem ($10^{-4}M$), (Figures 3, 4 and 5 respectively), suggesting that a Ca^{2+} influx is obligatory in stimulus-mitosis coupling. Calcium antagonists are widely believed to block Ca^{2+} entry into cells by interacting with voltage-operated Ca^{2+} channels (VOC's) (Spedding, 1987). The concept that VOC's are more sensitive to calcium channel blockers than ROC's (receptor-operated channels) is supported by the finding that contractions of vascular smooth muscle induced by calcium or potassium depolarization are potently inhibited by calcium antagonists whereas contractions induced by hormones such as noradrenaline are refractory to these drugs (Bolton, 1979; Cauvin et al, 1983). In contrast, several other studies indicate that in some tissues the ROC is more sensitive to calcium entry blockers than the VOC. Moreover, it appears that the ROC may vary from tissue to tissue in its sensitivity to calcium channel blockers (Van Breeman and Siegel, 1980). Thus, the ROC's of the cerebral circulation appear to be more susceptible to the action of calcium antagonists than those of the mesenteric arteries (Towart, 1981; Cauvin et al, 1983). Thus, the widely held belief that VOC's are more sensitive than ROC's to calcium antagonists does not always apply.

There are believed to be three classes of Ca^{2+} channels, a small transient current is carried by the T (for transient) channel; a larger current is carried by the N (neuronal) channel (only present in certain neurones) and a third class of channel the L (long) channel, a slowly inactivating channel. Of the three classes of Ca^{2+} channel only the L channel is thought to be susceptible to the classical calcium antagonists (Spedding, 1987). Because these organic Ca^{2+} channel blockers exert their actions at low concentrations and exhibit stereospecificity it appears likely that they are recognised by specific structures of the Ca^{2+} channel. The diversity of the molecular structures of Ca^{2+} channel blockers is consistent with differing modes and sites of action. Thus, it is believed that there are three different recognition sites on the L channel, one each for verapamil, diltiazem and the dihydropyridines (such as nifedipine) (Scharwtz and Triggle, 1984; Katz, 1985). Nevertheless, this does not necessarily imply that Con A cannot be exerting its effect by activating ROC's. Thus, the possibility exists that these calcium channel blockers inhibit Ca^{2+} entry via ROC or that ROC activation by Con A may indirectly lead to partial cellular depolarisation which automatically triggers the opening of VOC's (Cavero and Spedding, 1983). Unfortunately, radioisotopic $^{45}Ca^{2+}$ studies were unable to monitor this putative lectin-induced Ca^{2+} influx (see Results Section, Chapter 3). Although an increase in $^{45}Ca^{2+}$ uptake has been demonstrated in human peripheral blood lymphocytes stimulated with plant lectins (Whitney & Sutherland, 1972; Freedman et al, 1975; Greene et al, 1976) by a variety of methods, a more recent report indicates that Con A did not augment $^{45}Ca^{2+}$ uptake into T cells and even the calcium ionophore A23187 (1 $\mu g\ ml^{-1}$) only caused a slight increase in Ca^{2+} influx (at a concentration which was optimum for stimulating DNA synthesis in human Tonsillar T cells) (Komada et al, 1985). These workers suggest that even though enhancement of Ca^{2+} uptake may not be an absolute requirement, intracellular Ca^{2+} may act as an important messenger in T cell mitogenesis. Thus, it is possible that either the microcentrifugation technique used

in this study is inappropriate for estimating $^{45}Ca^{2+}$ influxes or that the mitogenic dose of lectin, 5 μg ml^{-1} and co-mitogenic concentration of A23187, 10^{-7}M do not enhance $^{45}Ca^{2+}$ uptake in murine splenocytes. However the data obtained from calcium antagonist studies make the first possibility more likely.

Rapid changes in the intracellular free calcium ion concentration $[Ca^{2+}]i$ in murine splenocytes certainly were detected by the fluorescent probe fura-2 in response to Con A (5 μg ml^{-1}). The resting $[Ca^{2+}]i$ in these cells was approximately 185 nM. Following lectin stimulation the $[Ca^{2+}]i$ rose rapidly to reach a maximal value by 1 1/2 minutes; the $[Ca^{2+}]i$ increased 1.45 fold over resting levels. Most other studies using fluorescent probes report a value of approximately 100-120 nM for the cytoplasmic free calcium concentration of resting lymphocytes (murine thymocytes) (Tsien et al, 1982; Metcalfe et al, 1985; Tsien & Poenie, 1986) which rose to between 150 nM and 200 nM following Con A treatment. The higher value for resting $[Ca^{2+}]i$ in splenocytes indicated in this study can probably be attributed to slight fura-2 leakage (see Section 2.3.1). The Ca^{2+} signal in response to Con A was partially diminished when Ca^{2+} was absent from the extracellular medium (Table 4). Subsequent Ca^{2+} addition to the media induced a further increase in $[Ca^{2+}]i$ suggesting that the Con A provoked rise in $[Ca^{2+}]i$ was due to both a mobilisation of intracellular Ca^{2+}ions and an influx of Ca^{2+} ions across the plasma membrane (or possibly an inhibition of Ca^{2+} extrusion). This Ca^{2+} signal persisted for at least one hour and possibly longer; indeed Hesketh and colleagues (1983) report that the lectin induced Ca^{2+} signal only returns to resting values after approximately 24 hours following the same time course as cap formation by Con A on the lymphocyte cell surface. This implies that the Ca^{2+} signal requires a continuous interaction of the mitogen with its receptor. The effects on the calcium signal of two calcium antagonists at concentrations known to significantly inhibit Con A induced DNA synthesis gave equivocal results (Tables 2 and 3). Thus, verapamil (5 x 10^{-5}M) significantly diminished the lectin induced

$[Ca^{2+}]i$ increase, suggesting that the Ca^{2+} influx component of the early Ca^{2+} signal may well be important in the molecular events following ligand-receptor interaction which ultimately lead to DNA synthesis. In contrast, diltiazem at a concentration ($10^{-4}M$) known to suppress [^{3}H]-thymidine incorporation into Con A stimulated splenocytes) had no effect on the early Ca^{2+} signal. However diltiazem might feasibly impair the persistent Ca^{2+}signal, since its effect on $[Ca^{2+}]i$ was only monitored for approximately 5 minutes following lectin treatment. Fura-2 studies thus demonstrate that lectin mediated increases in $[Ca^{2+}]i$ were remarkedly reduced but not abolished in the absence of extracellular Ca^{2+} or by the addition of a calcium channel blocker, verapamil. This is in agreement with other recent studies (Mills et al, 1985; Hesketh et al, 1985; Gelfand et al, 1986) using murine thymocytes and human T lymphocytes. This implies that following binding of Con A murine splenocytes, both intra- and extracellular mediated changes in Ca^{2+} contribute to the early Ca^{2+} signal. Moreover, this Ca^{2+} signal appears obligatory for subsequent DNA synthesis as witnessed by the abrogation of enhanced [^{3}H]-thymidine incorporation in Con A stimulated splenocytes by the calcium channel blockers.

It is not unreasonable to assume that if it is indeed this early rise in intracellular calcium which initiates the proliferogenic response to Con A then the next step in the sequence of events leading to DNA synthesis and ultimately mitosis is the association of Ca^{2+} with its specific binding protein, calmodulin. Therefore, if calmodulin is activated during the mitotic response of T lymphocytes to Con A then calmodulin inhibitors such as trifluoperazine (TFP) and M&B 13753 should compromise this response. This was confirmed when the lectin-enhanced DNA synthesis was inhibited by treatment with these calmodulin antagonists ($10^{-7}M$) (Figure 8). However, to attribute any effects of these drugs to the inhibition of calmodulin it is necessary to ascertain that the action results from inactivation of calmodulin and not from a general hydrophobic effect or calmodulin-independent

metabolic effect (Cheung, 1984). Calmodulin antagonists have been employed to identify calmodulin regulation of specific biological processes and indeed many different physiological processes have been inhibited following their use. Rather than indicating calmodulin's ability to regulate diverse enzymes this may infer that calmodulin antagonists have multiple sites of action (Veigl et al, 1984a). Thus, TFP ($\simeq$ 50 μM) reduces lymphocyte ATP levels, so that any enzymic process that involves hydrolysis of ATP will be sensitive to these changes irrespective of any calmodulin regulation of the process (Corps et al, 1982). Additionally TFP ($\simeq$ 50 μM) and other phenothiazines inhibit protein kinase C as well as inhibiting the function of Ca^{2+} calmodulin, and therefore cannot be considered as specific inhibitors of either activity (Schwantke et al, 1985). However, there is evidence that at least some of the effects of TFP are specific to calmodulin. Thus, the growth of a macrophage like cell line (J774) is normally inhibited by TFP whilst a variant of this line lacking in a specific calmodulin-dependent binding protein is unaffected by TFP although it has a normal calmodulin content. The enzymatic activity associated with the calmodulin binding protein, if any, is unknown (Speaker et al, 1982). Since lower concentrations of TFP inhibited lectin induced DNA synthesis in murine splenocytes than those indicated in the literature it seems likely that calmodulin is implicated in the signal-transduction pathway. Thus, calcium via the formation of calci-calmodulin conformers appears to have an obligatory role in triggering the cascade of mitotic events linked to Con A stimulation. Regulation of cell division by calmodulin is thought to be related to the effective concentration of the protein in the cell at any given time. Thus, it is believed that in certain cell types (see Section 1.5.2) an elevation in calmodulin concentration occurs at the G_1/S boundary of the cell cycle which is essential for progression of the cells into the S phase. Regulation of the cytoskeleton and protein phosphorylation, particularly histone phosphorylation by calmodulin might be crucial in the G_1/S transition (Chafouleas and Means, 1982).

The $[Ca^{2+}]i$ response to Con A in thymocytes was antagonised by membrane-permeant cyclic AMP analogues or agents that elevate intracellular cyclic AMP (Hesketh et al, 1985). However, in the present studies using murine splenocytes, neither dibutyryl cAMP nor 8-bromo cAMP impaired the Con A induced Ca^{2+} signal at any concentration tested. Moreover, only rather unphysiological concentrations of dibutyryl cAMP (10^{-4}M) significntly inhibited Con A induced DNA synthesis (Figure 9). However, forskolin, an agent which directly activates adenylate cyclase in a variety of cells (Seamon et al, 1981), inhibited lectin stimulated [^{3}H]-thymidine incorporation in a dose-dependent fashion (Figure 10). There are many reports suggesting that cAMP is indeed an inhibitory regulator of cell proliferation (see Section 1.5.1). Moreover, the inhibition of growth by cyclic AMP has been attributed to its ability to prevent excess calcium from entering cells at a critical growth restriction point in G_1 phase of the cell cycle. Thus, Con A activation of murine thymocytes requires Ca^{2+} and the influx of Ca^{2+} into Con A treated cells is inhibited by dibutyryl cAMP, as is DNA synthesis (Tsien et al, 1982; Hesketh et al, 1985). Further support for this hypothesis is that cyclic AMP also reduces Ca^{2+} availability in rat thymocytes by stimulating Ca^{2+} extrusion whilst the calcium ionophore A23187 can bypass the effect of cAMP and induce thymocyte proliferation (Ralph, 1983). These observations imply that normally cAMP acts to decrease intracellular Ca^{2+}. In this study the membrane-permeant cAMP analogues apparently had no effect on the early Ca^{2+} signal induced by Con A as monitored by the fluorescent probe fura-2 although it is impossible to know what their effect on the persistent signal might have been. However, an objection to the studies in which cAMP is implicated as a Ca^{2+} antagonist are that the inhibitory effects on the Ca^{2+} signal were elicited by high concentrations of cAMP analogues (5-10 mM) (Hesketh et al, 1985) and could therefore be described as non-specific. Certainly it is unlikely that endogenously generated cAMP ever reaches such proportions.

An alternative hypothesis to explain the inhibitory effects of forskolin on Con A induced murine splenocyte proliferation is that rather than reducing the Ca^{2+} availability, cAMP formation might antagonise protein kinase C activity. Indeed, the inhibitory effect of forskolin on human platelet secretion and aggregation induced by 5-hydroxtryptamine is attributed to its ability to inhibit protein kinase C activity (De Chaffoy De Courcelles et al, 1987).

The inhibitory role of cAMP has recently been challenged on the basis that cAMP elevating agents act synergistically with other mitogens to stimulate DNA synthesis in 3T3 cells (Rozengurt, 1985) (see Section 1.5.1). Murine splenocytes incubated for 48 hours with forskolin alone showed no enhanced [^{3}H]-thymidine incorporation, but these preliminary studies do not imply that cAMP is irrelevant in stimulus-mitosis coupling. Thus, it is possible that once cells have passed through the restriction point in G_1 (possibly Ca^{2+} dependent) then a cAMP signal might activate certain metabolic processes, thereby enhancing cell cycle progression (Ralph, 1983). Furthermore, it is possible that after G_1 phase growth has commenced, subsequent cAMP-dependent events may be obligatory in certain cells (including lymphocytes) before DNA synthesis can eventually occur. In addition, a later cAMP surge in G_2 may also be required before the cell is able to progress into mitosis (Whitfield et al, 1987).

Whilst the role of cAMP remains unclear the early Ca^{2+} signal demonstrated by the fluorescent probe fura-2 is strongly implicated in the signal-transduction pathway ultimately leading to DNA synthesis in lectin stimulated murine splenocytes. The early Ca^{2+} signal appears to involve both mobilisation of intracellular Ca^{2+} ions and also depends on extracellular calcium. There is a large body of evidence which demonstrates that mobilisation of Ca^{2+} in the cytoplasm of cells stimulated by agents acting via cell surface receptors is nearly always associated

with the hydrolysis of phosphatidylinositol 4,5,bisphosphate (PIP_2) to yield inositol trisphosphate ($InsP_3$) and diacylglycerol (DG). $InsP_3$ can induce the release of Ca^{2+} ions from the endoplasmic reticulum (Berridge and Irvine, 1984; Spat, 1986; Taylor, 1988). However, the release of calcium from intracellular stores is only one component of the early Ca^{2+} signal in lectin-stimulated splenocytes. When cells were suspended in Ca^{2+} free medium, the rise in $[Ca^{2+}]i$ was significantly reduced (Figure 7), suggesting that part of the intracellular $[Ca^{2+}]i$ increment originates from the extracellular medium. The transfer of extracellular calcium to the cell cytosol upon activation does not necessarily imply the existence of a calcium channel. Thus, a possible explanation is that calcium constantly leaks into cells down the inwardly directed concentration gradient (1mM —> 100nM) and that Ca^{2+} is constantly pumped out. Any inhibition of the 'Ca^{2+} pump' upon lectin stimulation would induce an increase in $[Ca^{2+}]i$ without any channel opening (Linch et al, 1987). If this hypothesis is indeed true for murine splenocytes, then fura-2 loaded splenocytes incubated in Ca^{2+} free medium should have demonstrated an increase in $[Ca^{2+}]i$ upon Ca^{2+} restoration to match that invoked by restoration of Ca^{2+} ions to lectin-stimulated splenocytes in Ca^{2+} free medium (Figure 7). Since this wasn't the case it seems likely that Con A stimulation of these cells does involve a Ca^{2+} influx, rather than a 'leak', which serves as one component of the early Ca^{2+} signal. There are several explanations advanced to explain how hydrolysis of phospholipids may lead to Ca^{2+} influx into cells (see Section 1.5.2). Thus, phosphatidic acid formed from DG might function as a Ca^{2+} ionophore, (Putney et al, 1980), or an increase in Na^+/Ca^{2+} exchange might be stimulated leading to Ca^{2+} uptake dependent on the Na^+ gradient (Philipson & Nishimoto, 1984). A more recent hypothesis suggests that $InsP_4$ (formed by phosphorylation of 1,4,5 $InsP_3$) might first regulate transfer of Ca^{2+} from the extracellular space to the intracellular ER pool. Subsequently $InsP_3$ would then

induce mobilisation of this Ca^{2+} from the ER into the cytosol (Irvine & Moor, 1986; Taylor, 1987; Imboden, 1988) (see Section 1.5.2.).

The two limbs of the inositol lipid signalling system can be mimicked by calcium ionophore A23187 and the phorbol ester TPA. Studies undertaken to establish a possible role for this signal transduction pathway in stimulated splenocytes demonstrated that A23187 (10^{-7}M) and TPA (10^{-9}M) did indeed act synergistically to induce DNA synthesis in these cells (Figure 14). Since the synergy could not be repeated with 4α-phorbol-12,13-didecanoate, a phorbol ester without protein kinase C (PKC) enhancing activity (Castagna et al, 1982) it appears that protein kinase C activation may also be implicated in T-lymphocyte proliferation. Since neither TPA nor A23187 alone were able to substantially enhance [^{3}H]-thymidine incorporation (Figure 14) it is apparent that both PKC activation and an increase in $[Ca^{2+}]i$ are necessary signals in murine splenocyte proliferation. In this model, it is believed that the co-mitogens by-pass the physiological pathway acting through PIP_2 hydrolysis, by increasing $[Ca^{2+}]i$ and activating PKC directly. These are assumed to be obligatory secondary responses for lectin stimulation. Indeed, Con A stimulates the breakdown of PIP_2 in murine thymocytes (Moore et al, 1984) leading to these dual signals.

Protein kinase C and Ca^{2+} are now believed to be synergistic signals in the control of many biological responses in a variety of cell types (Downes and Michell, 1985) although such activation is not a universal phenomenon. For example, Ca^{2+} alone controls K^+ efflux from parotid acinar cells (Putney et al, 1984) and stimulation of oxygen radical production in neutrophils appears to relate exclusively to protein kinase C activation (Di Virgilio et al, 1984). However, in murine splenocytes the hypothesis that both signals are obligatory for mitosis to occur is substantiated by studies which demonstrated that a Ca^{2+} signal alone was probably insufficient to

induce DNA synthesis. Thus, A23187 (10^{-7}M) alone, produced an early Ca^{2+} signal of greater magnitude than Con A but was not itself mitogenic (Figure 14). Furthermore this Ca^{2+} signal was unaffected by either TPA (10^{-9}M) or 4α12,13 phorbol didecanoate (10^{-9}M) although only TPA which activates PKC was able to synergise with A23187 (Table 7, Figures 14 and 17). These results imply that in addition to the early Ca^{2+} signal there must be another signal presumably PKC activation. Preliminary studies indicated that the co-mitogens A23187 and TPA did not enhance [^{3}H]-thymidine incorporation to the same extent as Con A, implying that the lectin promotes a further unidentified signal in addition to the Ca^{2+} and PKC signals. Indeed, studies with macrophage-depleted human peripheral lymphocytes demonstrate that these two pathways, protein kinase C activation and Ca^{2+} mobilisation are both essential and synergistic for promoting DNA synthesis. However, in addition to the appropriate concentrations of TPA and A23187 to mimick these signals, a small quantity of plant lectin, phytohaemagglutinin (PHA) is also required for maximal DNA synthesis to occur (Kaibuchi et al, 1985). Studies with murine splenocytes in contrast demonstrated that no concentration of Con A tested further enhanced DNA synthesis stimulated by the co-mitogens TPA and A23187 (Figure 18). Moreover, in these later studies, the stimulation achieved by A23187 and TPA matched that of Con A (5 μg ml^{-1}) implying that PKC activation and an increase in [Ca^{2+}] are the only signals involved in lectin stimulation which mediate the cellular responses leading to DNA synthesis. However, earlier studies demonstrated that receptor occupancy of Con A was necessary for 4-6 hours whilst extracellular Ca^{2+} was only required for the first 4 hours (Figures 13 and 11 respectively). This implies that receptor occupation by Con A performs another function in addition to the generation of $InsP_3$ and diacylglycerol which leads to Ca^{2+} mobilisation and PKC activation. Indeed, it is believed that Con A-promoted cross-linking (achieved by

binding to and linking components of the T cell receptor through their carbohydrate moieties) is obligatory for the maximal mitotic response in T cells (Metcalfe et al, 1985).

Synergism between protein kinase C and Ca^{2+} signals has been amply demonstrated in the proliferation of murine splenocytes by the appropriate combinations of TPA and ionophore. However fura-2 studies demonstrate that pre-treatment of these cells with a higher concentration of TPA (10^{-7}M) resulted in a diminution of the Ca^{2+} signal induced by Con A (Tables 8 & 10, Figure 19). Since 4α 12,13 phorbol didecanoate, which lacks the ability to activate protein kinase C had no such antagonistic effect (Table 9) it appears that TPA via its protein kinase C enhancing activity can at high concentrations (10^{-7}M) suppress the lectin-induced increase in $[Ca^{2+}]i$ whilst at lower concentrations (10^{-9}M) can synergise with A23187 to induce proliferation of murine splenocytes. A similar situation exists in antigen-stimulated RBL basophilic leukaemia cells. Thus, A23187 and TPA synergise in producing a secretory response whilst TPA at higher concentrations suppresses the antigen-activated mobilisation of Ca^{2+}. In this manner, TPA can act either as a potentiator or inhibitor of secretion, depending on its concentration and when it is added (Di Virgilio et al, 1984). There are additional reports in the literature that TPA can antagonise the Ca^{2+} signal generated by various stimuli in different cells. Although TPA is thought to exert its affects on the $[Ca^{2+}]i$ via protein kinase C activation in all cases, the way in which Ca^{2+} homeostasis is affected differs. Thus, TPA is believed to stimulate Ca^{2+} efflux from human platelets to restore $[Ca^{2+}]i$ to resting levels following activation. PKC activation leads to Ca^{2+} extrusion probably by Ca^{2+}-ATPase and possibly by enhanced Na^{+}-Ca^{2+} exchange (Pollock et al, 1987). In contrast, TPA is thought to mediate its effects on Ca^{2+} homeostasis in PC12 and RINm5F cells by inhibition of the voltage-gated Ca^{2+} channel. Thus, in these two cell types, TPA (presumably via PKC activation) inhibits the cytosolic-free

Ca^{2+} concentration rise induced by depolarizing agents (Di Virgilio et al, 1986). Other reports suggest that TPA interferes in the stimulus-response sequence at a very early point which precedes the mobilisation of calcium. Thus, it is possible that the antagonistic effect of TPA on fMet-Leu-Phe-stimulated Ca^{2+} mobilisation in rabbit neutrophils is due to phosphorylation of proteins, such as the receptors themselves or the G proteins of the receptor coupling systems which subsequently inhibits the generation of the signal, $InsP_3$, responsible for the mobilisation of Ca^{2+} (Naccache et al, 1985). This particular hypothesis is substantiated by other studies which suggest that phorbol esters disrupt coupling of the activated G protein to phospholipase C (Muldoon et al, 1987; Smith et al, 1987). Furthermore, it seems likely that this phorbol ester-mediated effect may mimic a negative feedback signal induced by protein kinase C activation stimulated by DG generated upon activation of phospholipase C. Thus it is suggested that PKC activation by Ca^{2+} and DG may be responsible for the normal decrease in $InsP_3$ release, possibly by a modification resulting in the down-regulation or inhibition of the G protein responsible for relaying the mitotic signal from the cell surface receptor to the phospholipase C activity (Muldoon et al, 1987).

TPA pre-treatments have been demonstrated to depress Con A induced increases in $[Ca^{2+}]i$ but rather than inhibiting the mitogenicity of Con A, TPA enhanced the lectin stimulated DNA synthesis in murine splenocytes (Figure 20).

Since the inactive phorbol ester, 4α 12,13 phorbol didecanoate had no such effect it implies that PKC activation is responsible for the enhancement of DNA synthesis in lectin-stimulated cells. These results reaffirm the hypothesis that the Ca^{2+} signal alone is insufficient to trigger splenocytes to divide following stimulation, since the magnitudes of the rises in $[Ca^{2+}]i$ induced by Con A alone, or Con A and TPA in combination (Figure 19) did not correlate with their relative mitogenicity in the

[^{3}H]-thymidine assay (Figure 20). Thus, rises in [Ca^{2+}]i cannot by themselves account for the stimulation of DNA synthesis in murine splenocytes. However, since the Ca^{2+} signal was not completely abolished by pretreatment with TPA the hypothesis that PKC activation and elevated [Ca^{2+}]i act synergistically to induce proliferation in murine splenocytes remains viable.

In summary therefore, Con A stimulation of murine splenocytes is believed to lead to both PKC activation and an early [Ca^{2+}]i increment, subsequent cellular responses are set in motion ultimately leading to DNA synthesis. These two signals are assumed to be generated by hydrolysis of PIP_2 following lectin induced phospholipase C activity, under normal conditions this activity may be down-regulated by a negative feedback loop in which PKC disrupts the coupling of the activated G protein to phospholipase C. Generation of $InsP_3$ and DG will therefore cease together with the subsequent Ca^{2+} mobilisation and PKC activation and the proliferative response will therefore wane. However, when cells are pretreated with TPA, the subsequent protein kinase C activation will down-regulate the generation of $InsP_3$ and DG induced by Con A stimulation and therefore the Ca^{2+} signal will be impaired but whilst the physiological activator of PKC, DG may be suppressed, the continued presence of TPA which also activates PKC will bypass this negative feed-back loop and ultimately lead to proliferation. This super-stimulation of PKC by phorbol ester more than compensates for the reduced Ca^{2+} signal since splenocytes pre-treated with TPA demonstrate a greater increase in DNA synthesis than cells stimulated by Con A alone (Figure 20). Nevertheless, since TPA alone does not match the mitogenicity of Con A it reiterates the hypothesis that both PKC activation and an increase in [Ca^{2+}]i are obligatory for murine splenocyte proliferation.

One of the additional effects of protein kinase C activation appears to be the activation of the amiloride-sensitive Na^+/H^+ antiport leading to cytoplasmic

alkalinization (Grinstein et al, 1985). In the present study, amiloride, an inhibitor of Na^+/H^+ exchange (Besterman et al, 1985) significantly inhibited [^{3}H]-thymidine incorporation into Con A stimulated murine splenocytes in a dose dependent manner (Figure 21).

An increase in pHi in response to Con A was detected using the fluorescent probe BCECF confirming other studies in which lectin stimulation of lymphocytes caused cellular alkalinzation (Gerson et al, 1982; Metcalfe et al, 1985). The pHi increase in response to Con A stimulation was slower than the Ca^{2+} signal and was only fully developed after ten minutes (Figure 22). Since, the co-mitogenic concentration of TFP (10^{-9}M) produced a comparable pHi increase it appears that protein kinase C activation does lead to cytoplasmic alkalinization probably due to stimulation of the Na^+/H^+ antiport in splenic lymphocytes. The Con A induced increase in pHi might result from Na^+/H^+ exchange initiated by protein kinase C activation or calci-calmodulin activation dependent on the earlier Ca^{2+} signal (Villereal et al, 1985). Certainly in antigen-stimulated T cells of the leukaemia line, HPB-ALL the rise in pHi was dependent on the presence of extracellular Ca^{2+}, suggesting that an elevation of $[Ca^{2+}]i$ mediates the stimulation of the antiport (Rosoff and Cantley, 1985). Many other agents that activate Na^+/H^+ exchange also induce an increase in cytoplasmic free Ca^{2+}. The change in $[Ca^{2+}]i$ generally precedes the activation of the antiport (Hesketh et al, 1985) which suggests a causal relationship. The effects of A23187 on cytoplasmic pH in rat thymocytes, an agent chosen to define the role of Ca^{2+} in the regulation of the Na^+/H^+ antiport, suggest that stimulation of Na^+/H^+ exchange is due at least in part, to cell shrinking and does not require stimulation of PKC (Grinstein and Cohen, 1987). Whilst it is clear that Con A stimulation of murine splenocytes leads to cytoplasmic alkalinization it is as yet unclear whether it is an obligatory signal in the series of events triggered by ligand binding which result in proliferation. Thus, it may represent a passive accompaniment intended to increase

H^+ ejection in anticipation of an increased metabolic rate or it might increase the osmotic content of the cells leading to cell swelling which might be an early requirement for cellular growth (Gelfand et al, 1987). Amiloride inhibition of DNA synthesis in lectin stimulated splenocytes seems to imply that enhanced Na^+/H^+ exchange leading to an increase in pHi might be an obligatory signal. However, amiloride is non-specific and has been demonstrated to block Ca^{2+}/Na^+ exchange (Kaczorowski et al, 1985), to inhibit protein synthesis (L'Allemain et al, 1984) and growth factor receptor tyrosine kinases (Davis & Czech, 1985). Intriguingly, when more potent and specific structural analogues of amiloride were investigated for their effects on human peripheral lymphocytes stimulated with phytohaemagglutinin, it was found that concentrations of the analogues 10 times higher than those required to inhibit Na^+/H^+ exchange had no effect on proliferation. This implies that neither activation of the antiport nor the associated intracellular alkalinization are essential for the proliferation of human T lymphocytes (Mills et al, 1986). Thus, whilst Con A does indeed initiate an increase in pHi in murine splenocytes it is as yet unclear whether it is an obligatory signal for proliferation to occur. Stimulation of Na^+/H^+ antiport is only one of many possible targets of protein kinase C, thus protein kinase C has a potential role in the control or modulation of many metabolic processes (for review see Kikkawa and Nishizuka, 1986).

In summary, both protein kinase C activation and a Ca^{2+} signal are implicated in the Con A stimulation of splenocytes. The Na^+/H^+ antiport may be a target protein for PKC, stimulation of the antiport leads to an increase in pHi which although monitored following Con A stimulation has not yet been irrevocably established as an essential signal for DNA synthesis to occur. The generation of pHi and Ca^{2+} signals in response to lectin binding is consistent with the hypothesis that both signals are generated following PIP_2 hydrolysis; $InsP_3$ thus produced can release intracellular Ca^{2+} whilst diacylglycerol may activate PKC which subsequently causes

the pHi increase.

Another intracellular messenger, ornithine decarboxylase (ODC) has been implicated in both T lymphocyte proliferation and in the generation of Ca^{2+} signals in other cells (see Section 1.7). However, preliminary studies with the ODC inhibitor DFMO (Tables 11 and 12) failed to resolve whether or not ODC plays a role in murine splenocyte proliferation since the inhibitor appeared to be lacking in activity (see Chapter 3).

Having delineated the mechanism of Con A induced murine splenocyte proliferation as far as possible, the effects of known physiological modulators of immunological proliferation were investigated for their ability to interfere with the various steps of the outlined mechanism. Of the many hormones known to modulate the immune system, regulation by gonadal steroids is a well documented fact. This contention is supported by both clinical and experimental evidence (Grossman, 1985) (see Section 1.7). Despite the controversies which exist in the literature most agree that sex steroids depress the cell-mediated immune response (Grossman, 1985; Myers et al, 1986). With a view to determine the mode of action by which sex steroids modulate the cell-mediated immune system, their effects on T cells, the major functional units of the cellular immune system were investigated. Thus, the ability of sex steroids to inhibit Con A-induced murine splenocyte proliferation (an experimental model considered to represent the proliferative aspect of the immune response) was examined. The results of this study demonstrate clearly that sex steroids and certain of their metabolites are able to inhibit mitogen-induced blast transformation (Figures 23 and 24) and therefore confirm observations made in other T-lymphocytes (Schiff et al, 1975; Mendelsohn et al, 1977; Neifeld et al, 1977; Wyle and Kent, 1977; Homo et al, 1980). Since inhibition of lymphocyte stimulation is only observed at pharmacological concentrations of sex steroids together with the fact that most studies indicate that steroid receptors are present only within the epithelial reticulum of the lymphoid organ

(Grossman et al, 1979) it suggests that the inhibitory effect of sex steroids on splenocyte proliferation is the result of a nonspecific, nonreceptor mediated event.

This hypothesis is further substantiated by the finding that α-oestradiol, a relatively rare and physiologically inert female sex steroid which does not bind to oestrogen receptors in target tissue (Noteboom and Gorski, 1965) is an equally effective inhibitor of lectin-induced proliferation.

There is a substantial amount of literature describing non-receptor-mediated actions of steroid molecules, many of these 'direct' effects of steroids relate to changes in membrane function, for a review see (Duval et al, 1983). Because of the analogy between the structure of the cholesterol molecule and that of steroids it was suggested that steroids could be inserted vertically in the phospholipid bilayer instead of the cholesterol moiety (Willmer, 1961). However it is now believed that steroids are not really incorporated in the membrane but lie flat or on their edge on the surface of the phospholipid polar heads (Khaiat et al, 1975). Accumulation of these hydrophobic steroid molecules around the membrane together with their interaction with membrane components is likely to produce alterations in membrane properties especially at concentrations above 10^{-5}M (Read & McElhaney, 1976). The characteristics of these interactions between steroids and the membrane are different from those of usual steroid-receptor associations (Duval et al, 1983); moreover the affinity of these compounds for the membrane site appears relatively weak since most steroids are only active at pharmacological concentrations (Godeau et al, 1978). Steroid recognition sites may be formed by phospholipids or phospholipid clusters. This hypothesis is based on the similarities demonstrated between the effects of steroids and anaesthetics on the membranes. Administration of an anaesthetic molecule or a steroid will induce a change of this lipid core towards a liquid state and thus alter functions of certain membrane proteins (Lee, 1976). The anaesthetic properties of steroids have been recognised for a long time; (Selye, 1941). The structure-activity relationship of

steroids with anaesthetic activity is not clearly defined although it is believed that only hormonally active steroids have an anaesthetic effect (Selye, 1941) which might explain why testosterone glucuronide, a conjugated metabolite of testosterone and cholesterol had no inhibitory effect on lymphoblast transformation (Figures 23 and 25).

Interestingly, not only do Ca^{2+} ions play an important role in controlling the physical property of the lipid and anaesthetic action but they also appear to be involved in steroid membrane interactions (Papahadjopoulos, 1972). Indeed, steroids (although not sex steroids) cause enhanced $^{45}Ca^{2+}$ uptake into mouse thymocytes (Homo & Simon, 1981). Testosterone (10^{-4}M) had no effect on the lectin-induced Ca^{2+} signal (Table 13) implying that its inhibitory action on murine splenocytes must be mediated elsewhere. Since high concentrations of sex steroids (10^{-4}M) have been shown to inhibit glucose transport within 1-2 minutes of administration in adipocytes (Livingston and Lockwood, 1975) it is possible that by interfering with glucose transport following lectin stimulation, testosterone inhibits murine splenocyte proliferation. Another direct effect of steroids rather than by a classical receptor-mediated event is their reported rapid inhibition of nucleoside transport in isolated mouse thymocytes. Sex steroids at high concentrations (10^{-5}M and above) exerted a rapid inhibition of tritiated uridine uptake (Gagne et al, 1980). These rapid effects on precursor nucleoside pools could explain the inhibitory effect of sex steroids on lectin-induced stimulation, since testosterone (10^{-4}M) caused a significant inhibition of DNA synthesis in murine splenocytes even when administered 44 hours following Con A treatment (Figure 26).

These *in vitro* studies demonstrate that the inhibitory effects of sex steroids are only observed at pharmacological concentrations. However there is a large volume of literature which suggests that gonadal steroids interact with the immune system under physiological conditions (see Section 1.7). The evidence is based on several

observations including, the sexual dimorphism which exists in the immune response, alterations in the immune response noticeable during pregnancy and the presence of specific receptors for gonadal steroids in the organs responsible for the immune response (Grossman, 1985). Results from this study demonstrate that gonadectomy does indeed have dramatic effects on the lymphoid organs of mice. Orchidectomy resulted in significant increases in both the spleen and thymic indices (Figure 28) whereas ovariectomy only caused thymic enlargement (Figure 27). These observations confirm earlier studies (Dougherty, 1952; Chiodi, 1976) and imply that the gonadal steroids, particularly testosterone are involved in involution of the lymphoid organs. Indeed, regeneration of the thymus after orchidectomy has been shown to be inhibited in a dose-related fashion by testosterone implants which release physiological concentrations of testosterone. These findings illustrate the important physiological link between the endocrine and immune systems (Greenstein et al, 1986; Fitzpatrick & Greenstein, 1987).

Orchidectomy did not appear to potentiate T cell function since splenocytes derived from orchidectomised mice responded to lectin stimulation in a comparable fashion as cells from control animals (Figure 29). In contrast, preliminary studies demonstrated that ovariectomy impaired the spleen cell mitogen response (Figure 30) implying that oestrogen potentiates T cell function in the intact animal. Interestingly, however, there are reports in the literature which suggest that females have decreased cellular immune responses as compared to males due in part to the presence of oestradiol (Huber et al, 1982) (see Section 1.7). Moreover, it is suggested that oestradiol rather than directly suppressing T cell function may depress the cell-mediated immune response indirectly through the generation of thymic serum factors (Grossman et al, 1982; Grossman & Roselle, 1983; Grossman, 1985; Myers et al, 1986). Furthermore, it is believed that removal of sex steroids in the male rat by castration results in an increase in thymus gland size and in the production of a serum

substance which enhances the response to the mitogen, Con A. Administration of physiological levels of oestradiol (it was suggested that testosterone is aromatized into oestradiol in the male rat) reversed these effects. Since thymectomy abolished the enhancing affects of castration on T cell transformation the thymus is likely to be the source of the active serum factor (Grossman et al, 1982). Indeed, the hypothesis is that oestradiol interacting with receptors in the thymic epithelial reticulum depresses the production and/or release of a thymic source of a serum factor that enhances proliferation induced by Con A. However, preliminary investigations in this study dramatically demonstrated that mouse serum fractions from any source (viz. control, gonadectomised and sham-operated animals) and at any concentration tested failed to support the enhanced DNA synthesis in murine splenocytes, normally achieved by lectin stimulation (Figure 31). Moreover, it is apparent that not only does mouse serum not support splenocyte proliferation but it actually inhibits in a dose-related fashion DNA synthesis in murine splenocytes stimulated with Con A in the presence of 10% foetal calf serum (FCS) (Figure 32). These results are substantiated by reports which suggest that mouse serum does not support a primary *in vitro* immune response to sheep red blood cells (Mishell & Dutton, 1967). In addition, there are indications that mouse serum exerts an inhibitory effect on the immune response in cells cultured in foetal calf serum supplemented media. A similar inhibition was obtained with gerbil, rat and guinea pig sera but not with human or rabbit serum (Veit & Michael, 1973; Schreier & Nordin, 1977). Indeed, many investigators using the immune response *in vitro* (the proliferative aspect of which is considered to be mimicked by lectin stimulation), agree that FCS has a pivotal role (Schreier & Nordin, 1977). Serum, as a medium supplement supplies macromolecules which are protective or promote growth in some ill-defined manner, small molecules, eg. nucleosides, vitamins, hormones and co-enzymes which supply essential trace nutrients not present in the medium, factors (including antibodies) which neutralise or combine with the

stimulant and foreign antigens (Ling and Kay, 1975). The heterologous sera (FCS) used in this study obviously provided a variety of potential antigens and factors which were critical in promoting/supporting murine splenocyte proliferation. Despite its widespread use it is difficult to reconcile in the light of these findings how this lectin-induced *in vitro* proliferative model, which depends on heterologous serum-supplemented media can in reality provide information relating to the *in vivo* murine immune response. Results of these and similar *in vitro* experiments should be interpreted cautiously and critically when delineating the immune response in the intact animal, since it is clear that no simple extrapolation from the *in vitro* data can be made.

These studies with mouse serum fractions were unable to shed any light on the presence or absence of thymic serum factors which might mediate the effects of gonadal steroids on the immune system. However of all the sera types tested, female 'control' serum alone significantly enhanced DNA synthesis (albeit at a severely reduced level when compared with FCS supplemented cultures) in response to lectin in murine splenocytes derived from female animals (Figure 31). These findings taken together with earlier experiments which demonstrated that ovariectomy impaired the spleen cell mitogen response, suggest that oestrogen potentiates rather than suppresses T cell function. This hypothesis is in stark contrast to reports in the literature which suggest that females have enhanced humoral reactivity but decreased cell-mediated immunity compared to males (Ahlqvist, 1976; Bell, 1981; Huber et al, 1982; Myers et al, 1986). Nevertheless, controversy exists in the literature and there are other reports, in agreement with these preliminary findings, which indicate that females are reported to have a greater capacity for cellular immunity than males, (Eidinger and Garrett, 1972). Thus, females reject tumours and homografts with greater efficiency than males (Graff et al, 1969) and cells derived from females perform better in mixed lymphocyte cultures than cells derived from males (Lieber et al, 1969). A more recent study suggests that oestradiol treatment decreases a subpopulation of T cells (Lyt-2^+)

in the thymus and in the spleen whereas testosterone maintains this subpopulation of suppressor T cells (Ahmed et al, 1985). Oestradiol receptors have also been demonstrated on OKT-8^+ peripheral T cells (analogous to murine Lyt-2^+ cells), which have suppressor/cytotoxic function (Cohen et al, 1983). Thus, it is possible that the enhanced cellular immunity in females may relate in part to alterations induced by oestradiol in the number and function of suppressor cells (Ahmed et al, 1985). The tentative hypothesis based on preliminary investigations in this study that oestradiol potentiates T cell function could thus be due to the inhibition of the activity of suppressor T cells by oestradiol rather than to a direct mitogenic effect.

In contrast, the manner in which androgens modulate the immune system is difficult to discern, castration causes both thymus gland and spleen enlargment although the spleen cell mitogen response, on a cell for cell basis for lymphocytes derived from castrate animals was not significantly different from controls. This may be interpreted to mean that castration does not result in a net augmentation of individual T cell function, at least under the model system employed. However, the *in vivo* immune response of castrated animals may be enhanced due to an absolute increase in the number of lymphoid cells able to respond. Indeed, castration of males is believed to result in improved *in vivo* immune function (Graff et al, 1969; Castro & Hamilton, 1972).

The observation made in this study, that the inhibitory effect of sex steroids *in vitro* on murine splenocyte proliferation are only noticeable at high concentrations does not necessarily preclude a role of these hormones *in vivo* under some circumstances. Thus, progesterone concentrations close to 10^{-5}M may occur within human trophoblastic tissue and may therefore play a role in the failure to reject the embryo (Siiteri et al, 1977). Whilst only pharmacological concentrations of sex steroids exert an inhibitory effect on isolated murine splenocytes stimulated to divide by lectin, it is evident that physiological levels of sex steroids do modulate the immune

response *in vivo*(Grossman, 1985). This difference may be accounted for when it is understood that the sex steroid receptors are most likely present in the epithelial reticulum of the lymphoid organ (Grossman et al, 1979a,b) and that these non-lymphoid cells are eliminated in the preparation of lymphocyte suspensions (Sasson & Meyer, 1981). Since lymphoid reticuloepithelial cells not only possess sex steroid receptors but also undergo hypertrophy following gonadectomy and marked alterations following treatment with gonadal steroids, it is conceivable that such changes are mediated by steroid-receptor interactions. It is also believed that these cells whilst undergoing hypertrophy also produce and release substances into the blood, thymic serum factors (Grossman & Roselle, 1983). Indeed it is believed that sex steroids function within the hypothalamic-pituitary-gonadal-thymic axis (HPG-thymic axis) (Grossman, 1985). Thus, gonadotropin releasing hormone (GnRH) from the hypothalamus stimulates luteinizing hormone (LH) release from the pituitary and LH stimulates sex steroid production. Through a negative feedback mechanism this causes GnRH release from the hypothalamus to be depressed and LH release from the pituitary to be inhibited. Increasing levels of sex steroids from the gonads depress thymosin release, whereas decreasing levels of sex steroid are thought to induce thymosin release. In turn increased levels of thymosin stimulate GnRH release. T cell function is modulated by various thymosins which stimulate maturation of the different T cell subpopulations (Grossman, 1985). These reproductive-immunological interactions are likely to be very complex. Indeed a recent paper suggests that a thymic factor inhibits the secretion of testosterone by rat Leydig cells *in vitro* (Pedernera et al, 1986). This report emphasises the bidirectional nature of the HPG-thymic axis.

Given the difficulties of interpretation associated with the Con A model referred to in the preceding section, the mechanism of action of a more physiological mitogen Interleukin-2 was investigated with a view to produce a more relevant *in vitro*

model to examine proliferation. Thus, exposure of Con A blasts to human recombinant Human IL2 (100 U ml^{-1}) stimulated cell proliferation (Figure 33). Nifedipine suppressed this response to IL2 (Figure 34) implying that a Ca^{2+} influx is necessary for cell division to occur. This is in agreement with Birx et al (1984) and Gearing et al (1985) who also observed a reduction in IL2-induced proliferation in T lymphocytes treated with calcium antagonists. However, measurement of $[Ca^{2+}]i$ in Con A blasts following IL2 stimulation, although complicated by pronounced fura-2 leakage failed to detect a distinct Ca^{2+} signal (Figure 35). Whilst calcium is relatively firmly established as a mediator in lectin stimulation of T lymphocytes (Tsien et al, 1982; Hesketh et al, 1983; Gelfand et al, 1984), there are conflicting reports as to whether IL2 interaction with its cell surface receptor generates a change in $[Ca^{2+}]i$. Thus, some workers are convinced that cytosolic Ca^{2+} does increase following IL2 administration (Gearing et al, 1985; Farrar et al, 1986; Rossio et al, 1986) whilst others have demonstrated that increases in $[Ca^{2+}]i$ are not associated with IL2-IL2 receptor interaction (Weiss et al, 1984; Mills et al, 1985b; O'Flynn et al, 1985; LeGrue et al, 1987). Since TFP the calmodulin antagonist also prevented proliferation of Con A blasts (Figure 36) it appears that IL2-provoked mitosis is Ca^{2+} dependent , with a subsequent calci-calmodulin mediated protein phosphorylation ultimately leading to mitosis. However, these results should be interpreted cautiously since both nifedipine and TFP only exerted their inhibitory action on IL2 induced proliferation at a concentration ($10^{-5}M$), ten times greater than the concentration required to suppress Con A induced murine splenocyte proliferation (Figures 34 and 3, 36 and 8). Thus, the effects may relate to non-specific metabolic actions rather than to an indication of Ca^{2+} dependency.

Attempts to correlate IL2 ligand-receptor interaction with phosphatidylinositol turnover are just as equivocal (see Section 1.7). Amiloride, an agent used to inhibit the Na^+/H^+ antiport (believed to be activated by PKC) inhibited

IL2-induced proliferation (Figure 37), suggesting that PKC activation mediated by DG one of the products of phosphatidylinositol hydrolysis may indeed be involved in stimulus-mitosis coupling. IL2 induced cytoplasmic alkalinization via the Na^+/H^+ antiport has been monitored in lymphocytes (Mills et al, 1985a) although no alterations in labelled inositol phosphates were detected (Mills et al, 1986) or in the intracellular distribution of PKC (Gelfand et al, 1987). In contrast, other workers suggest that the IL2 signal does involve the activation of inositol phospholipid turnover (Bonvini et al, 1986; Farrar et al, 1986) and PKC translocation (Farrar and Anderson, 1985; Evans & Farrar, 1987). Thus, the precise biochemical and molecular events associated with IL2 regulation of lymphocyte proliferation await clarification. In peripheral T lymphocytes IL2 does not directly stimulate cell division, thus an antigenic or mitogenic stimulus is first required for cellular activation and expression of IL2 receptors. IL2 then provides a second signal which enables progression of activated cells from G_1 to the replicative S phase (Stadler et al, 1981). Activation of lymphocytes by mitogenic lectins leads to proliferation, differentiation and secretion of lymphokines (Gillis, 1983). Mitogenic lectins or antigens in conjunction with monocytes initiate the sequential secretion of IL1 from activated monocytes and IL2 from activated T lymphocytes (Gillis, 1983). In a distinct activation pathway, mitogens or lectins promote IL2 receptor expression (Gillis, 1983; Robb, 1984) and binding of IL2 to this receptor then leads to cell proliferation. The lectin induced Ca^{2+} increase demonstrated in this study is believed to be required for IL2 production but not for IL2 receptor expression (Mills et al, 1985a). In the absence of a Ca^{2+} influx and subsequent IL2 production, lectins are unable to trigger DNA synthesis and cell proliferation.

Since the sex steroids inhibited IL2-induced proliferation at the same pharmacological concentrations necessary to suppress Con A-induced proliferation (Figure 38), it appears that Con A blasts are equally sensitive in terms of inhibition of

DNA synthesis as murine splenocytes. Thus, signalling of cells after the binding of IL2 to its receptor, one of the final steps in the interleukin cascade leading to lymphocyte proliferation is as vulnerable to sex steroid as is activation of lymphocytes by lectin. It seems unlikely on the basis of this preliminary evidence that sex steroids mediate their inhibitory effects on lymphocyte proliferation by blocking the production of IL2 by a T cell subset in an analogous fashion to glucocorticoid inhibition of T lymphocyte activation (Smith et al, 1977). Indeed, since similar concentrations of sex steroid also inhibit B lymphocyte proliferation (Figure 42) it seems likely that their effect is the result of a nonspecific non-receptor-mediated event. Possibly, the incorporation/interaction of sex steroids with membrane lipids leads to a modification of membrane properties (eg. fluidity, permeability and membrane enzyme activities) which in turn could abolish critical membrane events, such as inhibition of nucleoside or glucose transport, necessary for DNA synthesis to occur.

The activation of quiescent T lymphocytes requires two stimuli: binding and cross-linking of the T cell antigen-receptor complex by antigen-bearing accessory cells or mitogenic lectins (Zanders, 1985; Isakov, 1987), and the accessory cell-derived hormone, IL1 (Mizel, 1982). Of the two members of the IL1 family IL1α and IL1ß, only IL1ß induced a significant increment in DNA synthesis when administered alone (Figured 39 and 40) and neither IL1 enhanced the proliferation induced by low concentrations of Con A (0.35 $\mu g\ ml^{-1}$), presumably the dose of Con A chosen did not provide an adequate signal in the form of receptor cross-linking, although further investigations are required to substantiate this hypothesis. IL1 responsive lymphocytes bear a single class of high affinity receptors for IL1 (Mizel et al, 1987; Dower and Urdal, 1987). In the presence of IL1, the IL1 receptor undergoes extensive ligand-induced down regulation, the internalized IL1 receptor is not recycled but is either degraded or redirected to sites within the cell (Mizel et al, 1987). The

mechanism of IL1 action is unclear but does not appear to involve PIP_2 hydrolysis (Abraham et al, 1987), indeed the accumulation of relatively high levels of IL1 in the nucleus raises the possibility that IL1 may mediate its effects via interaction with nuclear receptors (Mizel et al, 1987).

The findings presented in this study emphasize the role of a rapid rise in $[Ca^{2+}]i$ and activation of protein kinase C as intracellular messengers in the signal transduction pathway following lectin stimulation of lymphocytes. Hydrolysis of PIP_2 is likely to be responsible for these two signals, since it yields IP_3 which mobilises intracellular Ca^{2+} and DG, which activates PKC. PKC may subsequently enhance Na^+/H^+ exchange leading to cellular alkalinization. The lectin seems to provide an additional signal, possibly receptor crosslinking which is obligatory for T cells to progress from G_0, the resting stage of the cell cycle into the G_1 phase and subsequent completion of the mitotic cycle. Transition of activated lymphocytes from G_1 into the S phase occurs as a result of IL2 production and binding to its high affinity receptors. The signals generated by IL2-IL2 receptor interaction remain elusive and await further clarification. Proliferation of T lymphocytes *in vitro* is vulnerable to pharmacological concentrations of sex steroids which mediate their effect probably by a non-specific, non-receptor-mediated event possibly leading to alterations in membrane properties. The mechanisms by which gonadal steroids modulate the immune system in the intact animal remain speculative and there are still many missing parts in the puzzle that must be resolved in the future.

APPENDIX

(A) Determination of Viability by Trypan Blue Exclusion

The number of viable splenocytes was determined by staining cell populations with trypan blue. Viable cells exclude the dye while nonviable cells take up the dye.

Equal volumes of spleen cell suspension and trypan blue saline solution (4 parts of 0.2% trypan blue with 1 part of 4.25% sodium chloride (w/v) were mixed and the number of unstained (viable) and stained (dead) cells were counted (at least 200 in total) within three minutes.

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