Stimulus-Mitosis Coupling In the Rat Thymic Lymphocyte

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A thesis presented for the degree of

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Quiescent thymocytes are stimulated to divide by a variety of compounds. These may be subdivided into four categories.

The first group includes parathyroid hormone, vitamin D_3 and its metabolites and raised extracellular calcium concentrations. Enhanced calcium influx provoked directly or possibly via cGMP production is the prelude to calmodulin activation which subsequently triggers cell arousal by a process which is independent of magnesium ions. High oestradiol concentrations by further increasing intracellular calcium concentrations beyond a threshold inhibit this response.

High extracellular magnesium concentration and magnesium-dependent agonists (e.g. isoprenaline) belong to a second group. These agents probably raise intracellular cAMP and magnesium levels following their combination with cell surface receptors. High concentrations of testosterone prevent this process although the mechanism is unknown. Modulation of calcium metabolism and calmodulin activation plays no part in their action.

The third group comprises agents which promote calcium influx or intracellular mobilization but to be effective extracellular magnesium is required. Thus low concentrations of alpha- and beta-oestradiol and tamoxifen elevate intracellular calcium and activate calmodulin which initiates the proliferogenic response(s). The calcium changes occur independently of gene derepression and protein synthesis.

The final category of compounds modify intracellular sodium concentrations. Ouabain and high extracellular sodium raise intracellular sodium. Thus by enhancing calcium mobilization and/or preventing its efflux a calmodulin dependent process ensues. At certain mitogenic concentrations magnesium is required during such events.

There are clearly two major pathways (controlled by intracellular calcium or magnesium concentrations) via which cells can proceed from quiescence to mitosis. Subtle interactions between the divalent cation homeostatic mechanisms may occur, influenced by cyclic nucleotides and monovalent cations. Abnormalities in any of the discrete or interrelated events governing the cell's divalent cationic status could alter proliferation and should be considered in any survey of normal (or abnormal) growth control.

Key words: Calcium
Magnesium
Calmodulin
Mitogenesis
Thymocyte Cultures

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1.0 STIMULUS RESPONSE COUPLING

1.1 THE MEMBRANE

Although the plasma membrane behaves as a boundary, it is not a totally inert barrier. In fact, the membrane is a delicately balanced functional organelle which plays an important part in regulating the overall behaviour of the cell. The different functions of the plasma membrane and the variations in properties between the membranes of different cell types are reflected in a corresponding complexity of structure and organisation.

1.1.1 Membrane Structure and Function

The Singer-Nicholson model of the plasma membrane views it as a central, liquid bilayer of lipid molecules that forms the backbone of the membrane and serves as its primary permeability barrier. The three classes of lipids found in the membrane, phospholipids (which comprise the majority), neutral lipids (mainly cholesterol) and glycolipids usually constitute up to fifty percent of the total plasma membrane mass. The bulk of the lipids exist in the bilayer with polar groups forming a hydrophilic exterior. The phospholipids are capable of lateral diffusion in two dimensions but rarely migrate from one monolayer to the other (Kornberg & McConnel, 1971). The other major component of the membrane is protein. Some proteins are 'extrinsic' in that they are superficially attached to or partially embedded in the lipid bilayer. The others are 'intrinsic' and extend across the bilayer forming an integral part of the structural mosaic of the plasma membrane, (Singer, 1974); see diagram (1).

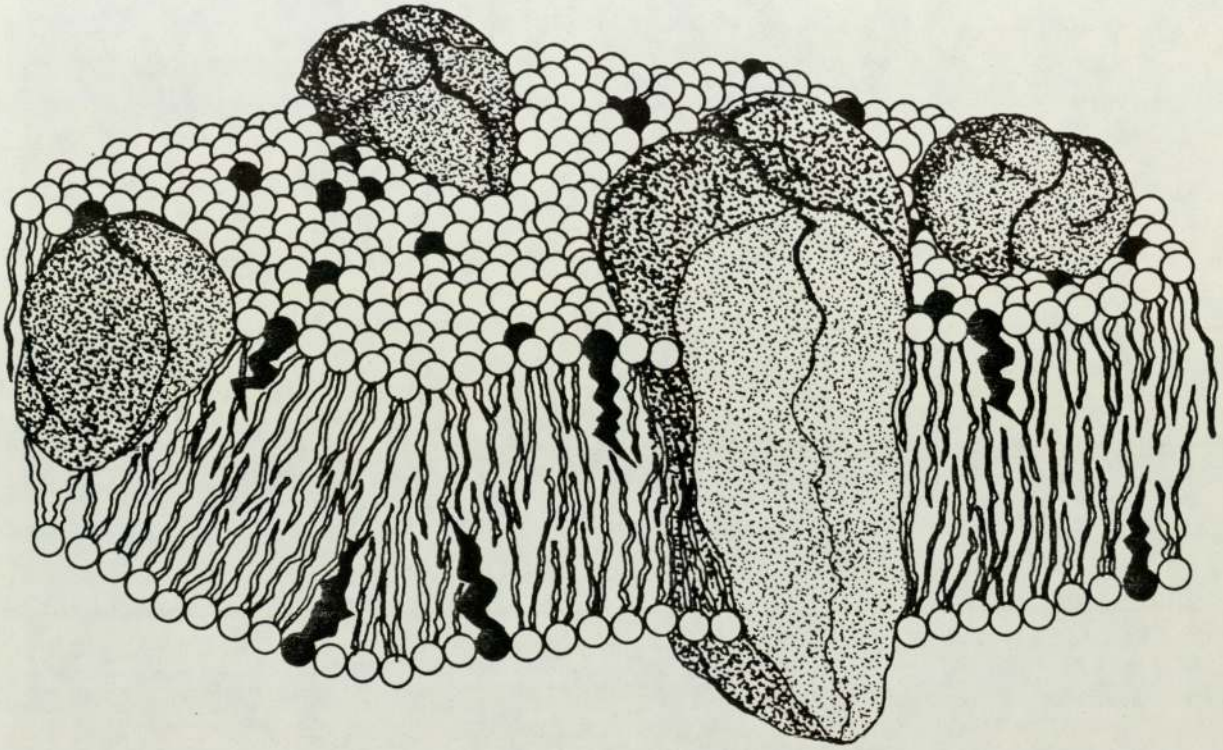


Diagram 1 Diagrammatic Representation of the Plasma Membrane "Fluid Mosaic".

The model shows a lipid bilayer composed of polar phospholipids and steroids (shaded molecules) in which is embedded a number of proteins. (from Singer & Nicholson, 1972).

Differences in chain length and degrees of unsaturation of the phospholipids are able to modulate the permeability of the membrane. In general, the presence of relatively short chains or highly unsaturated lecithins increases the permeability whereas the presence of cholesterol tends to decrease it (Bretscher & Raff, 1975). Similarly, some particularly hydrophobic protein components of the plasma membrane may be structural elements that contribute to its barrier properties. The three-dimensional structure of some relatively large membrane proteins may enable them to provide hydrophilic channels that extend from the extracellular to intracellular environment, while other proteins can behave as carriers (Diedrich, 1966). Although the carbohydrates of the plasma membrane rarely constitute more than ten percent of the total mass, they appear to be functionally very important. Glycosylation of some membrane proteins serves to prevent their diffusion back into the cytoplasm and thus to maintain specific proteins in appropriate membranes (Bretscher, 1973). The oligosaccharide portion of these glycoproteins (and the glycolipids) vary in their complexity and reside only at the outer surface of the membrane. Here they are responsible for much of the cells antigenic activity and also function as specific cell surface receptors (Critchley & Vicker, 1977).

1.1.2 Receptor Binding and Regulation

The effective expression of specific potential demands that the cell be responsive to the influence of its extracellular environment. Cells that are targets for particular external signals therefore contain specialised molecules (generally glycoproteins) that bind the signal molecule or ligand and consequently mediate its

cellular actions. Certain external ligands are able to permeate the plasma membrane (e.g. the lipid soluble steroid hormones) and thus are able to bind to specific cytosolic receptors. These may then be translocated to and become complexed with the nuclear chromatin which results in protein translation and mediation of the response (Jensen & DeSombre, 1973).

For non-penetrating ligands however, the receipt and transduction of external signals is largely a cell surface phenomenon and is dependent upon the existence of membrane associated receptors. Most of these receptors possess a random distribution in the surface and a lateral mobility which can be modified to different degrees by protein-protein interactions or the involvement of cytoskeletal elements (Nicholson, 1976). Microtubules reversibly anchor the receptors whereas the microfilaments are responsible for the systematic redistribution of surface receptors into clusters, often seen as a consequence of multivalent ligand binding and as a prelude to the appropriate cellular response (Edelman, 1976). However, regardless of the receptors' distribution, it is imperative that they have the ability to discriminate signal from 'noise'. Thus, the receptor must have an affinity for the hormone (or ligand) that is high in relation to the concentration of available hormone and must have an appropriately lower affinity for potentially misleading signals, (i.e. it must show specificity). The affinity of hormones for receptor is due to non covalent binding involving electrostatic interactions which include hydrogen bonding, hydrophobic interactions and van de Waals forces (Baxter & Funder, 1979). It is generally accepted that only fractional receptor occupancy is needed to elicit maximal response. The excess binding sites are generally termed

'spare receptors'. This terminology however, is ambiguous since these receptors are not superfluous but can in some way enhance the speed of the ligand response and increase cellular sensitivity to low concentrations of ligand (Baxter & Funder, 1979).

The sensitivity of the cells may also be modulated by ligands specific for a particular receptor. This self regulation can be achieved by modification of receptor numbers or alternatively by alteration of their affinities for ligand binding. Cells thus may respond to high doses of ligand by reducing the density of their receptors. Chronically elevated levels of insulin cause a decrease in the numbers of receptors to this ligand in all of its target cells (e.g. hepatocytes and adipocytes). This process is commonly termed down regulation (Chang & Polakis, 1978; Krupp and Lane, 1981). Similarly, epidermal growth factor (EGF) produces a reduction of up to 90% in the density of its own receptors when added to confluent fibroblasts (Das & Fox, 1978).

A related form of regulation is the acquisition of new receptor specificities as a consequence of ligand binding and by definition is termed up-regulation. There is a display of elevated numbers of functional acetylcholine receptors in muscle after loss of innervation, reflecting a compensatory positive feedback response (Gardner & Fambrough, 1982). Up-regulation and down-regulation are achieved by influencing the rates of internalization and degradation of hormone receptors or alternatively the rates of receptor synthesis and re-entry to the surface. In addition to altering receptor synthesis, there may also be a pool of newly synthesised receptors which is directly available for incorporation into the plasma membrane. In muscle such a population of receptors (for ACh) is at least 10%

as large as the surface ACh receptor total (Gardner and Fambrough, 1979; 1982).

A change in receptor affinity will also modulate the cellular sensitivity to ligands. The affinity of receptors appears not to be fixed and will decrease as occupancy of receptor increases. Insulin receptors may have a multimeric structure capable of binding several insulin molecules and when no site is occupied, all sites appear to have a high affinity for the ligand. Binding of insulin however so changes the conformation of the receptor that the affinity of the remaining sites falls and a fast dissociating state is induced (DeMeyts, Bianco & Roth, 1976). This phenomenon is known as 'negative cooperativity'. Receptors may exist in clusters prior to, or as a consequence of occupancy and such an induced low receptive state might therefore be transmitted to other receptors in the cluster, (Levitzki, 1974); this suggests that aggregation may be associated with a dampening of the effect which can thus prevent over-stimulation. This contrasts with the positive cooperativity of haemoglobin binding to oxygen where a greatly enhanced affinity is evident following the association of O_2 molecules.

Thus, fundamental biological processes may be regulated by altering the sensitivity of the cells to specific ligands at the surface membrane. This modulation may be a result of:-

1) influencing the metabolism of the receptors and hence the absolute numbers evident at the surface, and/or

2) modulating the affinity of existing receptors for a specific ligand.

1.2 TRANSMEMBRANE SIGNALLING

Ligand binding is merely the primary step of signal-response coupling. As a consequence of this, information must now be transmitted across the plasma membrane into the cell in order to trigger the appropriate response. The inward flow of information can be direct in nature, such as entry of signalling ligands into the cytoplasm, or indirect occurring through structural or enzymatical modifications within the membrane.

1.2.1 The Role of Calcium Ions

The concentration of free Ca^{++} ions in the cytosol appears to have a central role in signal transmission in many cells. It is significant thus, that the distribution of free ions across the cell membrane (which is essentially impermeable to Ca^{++}) is far from equilibrium, the concentration of intracellular calcium being normally low (10^{-7}M), while the extracellular level is much greater (10^{-3}M) (Blaustein, 1974). It is this large calcium gradient that provides for a rapid intracellular signalling system. Any alteration in membrane permeability thus, allows a rapid influx of Ca^{++} ions into the cytosol where it acts to trigger a wide variety of cellular processes. The calcium ion is uniquely suited to this role since it forms rapidly reversible cross linkages which subsequently modifies protein conformations. The structure of Ca^{++} - complexes varies from 6 - 12 coordinate, the geometry being irregular both in bond length and bond angle. This flexibility provides many opportunities for interaction with a number of cellular components within a wide variety of cell types (Levine & Williams, 1982).

1.2.1a Intracellular Calcium Regulation

Despite membrane discrimination restricting calcium entry the asymmetric distribution causes some passive influx of ions into the cell. The observed maintenance of the calcium concentration difference thus, dictates that there must exist specific mechanism(s) for the removal of intracellular Ca^{++}. Calcium influx is counteracted ultimately by two mechanisms associated with the plasma membrane, namely, Na^{+}/Ca^{++} exchange or by a calcium pump. It is generally assumed that most cells feature a calcium pump in their surface membrane but the prevalence of Na^{+}/Ca^{++} exchange in those cells which are unable to be stimulated electrically is still unclear. However, in cells where both mechanisms are evident (e.g. electrically excitable nerve axon cells) it is accepted that in the resting cell, efflux is predominantly powered by an ATP - driven pump. This calcium pump has been extensively studied in erythrocytes but most other cells or tissues exhibit a comparable extrusion system (Haynes, 1983; Barritt, 1981; Sarkadi, Enyedi, Szasz & Gardos, 1982; Chiesi & Inesi, 1981; Gimble, Goodman & Rasmussen, 1981; Stroobant, Dame & Scarborough, 1980). Binding of intracellular calcium to the pump in the presence of intracellular magnesium ions evokes an ATP - dependent phosphorylation of the transport enzyme which results in transmembrane calcium movement (Sarkadi, et al., 1982). Mg^{++} appears to increase the affinity of the enzyme for ATP and to increase its maximal rate of activity (Schatzman & Burgin, 1978). However, at increasing Mg^{++} concentrations, enzyme activity and its affinity for Ca^{++} appear to decrease (Klinger, Wetzker, Fleischer & Frunder, 1980) and thus it has also been suggested that magnesium may somehow be involved in the enhancement of pump dephosphorylation and hence continuous working of the pump (Sarkadi,

1980). The pump is also influenced by the action of the Ca^{++} - dependent regulatory protein, calmodulin (Klinger et al., 1980; Luthra, Watts, Scherer & Kim, 1980). This protein in erythrocytes increases the maximum transport rate of the pump and also increases the affinity for intracellular calcium ions (Vincenzi & Larsen, 1980; Carafoli, 1981; Muallem, & Karlish, 1982; Wetzker, Klinger, Cumme, Hoppe & Frunder, 1982). In the activated cell however, when internal calcium levels become elevated it is the second mechanism, a Na^+/Ca^{++} coupled exchange system, which becomes the predominant extrusion process (Dipolo & Beague, 1979). This coupled exchange is not entirely energy-independent since it relies upon an asymmetric distribution of sodium ions across the membrane which is itself maintained by an ATP - driven Na^+ pump (Blaustein, 1974). After extrusion, Na^+ can diffuse back into the cell either by an uncoupled passive pathway or via the forementioned Na^+/Ca^{++} exchanger. In the latter pathway entry of Na^+ is obligatorally coupled to the exit of calcium ions with a proposed Na^+/Ca^{++} stoichiometry of 3:1 (Blaustein, 1974) or 4:1 (Mullins, 1977). Mullins has proposed that the binding of 4 Na^+ to the carrier induces a reversible high affinity calcium binding site on the opposite side of the membrane, which precedes the subsequent reception, translocation and then dissociation of Ca^{++}. Decreased (Na^+/K^+)-ATPase activity would thus reduce the Na^+ gradient driving force for Ca^{++} extrusion, resulting in increased cytoplasmic calcium levels (Fleckenstein, 1977). It is accepted that such a process operates in most electrically excitable cells but it is thought by many authors that the observed external sodium (Na^+o)- dependent Ca^{++} efflux of non-excitable cells is not strictly Na^+/Ca^{++} exchange (Dani, Cittadini, Calviello, Festuccia & Terranova, 1978). Calcium efflux it is suggested, is probably activated by extracellular

sodium but energised directly by ATP (Borle, 1981). Krieger & Tashjian (1980) however, have shown that parathyroid hormone stimulates Ca^{++} release from bone cells via Na^{+}/Ca^{++} exchange mechanism dependent upon the Na^{+} gradient. A similar Na^{+}/Ca^{++} exchange system has also been demonstrated in isolated hepatocytes (Kraus - Friedmann, Biber, Murer & Carafoli, 1982).

Maintenance of low cytosolic free calcium can, in addition to the two plasma membrane features, be achieved by Ca^{++} transport processes operating in the mitochondrial and endoplasmic reticular membranes. Except in erythocytes and skeletal or cardiac muscle fibres, the mitochondria represent the main controllers of cytosolic calcium activity (Carafoli, 1979). It is generally agreed that the influx of Ca^{++} into mitochondria is electrophoretic in response to the negative membrane potential generated inside by either the hydrolysis of ATP or electron transport, as shown in diagram 2 (Racker, 1980; Fiskum & Lehninger, 1982). Charge compensation is achieved by respiration - dependent ejection of two hydrogen ions ($2H^{+}$). Simultaneous transport of phosphate occurs via an independent $H_2PO_4^{-} - H^{+}$ symporter in response to the alkaline inside transmembrane pH gradient created (Fiskum & Lehninger, 1982). The ability of mitochondria to act as a 'sink' to remove calcium from the cytosol stems from its unlimited capacity to sequester calcium ions by precipitation as rapidly exchangeable phosphate salts (Brierley & Slautterbach, 1964).

The endoplasmic reticulum and sarcoplasmic reticulum fulfil a similar Ca^{++} storage function serving to diminish any increased cytosolic levels of calcium. Calcium transport in reticulum has been widely studied in mammalian skeletal muscle (Miyamoto & Racker,

MITOCHONDRIAL REGULATION OF INTRACELLULAR CALCIUM

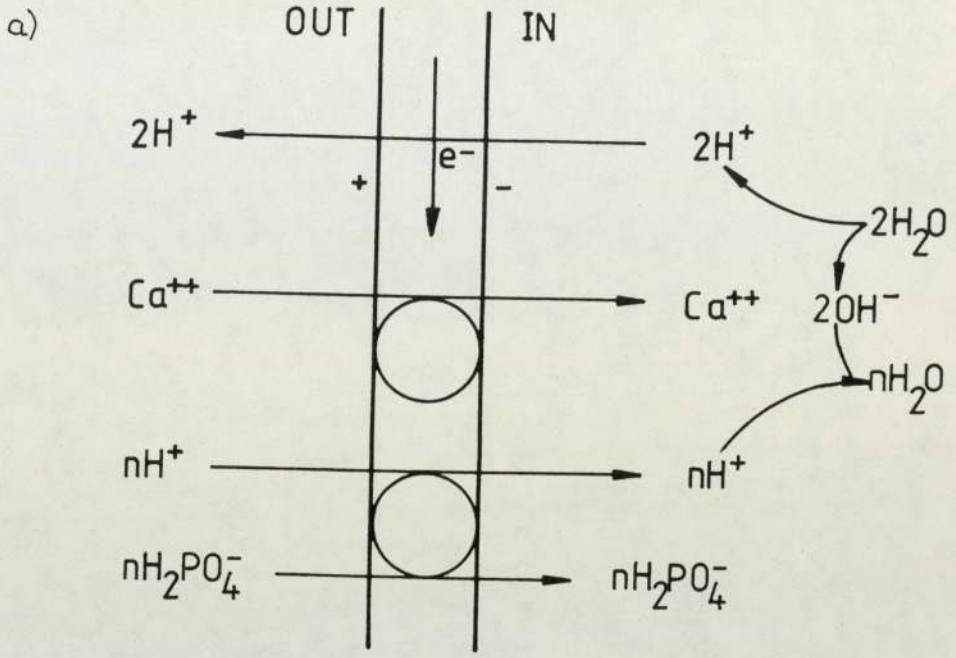
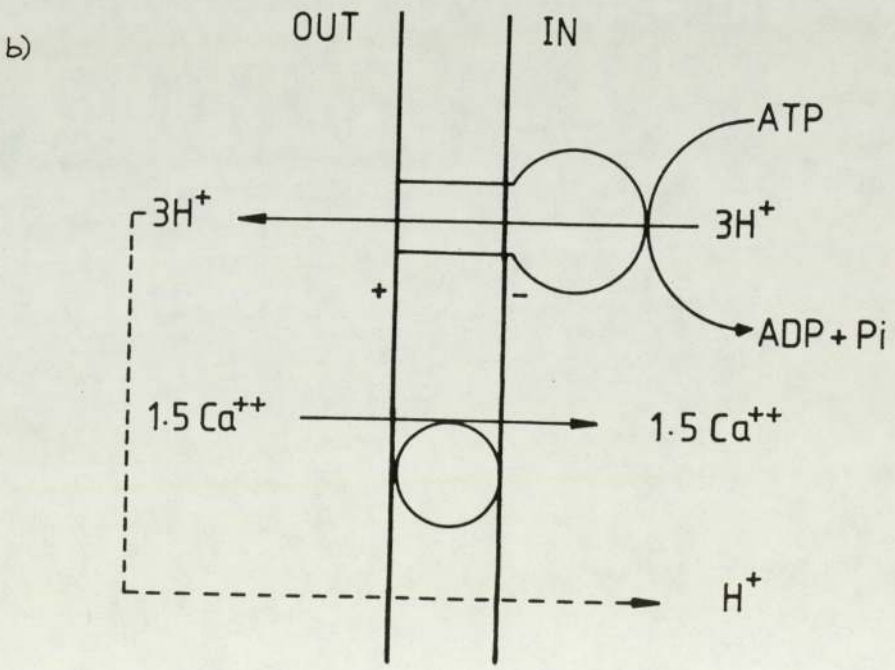


Diagram 2 Calcium Sequestration by the Mitochondria.

a) Respiration-dependent mitochondrial uptake of Ca^{++}.

b) ATP hydrolysis-dependent mitochondrial uptake of Ca^{++} (utilized when respiration is interrupted).



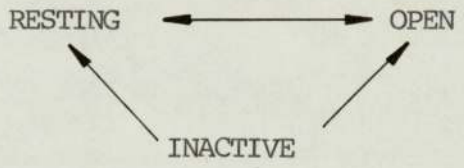
1982; Haynes, 1983; Takenaka, Alder & Katz, 1982; Takisawa & Makinose, 1981; Yamamoto & Kasai, 1982a; b; c) and cardiac muscle (Braunwald, 1982; Kirchberger & Antonetz, 1982; Carafoli, 1981). Calcium uptake by the sarcoplasmic reticulum is driven by a (Ca^{++}/Mg^{++})-ATPase pump in which the uptake of 2 mol of calcium is coupled with the hydrolysis of 1 mol ATP (Takenaka et al., 1982). The normal transport cycle has been characterized; binding of two Ca^{++} and Mg-ATP to external sites with high affinity promotes enzyme phosphorylation and inward translocation of the Ca^{++} laden sites. Ca^{++} is subsequently released inward and ADP outward. Dephosphorylation of the translocator is achieved by Mg^{++} binding to an allosteric regulatory site or in some cases K^{+} and H^{+} binding and consequently the translocator is reorientated in the membrane. Dissociation of Mg^{++} (or K^{+} and H^{+}) completes the cycle (Haynes, 1983). The stimulation of Ca^{++} uptake by cardiac sarcoplasmic reticulum (S.R.) may also involve a complex calmodulin - dependent system operating in parallel with a regulatory process dependent on cAMP and a specific protein kinase. Together it seems, they phosphorylate phospholamban resulting in ATPase activation (Carafoli, 1981; Lamers, Stinis & DeJonge, 1981). However, although calmodulin has been detected in S.R. from skeletal muscle it appears not to be involved in the stimulation of this Ca^{++}-ATPase pump (Carafoli, 1981). Ca^{++} transport has now been documented in the endoplasmic reticulum (E.R.) of various non-muscle cells and it appears that uptake proceeds with a high affinity for Ca^{++} but is much slower and the capacity of storage much less than in S.R. (Borle, 1981). As in cardiac S.R. there are also two regulatory pathways for Ca^{++} uptake in the E.R., one c-AMP-dependent and the other calmodulin-dependent which similarly may be operating in parallel (Carafoli, 1981).

Thus a host of extrusion and sequestration mechanisms tightly control intracellular Ca^{++} levels at very low concentrations. Nevertheless changes in membrane permeability, the consequence of ligand-receptor binding, can temporarily overwhelm these homeostatic devices. The resultant increase in Ca^{++} above the usual low background can thus serve as the stimulus for cellular activity.

Specific mechanisms exist in the cells which serve to elevate the concentration to the appropriate levels. The plasma membrane is essentially an ion impermeable lipid bilayer. In resting cells however, some Ca^{++} does slowly move down an electrochemical gradient across the membrane into the cytosolic environment. This steady state calcium influx is a function of the extracellular Ca^{++} concentration and is a saturable process suggesting that it is a carrier - mediated transport system (Borle, 1981), which is specific for Ca^{++} since Mg^{++} is not a competitive inhibitor (Borle, 1971). This diffusion process appears to be facilitated by the existence of a number of macromolecular proteins that traverse the plasma membrane and act as selective pores for the ionic transport across the barrier.

In addition there are also ion channels whose opening and closing can be clearly regulated by specific stimuli. There are two types of calcium entry routes in the plasma membrane which are controlled thus; the voltage dependent channels (VDC) and the receptor operated channels (ROC), (Bolton, 1979). The VDC's have within them voltage sensors coupled with activation gates which determine whether or not the channel permits ionic translocation (Glossmann, Ferry, Lubbecke, Mewes & Hofmann, 1982; Reuter, 1983). The large transmembrane concentration gradient of Ca^{++} dictates that the opening of such a channel (with the appropriate selectivity filters) will

result in elevated cytosolic calcium levels. Membrane depolarization induces calcium entry either via the fast sodium entry channel or the slow calcium specific channel (Triggle, 1980; Nayler & Grinwald, 1981). The calcium channel is assumed to be present in all cell types but has only been extensively studied in electrically excitable tissue. The "voltage dependent" calcium channels of the sarcolemma can exist in at least three states; open, inactive and resting. The transitions between these states (visualised below) are voltage dependent and time dependent and are controlled by two types of gates.



The activation gates lie on the extracellular side of the membrane and the inactivation gates lie on the cytosolic side (see diagram 3). In the resting channel the activation gates are closed and the inactivation gates are open. Depolarization of the membrane induces the opening of the activation gates, thus allowing calcium influx. Membrane depolarization also causes the inactivation gate to close, though at a slower rate than that at which the activation gates had opened. In this inactive state the channel can no longer respond to physiological depolarizing stimuli, i.e. the channel becomes refractory (Katz, Messineo & Herbette, 1982). The activation gate recloses and the inactivation gate reopens when the interior of the cell has regained electronegativity and the resting transmembrane potential has been restored. The channel thus resumes its resting state. Many calcium channels in

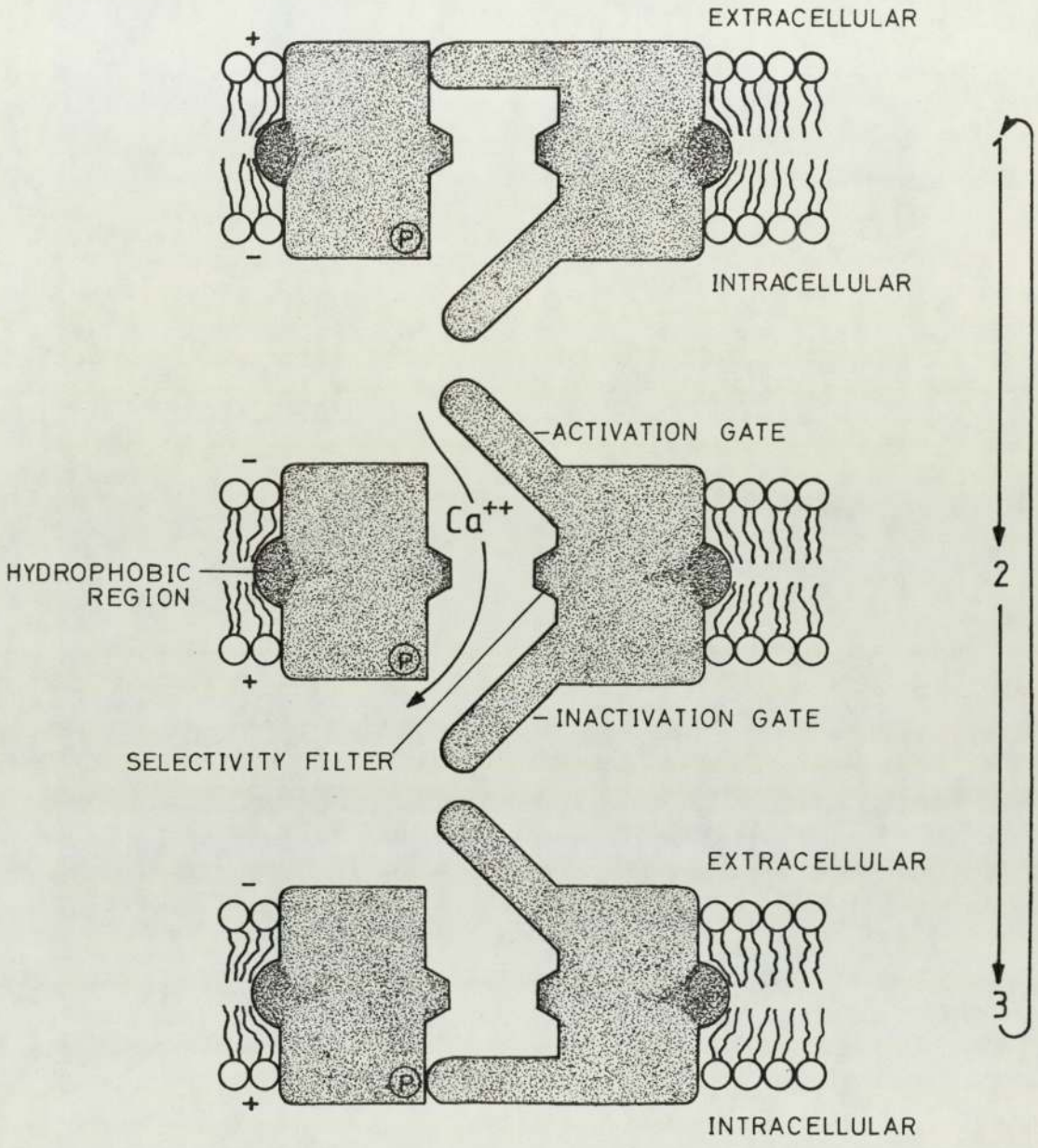


Diagram 3 Diagrammatic Model of the Calcium Channel in the Plasma Membrane.

Part 1 represents the resting state; part 2 is the activated (or open) state and part 3 depicts the inactivated or refractory phase of the cycle. The symbol P represents a putative phosphorylation site at which cAMP regulates its function.

the sarcolemma are sensitive to control by surface receptors. It is well established that beta-1-agonists in cardiac muscle and alpha-adrenergic agonists in vascular smooth muscle can influence Ca^{++} influx via the slow inward current. It has been suggested therefore that in a proportion of calcium channels the inactivation gate opens as a consequence of a c-AMP dependent phosphorylation and that Ca^{++} channel conduction depends on this and the simultaneous voltage stimulated opening of the activation gates (Reuter, 1979). Other channels may rely wholly on adenylate cyclase receptor occupation and subsequent C-AMP facilitated phosphorylation to mediate the opening of the channels (Braunwald, 1982). These being termed 'Receptor operated' channels (ROCs). A further example of discrete biochemical events serving to couple receptor occupation to the ROCs opening may be provided by phosphatidylinositol (PI) turnover. This process is associated with a wide variety of receptors in most animal cells studied (Sawyer & Cohen, 1981; Putney, Dewitt, Hoyle, McKinney, 1981; Cockcroft, 1981; Koutouzov & Marche, 1982; Meltzer, Weinreb, BellorinFont, Hruska, 1982; Billah & Lapetina, 1983; Downes & Michell, 1982). In the PI cycle, the breakdown of PI follows ligand binding. Phospholipase C catalyses this breakdown into diacylglycerol and inositol-phosphates, the latter of which may serve as second messengers. The diacylglycerol is rapidly phosphorylated to form phosphatidic acid which is finally reconverted into PI (see diagram 4). Michell, (1975) showed that PI metabolism is triggered only by those receptors that control a rise in cytosolic calcium. He went on to propose that the PI breakdown was a consequence of receptor occupancy, preceded Ca^{++} influx and therefore may be an event intrinsic to the calcium channel activation process. Evidence derived from a number of cell types has generally supported this theory. Vaso-

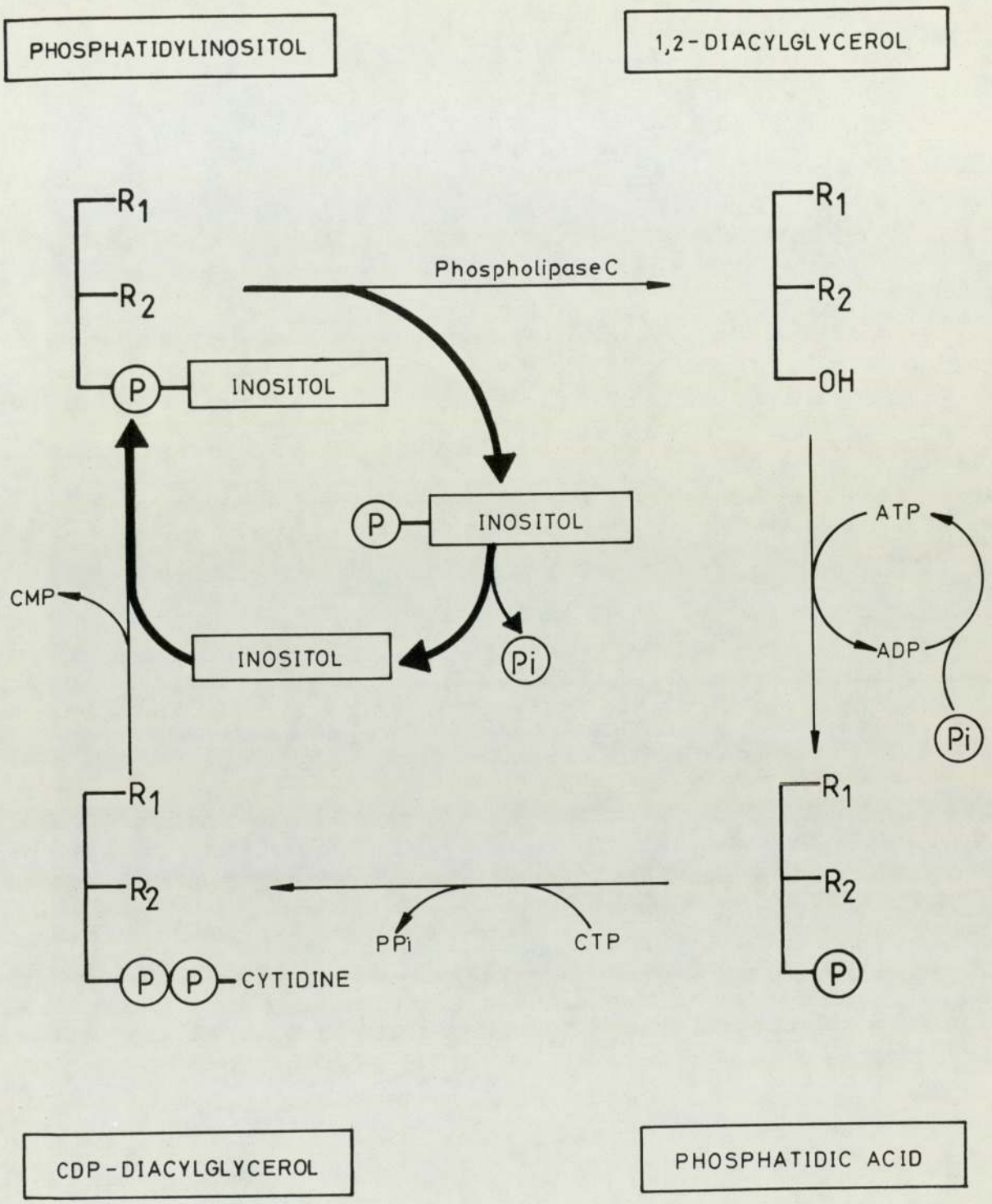


Diagram 4 Schematic Representation of the Cyclic Pathway of Phosphatidylinositol Turnover.

pressin - induced PI turnover in rat hepatocytes for example is followed by an elevation of intracellular calcium (Takenawa, Homma & Nagai, 1982). Receptor occupation appears primarily to stimulate the PI pathway and hence the accumulation of phosphatidic acid (PA) and since PA has been shown to directly promote calcium translocation in liposomes (Serhan, Anderson, Goodman, Dunham & Weissman, 1981; Serhan, Fridovich, Goetzl, Dunham & Weissman, 1982), it is possible that a transient elevation in this acid in the cellular membranes increases the calcium permeability of these membranes. Likewise, recent studies have suggested that platelet activating factor (PAF) induces a rapid mobilization of calcium ions in platelets probably as a consequence of rapid PI degradation (Billah & Lapetina, 1983). PI reformation and therefore reduction in PA might prevent excessive calcium accumulation and consequently overstimulation of the cell. PI may also bind divalent cations and enhance the Ca^{++} extrusion pump activity (Dawson, 1965; Billah & Lapetina, 1983). However, despite the close relationship between cell activation and PI turnover, some authors dispute the general applicability of Michell's theory that PI metabolism is causally related to elevated intracellular calcium levels. Cockcroft et al., (1980) have proposed that in neutrophils it is the entry of Ca^{++} into the cytosol which promotes PI breakdown. Similarly it has been reported that an epidermal growth factor (EGF) -induced calcium influx may trigger the stimulation of PI turnover in A-431 cells (Sawyer & Cohen, 1981). An obligatory role for PI metabolites in Ca^{++} mobilization in parotid and lacrimal glands also seems unlikely (Putney et al., 1981). In such cases the surface receptor may be directly linked to calcium movement in a way that receptor occupation induces a conformational change in membrane lipid and protein structure resulting in the development of a transmembranal transport system i.e. an open ROC(s) (Cullis, De Kruijff, Hope, Nayar & Schmid, 1980).

The existence of these various transmembrane Ca^{++} entry routes however, does not preclude the possibility that in some way calcium ions may be displaced from intracellular loci or that receptor occupation may serve directly to make available membrane-bound calcium ions. Substantial quantities of Ca^{++} are reversibly associated with external surface phospholipids and negatively charged groups of the glycocalyx. Calcium ions derived principally from the membrane bound pool can indeed enter the cytosol as a consequence of cellular stimulation (Langer, 1978; Poggioli & Putney, 1982). The cytosolic face of the plasmalemma, similarly, is known to interact with calcium ions. It has been suggested that here calcium binds tightly to membrane proteins but less so to negatively charged phospholipids and thus lipid calcium can be displaced by certain stimuli (Szasz, Sarkadi & Gardos, 1977).

The sarcoplasmic reticulum sequesters calcium ions and thus, is potentially an intracellular source for cation release during cellular activation. Indeed the existence of calcium efflux from SR has been demonstrated (Feher & Briggs, 1982; Chu, Tate, Bick, Van Winkle & Entman, 1983). Miyamoto and Racker (1981) have proposed two main hypotheses for the mechanism of release. The action potential may be transmitted via the transverse tubules of the cell to the SR membranes, which consequently become depolarized and permit calcium efflux via a voltage-dependent channel. Alternatively, calcium ions that cross the sarcolemma may induce Ca^{++} release from SR via a Ca^{++}-gated cation channel (Yamamoto & Kasai, 1982a, b; Miyamoto & Racker, 1982). Ca^{++}-induced Ca^{++} release will generate an inside negative membrane potential which may also open the voltage gated channel and hence both mechanisms might work together to increase cytosolic Ca^{++} levels. A similar process may operate in nonexcitable cells but it has been less widely studied in endoplasmic reticulum.

The mitochondria also play a critical role in cellular Ca^{++} homeostasis. In a similar manner to SR and ER, the mitochondria can reverse its sequestration process and act ultimately to raise the intracellular calcium levels. The existence of two mitochondrial efflux systems has been verified, one for predominantly excitable cells and the other for non-excitable tissues (Racker, 1980; Borle, 1981). Calcium release may be mediated by a Ca^{++}/Na^{+} antiport system which appears to be coupled to a Na^{+}/H^{+} countertransport so that charge compensation for Ca^{++} efflux comes ultimately from the H^{+} influx. (Fiskum & Lehninger, 1980; 82). In non-excitable tissues however, release of Ca^{++} from mitochondria operates via an electroneutral $Ca^{++}/2H^{+}$ antiport process coupled to H^{+} - phosphate symport to maintain the intra - mitochrondrial pH (Fiskum & Lehninger, 1980). However, mitochondria from tissues such as adrenal cortex, parotid gland and brown fat show exception to this generalisation in that calcium release is significantly stimulated by the presence of sodium ions (Crompton, Moser, Ludi, Carafoli, 1978; Al-Shaikhaly, Nedergaard & Cannon, 1979). In addition, recent evidence indicates that this Ca^{++}/Na^{+} antiport process is also present in other non-excitable tissues but it appears to operate at a much lower maximal activity (10-20%) than that in most excitable cells (Nicholls, 1978; Haworth, Hunter & Berkoff, 1980). The cyclic nucleotides may also influence the mitochondrial function. Cyclic AMP at 5×10^{-7} - 5×10^{-6}M raises the concentration of extramitochondrial calcium by triggering the release of the ion from mitochondria isolated from several tissues (Borle, 1973). Furthermore, this cyclic nucleotide may inhibit the Ca^{++} uptake process of the mitochondria. However, this remains a contentious issue since other laboratories have failed to reproduce

the effect (Scarpa, Malmstrom, Chiesi & Carafoli, 1976). The preceding studies demonstrate the extent of compartmentalisation of intracellular calcium and the mechanisms by which it may be regulated. The processes are summarized in a generalised model of cellular calcium homeostasis depicted in diagram (5). It is important to note however that it is most unlikely that all mechanisms included in the model will be featured in any one cell type studied.

1.2.1b Signal Transmission by Ca^{++}

It is now recognised that most regulatory roles attributed to the free calcium ion, require the association of the ion with one of a series of specific binding proteins which include troponin C of skeletal and cardiac muscle, regulatory and essential myosin light chains, the parvalbumins, vitamin D - induced binding proteins and the ubiquitous calmodulin, (Moore & Dedman, 1982; Van Eldik, Zendegui, Marshak, & Watterson, 1982). Sequence determinations have revealed important structural homology between these proteins, consistent with their origin from a single ancestral protein that bound a single calcium ion (Demaille, 1982). Moreover, this structural homology is paralleled by a functional homology since it has been shown that one protein can substitute successfully for another in the activation process (Amphlett, Vanaman & Perry, 1976). Calmodulin (CaM) is doubtless the most versatile of this group of proteins. It has a widespread distribution in eukaryotes (being found in both plant and animal tissues, from the most complex to the smallest single cell organism), has a highly conserved structure and has the capacity to modulate many different enzymes and cellular processes as listed over-page (Means, Tash & Chafouleas, 1982). Calmodulin is a small, acidic, heat stable, globular protein with a molecular weight of 16,700.

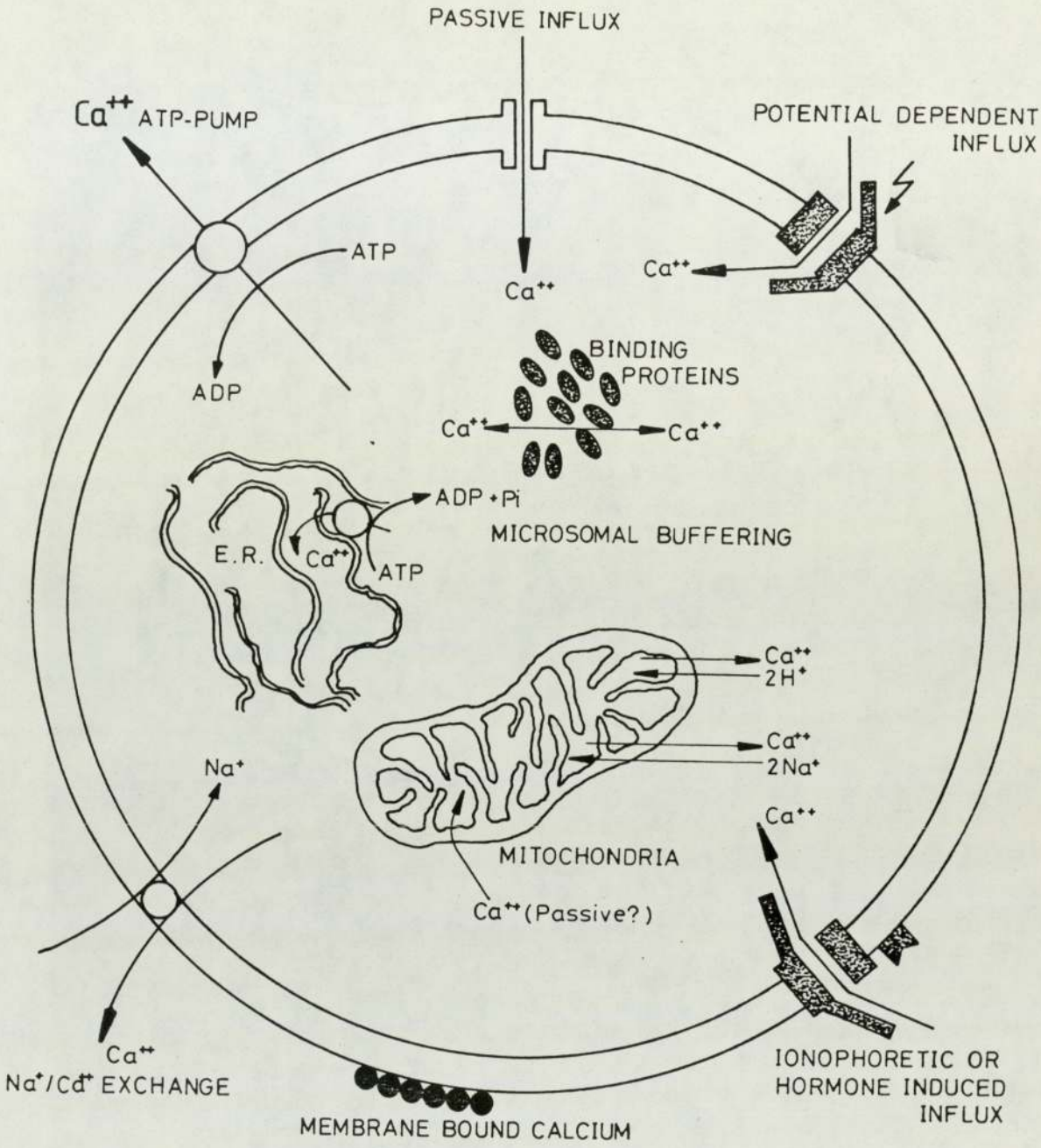


Diagram 5 Diagrammatic Representation of Cellular Calcium Homeostasis.

Depicts possible modes of calcium entry into and exit out of a generalised cell and mechanisms of intracellular buffering of the ion. (N.B. It is unlikely that all mechanisms included in this model will be featured in any one cell type studied).

Table (i) List of some of the Calcium-calmodulin regulated enzymes and cellular processes so far known in mammalian tissue.

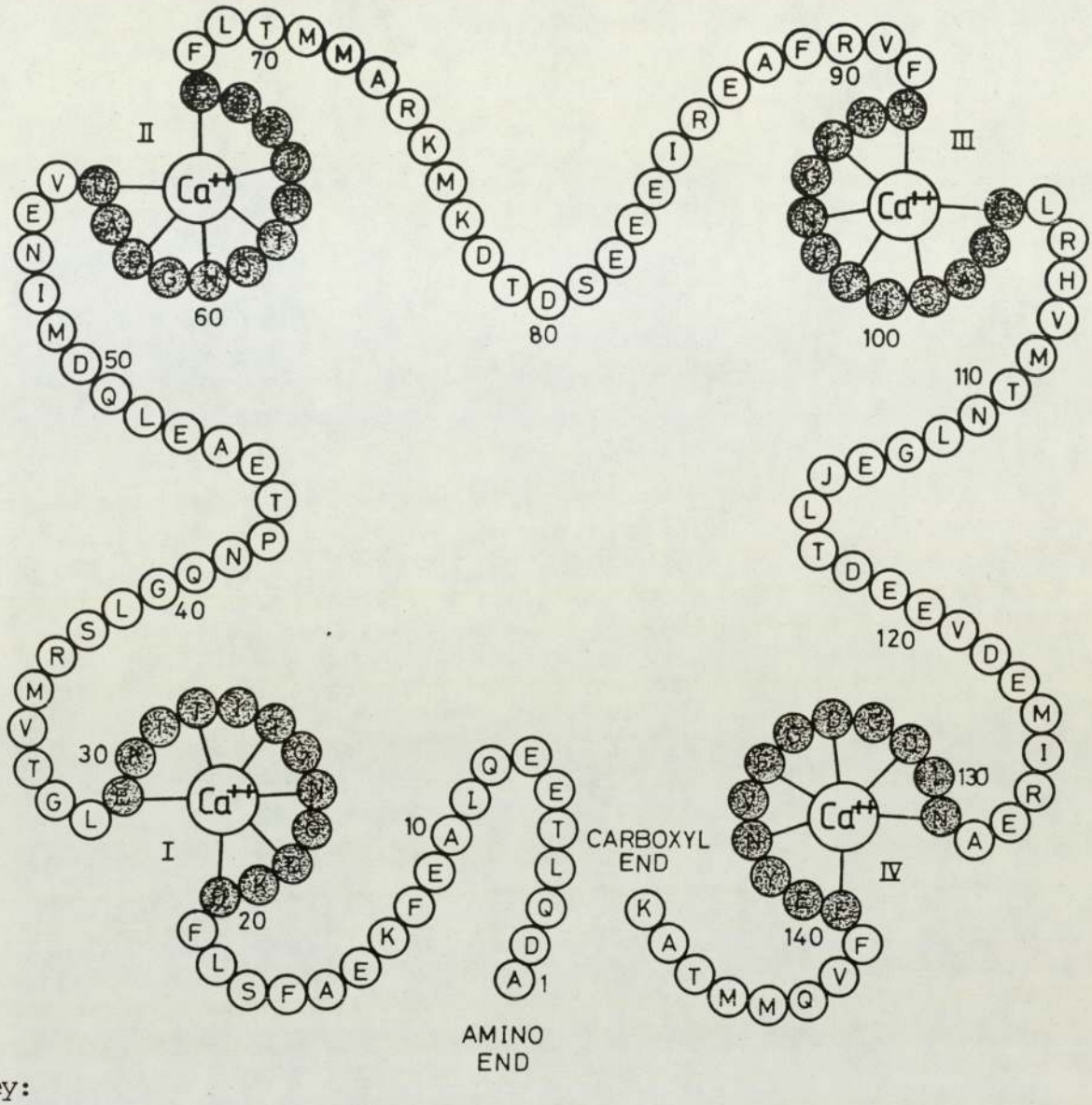
Ca^{++}-CaM sensitive enzymes and processes	
1.	Adenylate cyclase
2.	Soluble cyclic nucleotide phosphodiesterase
3.	Skeletal muscle actomyosin ATPase
4.	Myosin - light chain kinase
5.	Phospholipase A_2
6.	Phosphorylase kinase
7.	Synaptic membrane phosphorylation
8.	Microtubule assembly
9.	Erythrocyte membrane (Ca^{++}-Mg^{++})-ATPase
10.	Methyl transferase
11.	NAD kinase
12.	Protein kinase
13.	Neurotransmitter release
14.	Glycogen synthetase phosphorylation

Its tertiary structure is such that it contains four calcium binding domains, two of which are thought to be high affinity sites and the other two are of slightly lower affinity (Demaille, 1982). See diagram (6). It appears that physiological Mg^{++} levels can decrease the affinity of all sites for Ca^{++} (Klee, Crouch, & Richman, 1980; Haiech, Klee, & Demaille, 1981), but due to a considerable number of discrepancies with respect to the affinity of the binding sites for Ca^{++} and Mg^{++} (Demaille, 1982), the physiological significance of this observation is still unclear. Upon binding with Ca^{++}, calmodulin undergoes a conformational change towards a more helical structure that exposes a hydrophobic domain believed to be a binding site for CaM acceptor proteins, generally enzymes and termed here 'response elements' (Cheung, 1981; Lin, 1982). Biochemical analysis has demonstrated that the major conformational transition occurs at less than full occupancy of the Ca^{++} binding sites (Klee, 1977). It has similarly been suggested that there may be two (or more) Ca^{++} dependent transitions in CaM (Klee et al., 1980). The Ca^{++}-dependent interaction of calmodulin with the response element (RE) under its control thus, appears to occur in three steps (See diagram 7). The first step is the co-operative binding of calcium to the first two sites on calmodulin, (once the first calcium is bound to CaM, the next binds with a greater affinity or a lesser dissociation constant possibly due to a structural alteration).

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

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

The second step is the association of Ca_2.CaM with the response elements.



Key:

A	Alanine	J	Trimethyl Lysine	R	Arginine
D	Aspartate	K	Lysine	S	Serine
E	Glutamate	L	Leucine	T	Threonine
F	Phenylalanine	M	Methionine	V	Valine
G	Glycine	N	Asparagine	Y	Tyrosine
H	Histidine	P	Proline		
I	Isoleucine	Q	Glutamine		

Diagram 6 Schematic Diagram of the Calmodulin Molecule Which Mediates Many of the Regulatory Functions of Calcium Ions.

The molecule consists of a single protein chain of 148 amino acid sub-units with four calcium binding domains. Each binding site is a loop (shaded area) flanked by a helical region. Each calcium ion appears to be bonded to 6 amino acids. The diagram gives the amino acid sequence of calmodulin from bovine brain.

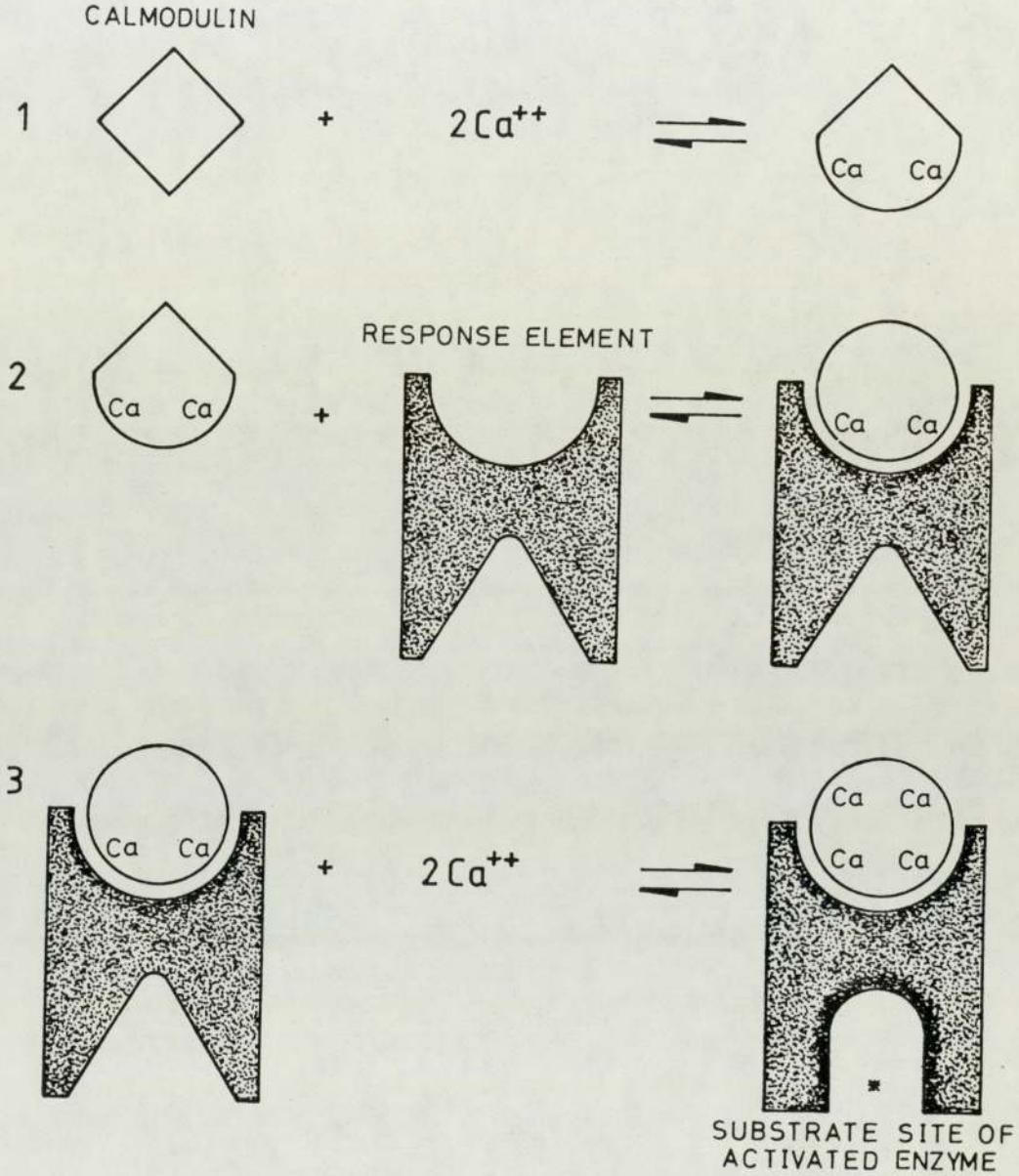


Diagram 7 A Topological Model of the Cooperative, Ordered Mechanism of Activation of a Calmodulin - Regulated Enzyme.

A change in shape represents a change in protein conformation.

$$RE + Ca_2 \cdot CaM \rightleftharpoons Ca_2 \cdot CaM \cdot RE$$

The formation of this complex is of crucial importance in inducing a conformational change in CaM such that the affinity of the remaining two sites for calcium is markedly increased.

The third step is the binding of the remaining two calcium ions to this complex leading to the production of the activated form of the response elements*.

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

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

There now follows the binding of the enzyme substrate to this activated form and consequently, the specific reaction proceeds. It has also been suggested that this system will display hysteresis so that after the formation of the activated complex it will remain in this configuration at a calcium ion level lower than that necessary to shift it there initially (Rasmussen & Waisman, 1983).

Although (as proposed in the model above) it is generally accepted that the active modulatory form of CaM is $Ca_4 \cdot CaM$ in most well characterized systems (Rasmussen & Waisman, 1983; Huang, Chali, Chock, Wang and Sharma, 1981), there is evidence available to suggest that the fractional occupancy of the Ca^{++} binding sites may dictate the particular process regulated by CaM and help to explain the protein's involvement in such diverse cellular events within the physiological system (Means & Dedman, 1980a, b; Cheung, 1982; Lin, 1982). For example, Wolff and Brostrom (1979) have proposed that the most active species for stimulation of phosphodiesterase in 1mM Mg^{++} is the tricalcium-monomagnesium complex, although it has been

suggested by some authors that this enzyme activation can occur maximally with only one Ca^{++} bound (Means & Dedman, 1980a). However, despite the discrepancies in the literature, with respect to such binding data there is general agreement that four sites for the binding of Ca^{++} exist. It is possible thus, that the stoichiometry of the active CaM.Ca^{++} complex varies (between one and four Ca^{++} per CaM molecule) with different calmodulin RE, so that CaM can translate a quantitative difference in Ca^{++} occupation into a qualitatively different cellular response.

It is apparent that cellular CaM is maintained at a specific level by a constant rate of synthesis and degradation of the molecule (Means & Dedman, 1980b). However, it appears that CaM can also be redistributed since C-AMP dependent phosphorylation of membrane systems within the cell can induce the reversible release of membrane bound CaM (Hanbauer, Pradham and Yang, 1980). The net effect is to increase cytosolic levels of CaM which acts to increase the concentration of Ca_2 . CaM at any fixed calcium level. In contrast, redistribution occurs from cytoplasm to membrane coincident with such processes as secretion (Means & Chafouleas, 1982). Sequential deactivation of CaM dependent enzymes may involve specific proteins which bind CaM. Little is known about the function or regulation of the levels of the CaM binding proteins found in cells but one such protein, calcineurin (found in nervous tissue and possibly erythrocytes) is able to inhibit CaM - dependent enzymes as a direct result of its Ca^{++} - dependent binding to CaM (Klee, Crouch & Krinks, 1979; Wallace, Tallant & Cheung, 1982). Many other CaM binding proteins with similar inhibitory propensities have been identified and thus are presumed to represent a means of chronically regulating cellular activity

(Vincenzi, 1981). Termination of stimulation, whatever the mechanism is always accompanied by the re-establishment of CaM distribution evident in non-activated cells (Means & Chafouleas, 1982).

Thus, CaM will respond directly to the elevated levels of intracellular calcium and as a result mediate the cell activation process. In addition, the molecule also appears to be involved in the limitation of duration of this response. Such self regulation by calmodulin occurs via activation of the plasma membrane Ca^{++} - pump and possibly indirectly by alteration of sarcoplasmic and endoplasmic reticulum Ca^{++} transport (Wetzker, Klinger, Cumme, Hoppe & Frunder, 1982; Vincenzi & Larsen, 1980; Carafoli, 1981; Vincenzi, 1981; Luthra, Watts Scherer & Kim, 1980) as discussed previously.

1.2.2 The Role of Magnesium

The magnesium ion, the most abundant cation in living cells, is also required as a cofactor by more intracellular enzymes than any other inorganic cation. The numerous enzymes that are dependent on intracellular free Mg^{++} include the (Na^{+}/K^{+}) transport ATPase (Mardh, 1982), the (Ca^{++}/Mg^{++})-ATPase of the SR (Hasselbach, Fassold, Migala & Rauch, 1981; Takakuwa & Kanazawa, 1982), adenylate cyclase (Somkuti, Hildebrandt, Herberg & Iyengar, 1982; Cech, Broaddus & Maguire, 1980), and phosphorylase kinase (King & Carlson, 1981).

1.2.2a Intracellular Regulation of Magnesium

In mammalian cells total intracellular Mg^{++} is maintained at about 10 mM over a broad range of external Mg^{++} levels, suggesting an

efficient homeostatic system operating to minimize cell loss in such conditions as Mg^{++} depletion. An inwardly directed Mg^{++} pump has thus been implicated, but has not yet been characterised in mammalian cell membranes (Sanui & Rubin, 1982a). This however, is in complete contrast to the Mg^{++} extrusion pump, powered by the Na^{+} gradient in vascular smooth muscle and cardiac muscle (Somylo and Somylo, 1981). A large percentage ($\sim$ 90%) of the intracellular Mg^{++} is complexed to internal membranes and nucleic acids and since the remaining free ions are also well buffered by various metabolic products such as ATP, citrate, amino acids and many phosphorylated molecules, free Mg^{++} concentration measurements have proved to be somewhat inconsistent. Despite the relatively wide range of reported free Mg^{++} levels most estimates are approximately 1 mM. Since this has been shown to be in the range of or just below the concentration pertinent for maximal activity of many enzymes it has been proposed that comparatively small changes in intracellular levels can potentially exert a large degree of metabolic control (Sanui & Rubin, 1982a).

It is apparent that many hormones can modify Mg^{++} transport. ACTH, epinephrine and norepinephrine cause an elevation in the accumulation of Mg^{++} by adipocyte plasma membrane vesicles, the catecholamine increase being mediated most probably by an alpha-adrenergic receptor (Elliott and Rizack, 1974). Similarly, insulin specifically provokes Mg^{++} uptake in fibroblasts and adipocytes (Cech et al., 1980). More recently it has been demonstrated that the rate of accumulation of Mg^{++} (but not Ca^{++}) is markedly depressed by the activation of the beta-adrenergic receptor - adenylate cyclase complex of S49 cells (Maguire & Erdos, 1978). This inhibition of Mg^{++} uptake however, is not mediated by cAMP suggesting that occupation of this receptor may initiate multiple specific intracellular responses by independent

pathways (Maguire & Erdros, 1980). Although the precise mechanism of Mg^{++} transport in this system is not known, a Mg^{++} carrier protein has been implicated as an integral part of the beta-receptor - cyclase complex. Alternatively the carrier may be influenced independently of the cyclase, by beta-receptor occupation (Cech, et al., 1980).

Thus beta-receptor occupation is associated with decreased Mg^{++} accumulation whereas alpha-receptor occupation promotes Mg^{++} influx. Since insulin and alpha-adrenergic agents can inhibit the effect of beta-receptor binding on Mg^{++} movement (Cech, et al., 1980) it is interesting to speculate that Mg^{++} may serve as the intracellular messenger for integration of such antagonistic signals within the physiological system.

It is generally accepted that the Mg^{++} status of the cell can modulate hormone mediated responses by:-

1) interfering with the Ca^{++} homeostatic system within the cell and thus ultimately by altering the intracellular Ca^{++} concentration, or

2) by influencing (at an earlier stage) the receptor function itself.

Extracellular Mg^{++} ions can induce an increase in the number of functional surface receptors available to an agonist (Pearlmutter & Soloff, 1979), or alternatively influence the responses mediated by alpha- and beta-receptors in many cell types. This may be achieved by differentially changing the intrinsic activity (maximal response) or the affinity of these receptors to their respective agonists. The sensitivity to agonist of beta-adrenergic receptors diminishes remarkably in the absence of Mg^{++} whereas physiological levels

induce a high affinity state of the beta-receptor in many cellular systems including isolated canine coronary arteries (Turlapaty & Altura, 1982), S49 cells (Maguire & Erdos, 1978) and uterine smooth muscle (Osa & Ogasawara, 1979). Extracellular Mg^{++} however, does not interfere with the binding of agonists to alpha-receptors, but rather influences the cellular response. Alpha-agonist activity in fact is greatly potentiated in the absence of Mg^{++} and is attenuated progressively as the Mg^{++} levels are increased, probably a result of an alteration in the threshold concentration required for a maximal response (Turlapaty & Altura, 1982). The enhanced intrinsic activity of alpha-agonist and possibly the decreased sensitivity of beta-receptor in the absence of Mg^{++} might be due to changes in the calcium status of the cell. Indeed data are available consistent with the premise that extracellular and intracellular Mg^{++} can modulate cytosolic Ca^{++} levels by influencing calcium entry, binding and translocation within the cell.

In smooth muscle acute reduction in the extracellular magnesium concentration ($[Mg^{++}]o$) is generally associated with an increased rate of Ca^{++} uptake due to enhanced membrane permeability, whereas an acute elevation of magnesium levels markedly depresses this influx rate (Altura & Altura, 1981; Turlapaty & Altura, 1978). Consistent with these findings is the observation that high $[Mg^{++}]o$ attenuates the calcium pump activity in the membrane and low Mg^{++} levels seem to promote Ca^{++} efflux (Turlpathy, & Altura, 1978), probably indirectly as a consequence of elevated intracellular Ca^{++} levels. Thus it is possible that Mg^{++} binding sites at the surface membrane act physiologically to regulate both Ca^{++} entry and exit processes.

In addition, cytosolic levels of Mg^{++} ($[Mg^{++}]i$), can interfere with intracellular regulatory mechanisms. In SR, magnesium exerts a biphasic effect. Low $[Mg^{++}]i$ in resting cells, produces a marked stimulation of Ca^{++} uptake. The ion serves as part of the true substrate (Mg.ATP) for the Ca^{++} uptake process in sarcoplasmic reticulum in which the enzyme becomes phosphorylated (Stephenson, 1981; Takakuwa and Kanazawa, 1982). In addition, subsequent Mg^{++} occupancy of an allosteric site on the outer surface of the SR membrane induces the acceleration of exchange between the bound Ca^{++} of the phosphorylated ATPase and the internal environment of the SR, by promoting enzyme dephosphorylation. This precedes the re-establishment of the ATPase resting state and re-orientation within the SR membrane ready for cytosolic Ca^{++} association and translocation (Chiesi and Inesi, 1981). Low levels of Mg^{++} may also inhibit local Ca^{++} - dependent Ca^{++} efflux from the SR probably by direct competitive binding at a calcium receptor on the Ca^{++}-regulated efflux channel (Stephenson, 1981). However, both of the mechanisms of Mg^{++} action have much less effect in high Ca^{++} conditions (i.e. during cell activation) and thus are most likely highly adaptive mechanisms specific for the maintenance of low resting cytosolic calcium levels (Stephenson, 1981).

We have already seen that high $[Mg^{++}]o$ inhibits Ca^{++} translocation into the cell. This increased Mg^{++} concentration gradient however dictates that intracellular magnesium level will also become elevated. Thus, it is significant that high intracellular Mg^{++} levels ($[Mg^{++}]i$) inhibit the Ca^{++} uptake process in SR and in this way may serve to raise the functional cytosolic calcium pool. Since high Ca^{++} can partially prevent this inhibition it may be due to competitive

binding between calcium and magnesium ions at the Ca^{++} binding site involved in enzyme phosphorylation or alternatively to displacement of calcium from the translocation sites of the ATPase (Chiesi & Inesi, 1981). High $[Mg^{++}]i$ also potentiates Ca^{++}-induced Ca^{++} release from SR (Takakuwa & Kanazawa, 1982) which too supports the contention that elevated intracellular Mg^{++} levels can induce a high cytosolic Ca^{++} concentration.

Therefore, there appears to be a differential range of effects of Mg^{++}, dictated possibly by the cell type and specific metabolic state of the cell as a consequence of the extent of plasma membrane permeability to Mg^{++} and/or the amount of SR and ER distributed within the cell.

Furthermore, mitchondrial calcium may also be modulated in a similar manner to SR. Resting $[Mg^{++}]i$ and elevated levels will inhibit Ca^{++} sequestration by mitochondria (Akerman, 1980; Fiskum & Lehninger, 1980; Becker, Fiskum & Lehninger, 1980), probably as a result of direct competition for negative charges at or near surface binding sites for Ca^{++} on the mitochondrial membrane. In some tissues however, (e.g. liver), Mg^{++} is a less efficient inhibitor of Ca^{++} uptake and thus in these cells it is a less likely candidate for promoter of Ca^{++} release from mitochondria (Carafoli, 1979).

That magnesium may act as a physiological regulator of cytosolic Ca^{++} levels within the cell, so that Ca^{++} may ultimately serve as the primary intracellular messenger, is accepted by many authors. The maintenance of compartmentalization of the Ca^{++} ion, (thus achieving a constant basal cytosolic Ca^{++} level) and the presence of specific binding proteins (that can receive the ion and successfully transmit the appropriate signals pertinent for cell

activation) favour such a theory. However, Grubbs and Maguire (1982), have recently submitted evidence to indicate the existence of at least two cytoplasmic compartments for magnesium, one of which consists of a small portion of cytoplasmic Mg^{++} that is intimately associated with the Mg^{++} transport system allowing entry into the cell. The mechanism and precise nature of the compartmentalization is unclear but its existence does demonstrate that cellular Mg^{++} has the potential to play an important regulatory role in cell function. It is well known that no specific Mg^{++} binding protein analagous to calmodulin has yet been characterised in mammalian cells. It is possible however, that Mg^{++} does not require any intermediary, to exert its co-ordinate control, but in fact produces its effects based entirely on its own intrinsic properties (Sanui & Rubin, 1982a).

1.2.3 The Role of the Cyclic Nucleotides

In addition to Ca^{++} (and possibly Mg^{++}) the cyclic nucleotides also appear to serve as universal intracellular messengers, with crucially important roles in the control of many cellular processes. There are a large number of operational similarities between the function of the cyclic nucleotide messenger system (particularly cAMP) and the calcium one (Rasmussen & Waisman, 1983). For example,

(i) The messenger operates within a very restricted concentration range,

(ii) Upon cell activation there is a transient increase in messenger concentration,

(iii) There are multiple mechanisms for the regulation of messenger formation,

(iv) There are multiple ways to regulate messenger termination,

(v) Both systems appear to display highly co-operative, ordered reaction sequences,

(vi) It is probable that both systems display hysteresis.

1.2.3a Formation of Intracellular cAMP

Adenylate cyclase is the enzyme responsible for catalysing the process of cAMP production (Rall & Sutherland, 1962). It is part of a complex regulatory system that mediates the actions of many hormones and neurotransmitters on their target cells. Structured within the lipid framework of the cellular membrane, the enzyme system appears to be composed of at least three distinct components (Ross and Gilman, 1980). Located at the outer surface is the hydrophobic protein or glycoprotein receptor (R) (which contains specific binding sites for attachment of hormones) and at the inner face of the membrane are the catalytic unit (C) and the nucleotide regulatory unit (N). The C-unit is thought to be composed of a hydrophilic portion with a small hydrophobic component for anchorage to the membrane (Neer, 1982), whereas the N-unit appears to be more loosely attached to the membrane than either the receptor or the catalytic portion.

Cell hybridization studies have revealed that the receptor can move freely in the plane of the outer lipid phase (Rodbell, Birnbaumer & Pohl, 1970), quite independently of the catalytic components (Orly & Schramm, 1976). Thus, hormone receptors of this enzyme from a variety of cell types can interact with a single catalytic unit (Schramm, 1979). The nucleotide regulatory unit (N) may serve to functionally couple the hormone receptors with the catalytic

moiety of the enzyme (Lefkowitz, Caron, Michel & Stadel, 1982; Rodbell, 1980; Levitzki, 1981; Rodbell, 1978). Such N components contain binding sites for GTP and are responsible for mediating the effects of GTP and the various hormones on the activity of 'C' (Rodbell, 1978). Two functional types of N-units have been distinguished, one mediating stimulation (termed Ns) and the other inhibition (Ni) of the enzymes by GTP. Separate classes of receptor are linked to the Ni and Ns nucleotide regulatory units for adenylate cyclase; for example beta-receptors are Ns-linked and alpha-receptors are Ni-linked (Rodbell, 1980; Levitzki, 1981). Rodbell (1980), has proposed that R and N exist in the membrane as a multimeric complex and in this form R inhibits interaction of GTP with N. When the hormone binds to R, N is released from this structural constraint and enhanced binding with GTP ensues. The multimers break down to monomers, thus permitting the formation of the holoenzyme (RNC) (See diagram 8). Depending on the type of R and N unit attached to C the holoenzyme exhibits either increased or decreased production of cAMP. Not all authors have adopted this model and some believe that R and C units may not have any direct contact but that N-GTP acts as a shuttle, conveying information between the two components (Lefkowitz et al., 1982). This might explain why N appears to be more loosely attached to the membrane than R and C. The basic pathway of the reaction however remains essentially the same (as shown in diagram 9). Incorporated in the cycle is a GTP-ase, associated with the deactivation of the holoenzyme reconverting it to its stable state. Here it is thought that GDP binding maintains the basal catalytic activity until such a time as hormone binding promotes the displacement of GDP by GTP in the activation cycle (Iyengar & Birnbaumer, 1979). Whatever the mechanism, the coupling of hormone and catalytic units

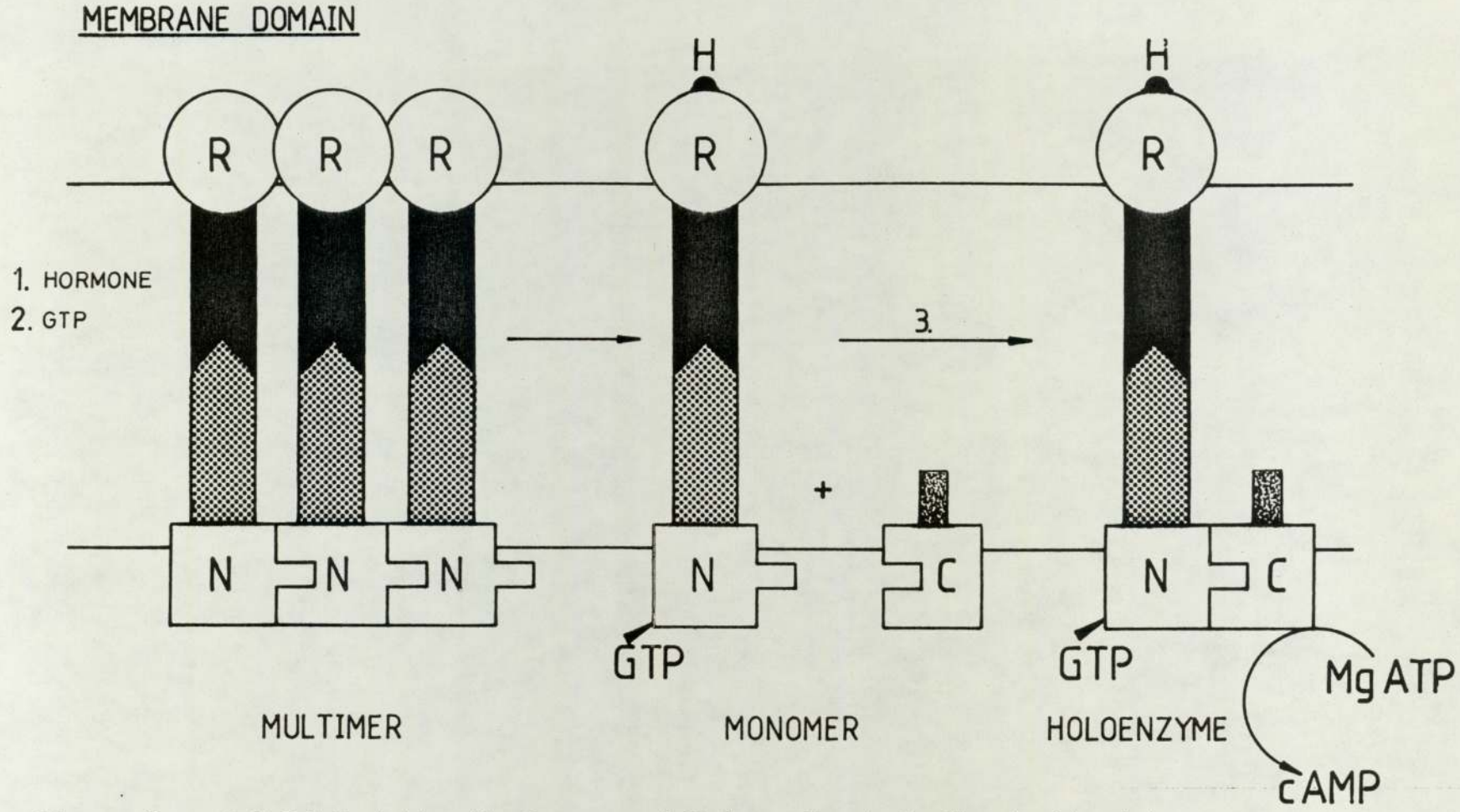


Diagram 8 A Model Depicting the Sequence of Membrane Events Leading to Adenylate Cyclase Activation.

R represents the receptor components, N the nucleotide regulatory units and C the catalytic unit of the enzyme.

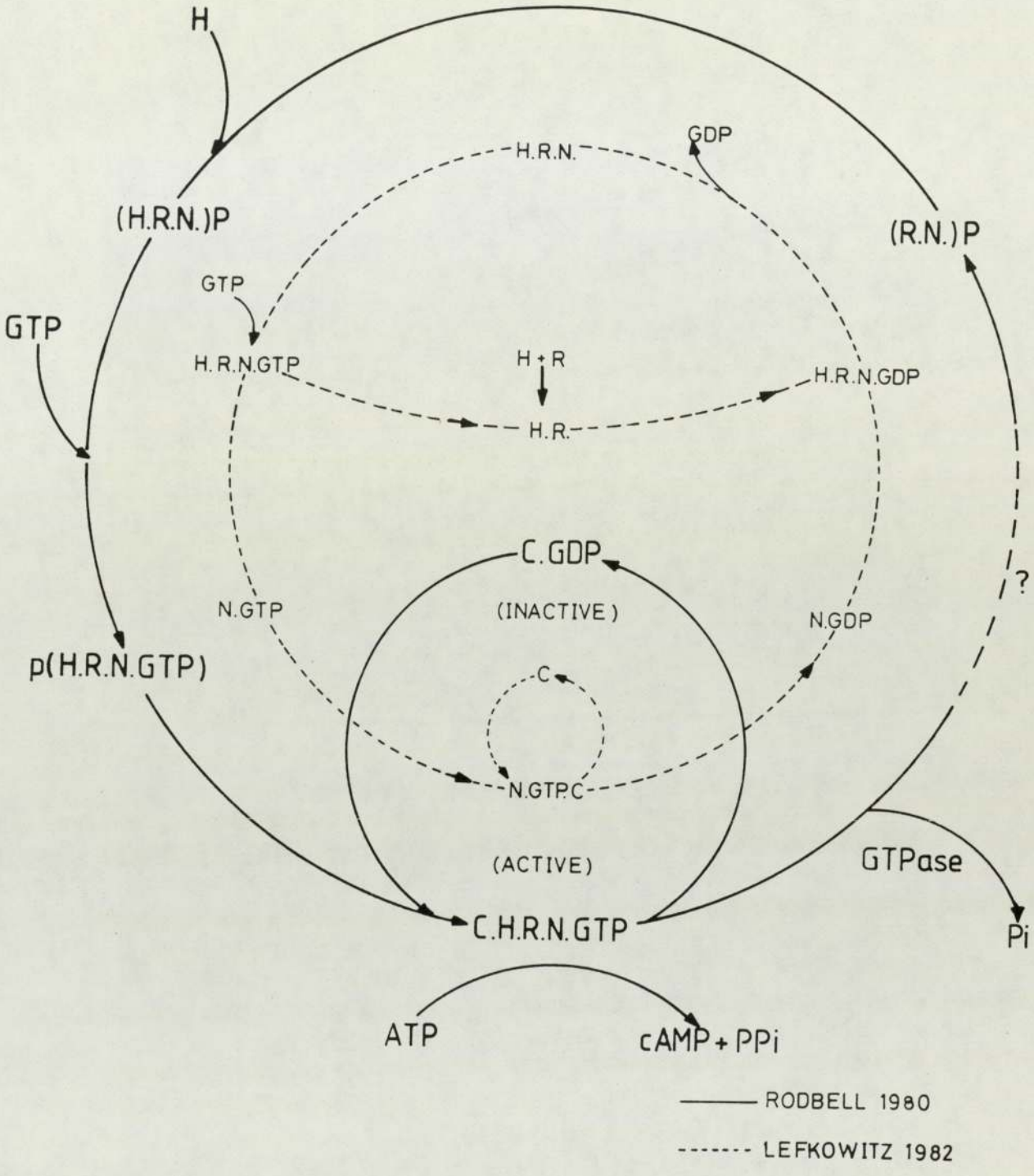


Diagram 9 Schematic Diagram Representing Two Proposals For the Reaction Cycle of Receptor Mediated Activation of the Adenylate Cyclase Enzyme.

H designates the hormone and R its specific receptor, N and C are respectively the nucleotide regulatory subunit and the catalytic unit of the enzyme. (RN)P represents the proposed multimeric complex.

will be influenced by membrane lipid fluidity. It is significant thus that beta-adrenergic agonists promote phospholipid methylation thereby increasing fluidity and thus probably mediate the subunit coupling by facilitating lateral movements of the appropriate components (Hirata, Strittmatter & Axelrod, 1979).

The cationic environment also influences the activation of adenylate cyclase and the formation of cAMP. The binding of Ca^{++} to a metal allosteric site on the C-component of the complex either causes an inhibition of catalytic activity or a non-interaction between the subunits which can be antagonised by Mg^{++}. Mg^{++} can not only remove this inhibition but also exerts its own stimulatory effect, suggesting an important role for changes in the relative local abundancy of both divalent cations in regulating the cyclase activity (Rodan, 1978). In addition to this apparently direct effect on the C-unit, calcium also influences the action of the regulatory proteins (N) which modulate adenylate cyclase activity. In some tissues, notably brain, Ca^{++} appears to exert a biphasic effect; low Ca^{++} concentrations stimulate while higher concentrations inhibit the catalytic formation of cAMP (Potter, Piascik, Wisler, Robertson & Johnson, 1980). Ca^{++} may exert its stimulatory action on brain adenylate cyclase directly, by facilitating the binding of GTP to the regulator protein (Mahaffe & Ontjes, 1980). However, other studies have implicated calmodulin in this process (Cech, Broaddus & Maguire, 1980; Potter et al., 1980). The Ca^{++} - calmodulin stimulation of adenylate cyclase seems to be independent of the nature of the guanine nucleotide present at the N binding site; i.e. activation will occur with either GDP or GTP and even possibly with no nucleotide at the binding site (Seamon & Daly, 1982). Activation of the enzyme by Ca^{++} - CaM, occurs at

very low concentrations of calcium ($< 10^{-7}M$) and thus, it has been proposed that the association of CaM (with Ca^{++} bound to one of its four sites) with the cyclase binding site might increase the affinity of the other three sites for Ca^{++} on the CaM molecule (Potter et al., 1980). This mechanism appears to be unrelated to the inhibition which occurs at high concentrations of calcium ($> 10^{-6}M$). Such enzyme inhibition by Ca^{++} in brain tissue, presumably involves interaction at a specific divalent cation site since calmodulin inhibitors do not influence this cyclase inhibition process (Seamon & Daly, 1982; Mahaffe & Ontjes, 1980). However, it is possible that inhibition is mediated by CaM bound to a distinct inhibitory metal site on the enzyme (separate from the activating site) which is inaccessible to the calmodulin inhibitors employed in these studies (Potter et al. 1980). Similarly, high Ca^{++} levels may inhibit cardiac adenylate cyclase via Ca^{++} - CaM mediation but activation of this cyclase by low calcium concentrations does not involve calmodulin, as is the case in brain tissue (Potter et al., 1980).

The hormonally stimulated adenylate cyclase enzyme activity is thus, clearly regulated by guanyl nucleotides and further modulated by the ambient divalent cationic environment, particularly by calcium ions.

1.2.3b Formation of Intracellular cGMP

Despite the similarities with the cAMP process, the production of cGMP appears to be a more complex and less well understood system. Cyclic GMP is formed from metal (Me^{++}) complexed GTP (Me^{2+} - GTP) by a manganese (Mn^{++}) dependent reaction catalysed by the enzyme guanylate cyclase (Hardman & Sutherland, 1969). The

characteristics of this enzyme clearly distinguish it from adenylate cyclase. It is located in both soluble and particulate fractions of homogenates of most mammalian tissues (Kimura and Murad, 1975a, b). The proportion of activity associated with the two fractions varies as a function of cell type and metabolic state (Sulakhe, Sulakhe, Leung, St. Louis & Hickie, 1976; Hardman, 1982). It appears that the soluble and particulate forms of guanylate cyclase are separate enzymes and not the same protein localised in different parts of the cell. In addition to their solubilities, the two forms have been known for some time to differ in their kinetic behaviour, sensitivity to activation and inhibition by divalent cations and other agents and apparent molecular weight (Garbers, Chrishman & Hardman, 1978; Murad, Mittal, Arnold, Ichihara, Braughler, El-Zayat, 1978). Probably the strongest published evidence that they are independent proteins comes from studies with antibodies raised to the particulate enzyme from sea urchin sperm. These antibodies cross react with particulate but not with soluble guanylate cyclase from several mammalian tissues (Garbers et al., 1978). The subcellular localisation of particulate guanylate cyclase has been reported in many tissues. It is associated with plasma membrane, endoplasmic reticulum, Golgi apparatus (Kimura & Murad, 1975b), nuclear membranes (Hardman, 1982), sarcoplasmic reticulum (White, 1975), mitochondria and synaptosomes (Nakazawa, Sand and Saito, 1976). The soluble form of the enzyme is assumed to be located in the cell cytoplasm, although the possibility that it is loosely associated with plasma membranes or other structures has not yet been ruled out (Hardman, 1980).

Several agents that can raise the cGMP levels in intact cells (e.g. acetylcholine, alpha-adrenergic agents, histamine and

lectins) show an absolute requirement for extracellular calcium ions to bring about their effects in numerous cells and tissues (Schultz, Hardman, Schultz, Baird & Sutherland, 1973; Goldberg & Haddox, 1977). Such agents thus, may mediate their action by provoking calcium ion influx into the cells or possibly by altering the intracellular distribution of this divalent cation. Soluble guanylate cyclase from rat lung appears to be activated by calcium in the presence of subsaturating manganese concentrations. Calcium alone also causes enzyme activation in this system but to a lesser extent, implicating a synergistic effect of calcium and manganese (Garbers et al., 1978). It is generally accepted that guanylate cyclase has two metal requirements for optimal activity, namely:-

1) That the substrate is Me^{++} -GTP (in particular Mn^{++} - GTP) rather than free GTP, and
2) The free Me^{++} interacts with the enzyme at an activator site.

Although Ca^{++} appears to have a lower affinity than Mn^{++} for the metal activator site and a lower intrinsic capacity to activate the enzyme when bound to this site (Murad, Arnold, Mittal & Braughler, 1979), it appears that in the presence of small amounts of Mn^{++}, Ca^{++} can replace some of the manganese in the Me^{++} -GTP complex. Thus, calcium may increase enzyme activity partially as a result of direct liberation of free manganese ions which may then bind to activation sites and partly because in the presence of some free Mn^{++}, Ca^{++} - GTP is at least as effective a substrate as Mn^{++}-GTP (Garbers, et al., 1978). The high calcium concentrations used in these studies however, suggest that this may not represent a physiological phenomenon. Intracellular

Mg^{++} levels however, are much higher than those of Ca^{++} and the stability constant for Mg^{++}-GTP is greater than that for Ca^{++}-GTP. Thus it is possible that Mg^{++} may be physiologically important for guanylate cyclase activation (Frey, Boman, Newman & Goldberg, 1977). This remains to be substantiated. Physiological concentrations of Ca^{++} have been reported to activate particulate guanylate cyclases from some mammalian sources (Levine, Steiner, Earp, Meissner, 1979). Also a Ca^{++}-CaM complex has been shown to activate membrane-associated guanylate cyclase from Tetrahymena (Nagao, Suzuki, Watanabe & Nozawa 1979), but calmodulin activation of soluble or particulate enzyme has not yet been reported in mammalian tissue.

A second category of agents that increase soluble cyclase activity in intact cells appears not to require calcium ions. These include a group of pharmacological agents such as azide, nitroprusside, nitroglycerin, nitrosoquanidine and nitrosureas (Murad et al., 1979), all of which act by liberating agents (such as nitric oxide (NO) or hydroxyl free radicals) that alter the redox state of the cell (Goldberg & Haddox, 1977), and thus cyclase activity. Oxidation of the enzyme or of some closely associated molecule also appears to modify its cation requirements. High concentrations (millimolar levels) of Ca^{++} are no longer able to stimulate cGMP production and the enzyme loses its requirements for Mn^{++}. The activated enzyme now seems able to utilize Mg^{++} as effectively as Mn^{++} as the sole cation cofactor (Murad et al., 1979; Goldberg & Haddox, 1977). The actions of these agents on the particulate enzyme fraction however are much less clear and only small effects have been reported thus far (Hardman, 1982).

On the contrary, both soluble and particulate fractions of the enzyme from several sources can be activated by lipids. Activity of guanylate cyclase in whole homogenates or in particulate and sometimes soluble fractions can be promoted by phospholipase A_2 (P.L.A_2) or by lysolecithin (Murad et al., 1979). Since lysolecithin is formed by phospholipase A_2, it has been suggested that intracellular guanylate cyclase can be indirectly stimulated by many agents through phospholipase A_2 activation (Shier, Baldwin, Nilsen-Hamilton & Thanassi, 1976). Indeed, several agents that increase cGMP levels in intact cells (including Ca^{++}, norepinephrine, acetylcholine, carbachol and serotonin) also enhance this enzyme activity. Fatty acids thus generated could raise the guanylate cyclase activity either directly or indirectly. Particulate cyclase can be activated by high levels of both saturated and unsaturated fatty acids. On the other hand, soluble guanylate cyclase can be stimulated by low concentrations of unsaturated but not saturated fatty acids (Hardman, 1980). It is possible thus that certain fatty acids (particularly the unsaturated acids) can interact directly with the cyclase to evoke activation (Ichihara El-Zayak, Mittal & Murad, 1978). Alternatively, fatty acid metabolites (such as fatty acid peroxides and prostaglandin endoperoxides) which give rise to hydroxyl radicals and also perhaps to some other oxygen species within the cell, (Murad et al., 1979; Hardman, 1980) may by influencing the redox potential, indirectly provoke cGMP production (Goldberg & Haddox, 1977). Ca^{++} may similarly mediate its action by such an oxidative process, possibly one involving ultimately the formation of oxidized metabolites of arachidonic acid (Goldberg & Haddox, 1977; Murad et al., 1979). Certainly, many phospholipases (including P.L.A_2) are calcium-sensitive and arachidonic acid and

its metabolites (which may subsequently activate the cyclase via formation of reactive free radicals) are released from various cells in response to agents which increase cytoplasmic Ca^{++} levels (Hardman, 1980).

Although, it is unknown which form of the enzyme (soluble or particulate) provides the major cellular catalytic activity, it is clear that hormonal effects on the cGMP levels may be mediated by a variety of mechanisms. Calcium level elevation, calmodulin - Ca^{++} complex formation, phospholipase A_2 activation and release of fatty acids or their metabolites, all may influence guanylate cyclase activity. Stimulation thus may be either by direct interactions with the enzyme or alternatively via the formation of a reactive oxygen species and consequently via oxidation of the cyclase.

1.2.3c Intracellular Action of Cyclic Nucleotides by Phosphorylation

The modification of protein activity by the enzymatic transfer of terminal phosphate from ATP to specific amino acids is an important mechanism for the regulation of numerous intracellular events. Cyclic AMP exerts many, if not all of its biological effects in eukaryotic cells by such phosphorylation, mediated by the activation of a specific protein kinase (Robison, Butcher & Sutherland, 1968). The cAMP-dependent protein kinase (A-kinase) appears to be mainly located in the cytosolic fraction of cells, but it has also been detected in nuclear and plasma membrane fractions of erythrocytes and brain tissue (Rubin, Rangel-Aldao, Sarkar, Erlichman & Fleischer, 1979). Two major types of A-kinase have been identified as types I and II by their relative elution patterns from anion exchange resins (Corbin, Keely, & Park, 1975). Both forms are tetramers which are

able to dissociate to form one regulatory subunit dimer (R_2) and two free catalytic (C) subunits (Hofmann, Beavo, Bechtel & Krebs, 1975). The holoenzyme is essentially catalytically inactive, but binding of cAMP reveals a latent activity by promoting its dissociation into the regulatory and catalytic forms to exhibit full enzyme activity

$$\underset{\text{(inactive)}}{R_2C_2} + 2\ cAMP \rightleftharpoons R_2.(cAMP)_2 + \underset{\text{(active)}}{2C}$$

(Nishizuka, Takai, Hashimoto, Kishimoto, Kuroda, Sakai & Yamamura, 1979). Apparently the catalytic subunits of both I and II are functionally and structurally very similar but the regulatory subunits (RI and RII) of the two species are immunologically distinct and structurally dissimilar (Doskeland & Ogreid, 1981). Type II A-kinase can catalyse the transfer of phosphate from ATP to two seryl residues in its own regulatory subunit thus promoting cAMP binding and consequently dissociation. Type I A-kinase is unable to mediate such autophosphorylation (Hofmann et al., 1975), although there is evidence that both RI and RII can be phosphorylated intracellularly (Geahlen & Krebs, 1980). A further distinction is that type I A-kinase only, binds Mg-ATP with high affinity (Hofmann et al, 1975). Under basal non-stimulated conditions the binding of Mg-ATP to allosteric binding sites on the holoenzyme decreases the affinity of the receptor units of RI for cAMP, (Bechtel & Beavo, 1974), not directly by competitive binding but indirectly by modulation of the interactions of the catalytic unit with the regulatory subunit thus preventing their dissociation (Hofmann, 1978). On the other hand, with type II A-kinase, MgATP will facilitate self phosphorylation of RII and increase cCAMP binding thus promoting the production of the active form of C.

Removal of MgATP will bring about the rapid recombination of isolated subunits to reform the inactive holoenzyme.

In a similar manner to cAMP the second cyclic nucleotide cGMP also mediates its biological actions via protein kinase activation. G-kinase is also widely distributed, present in highest amounts in the soluble fraction of homogenates of lung, cerebellum, small intestine pancreatic islets and heart (Doskeland and Ogreid, 1981). The enzyme may also be associated (to a lesser extent) with the nucleus (Steiner, Koide, Earp, Bechtel & Beavo, 1978) or with membrane fractions (Casenellie & Greengard, 1974). G-kinase exists as a dimer composed of two identical subunits (Nishizuka et al., 1979). Upon cGMP binding the enzyme becomes activated, but unlike the A-kinase R and C dissociation does not occur but rather the activation process appears to be a simple allosteric effect.

$$\underset{\text{(inactive)}}{E_2} + 2cGMP \rightleftharpoons \underset{\text{(active)}}{E_2.(cGMP)_2}$$

Comparable with the cAMP system, cGMP production and G-kinase activation also occur in specific subcellular compartments.

A further protein kinase quite independent of cAMP or cGMP has been recently described. This kinase possesses an absolute requirement for calcium ions and has been termed protein kinase C (or C-kinase). The enzyme is found in various tissues in mammals and in many of these its activity far exceeds that of A-kinase (Nishizuka, 1983). C-kinase (present as an inactive soluble form under normal conditions) exists as a single polypeptide with no apparent subunit structure. This chain of molecular weight 77,000 appears to be composed of two functionally distinct domains namely a hydrophobic membrane binding domain

and a hydrophilic domain carrying the catalytically active centre (Takai, Kishimoto & Nishizuka, 1982). The enzyme is able to phosphorylate many proteins, particularly those located in or just below the cellular membranes to which it may become reversibly attached. Activation of C-kinase requires Ca^{++} and specific membrane associated phospholipids. This reversible process is generally represented by:-

$$E + Factor + Ca^{++} \rightleftharpoons E.Factor.Ca^{++}$$

(inactive) (active)

(Nishizuka, et al., 1979)

The exact stoichiometry however is not clarified at this time. The reaction proceeds quite independently of calmodulin although it is possible that in some cases a calmodulin like moiety may be an integral part of C-kinase. Interaction with phospholipid may thus reveal the otherwise shielded calmodulin-like molecule and facilitate its association with Ca^{++} and subsequent enzyme activation (Zabrenetzky, Bruckwick & Lovenberg, 1981). In contrast, a further protein kinase which is specifically activated by Ca^{++}-CaM, can promote phosphorylation of histone, myosin light chain and phosphorylase kinase from many different sources. The CaM in this process cannot be replaced by phosopholipids (Nishizuka, et al., 1979).

At physiologically low Ca^{++} concentrations C-kinase possesses (in addition to its phospholipid dependence) a requirement for diacylglycerol (DG) which may be transiently derived from PI turnover in a signal-dependent manner (Nishizuka, 1983). Kinetically, DG sharply increases the affinity of the C-kinase for Ca^{++} and phospholipid and thus may render the enzyme fully active without net increases of Ca^{++} levels. Furthermore, DG may serve to reversibly anchor C-kinase in the membrane as a prelude to activation (Takai et al., 1982).

Individual phospholipids appear to exert positive or negative co--operativity on the kinase activity. In the presence of phosphatidylserine, enzyme activity is further enhanced by the addition of phosphatidylethanolamine (PE) in low Ca^{++} levels whereas phosphatidylcholine (PC) or sphingomyelin diminshes this activity. In general the latter phospholipids are extensively located in the outer membrane monolayer and therefore the lipid distribution within the bilayer may have a role in differentially influencing enzyme activation (Takai, et al., 1982).

Agents promoting PI turnover (largely alpha-agents) which may enhance C-kinase activity, also appear to be associated with elevated cGMP production and subsequent G-kinase stimulation. C-GMP induced protein phosphorylation is inversely correlated with the inhibition of DG formation and appears thus to act as a negative messenger limiting C-kinase activity and hence preventing overstimulation. Beta-agonists via A-kinase similarly block PI metabolism and will rapidly inhibit C-kinase activity (Takai et al., 1982). Since PC prevents C-kinase acitivity (Nishizuka, 1983), it is significant that methylation of PE to PC is intimately related to beta-receptor functions (Hirata, Strittmatter & Axelrod, 1979; Takai et al., 1982b). Phospholipid methylation may result from membrane perturbations or be an integral part of the receptor-cyclase activation mechanism and thus in some way may serve to stimulate A-kinase and/or inhibit C-kinase.

Thus, combinations of the effects of calcium, phospholipid and DG on the kinases are clearly complex and subject to both substrate and feedback inhibition. There may also be +ve and -ve co-operativity involved. The relationships between the transmembranal control mechanisms for the regulation of cellular activity are summarised in diagram (10), (modified from Takai, et al., 1982, a; b).

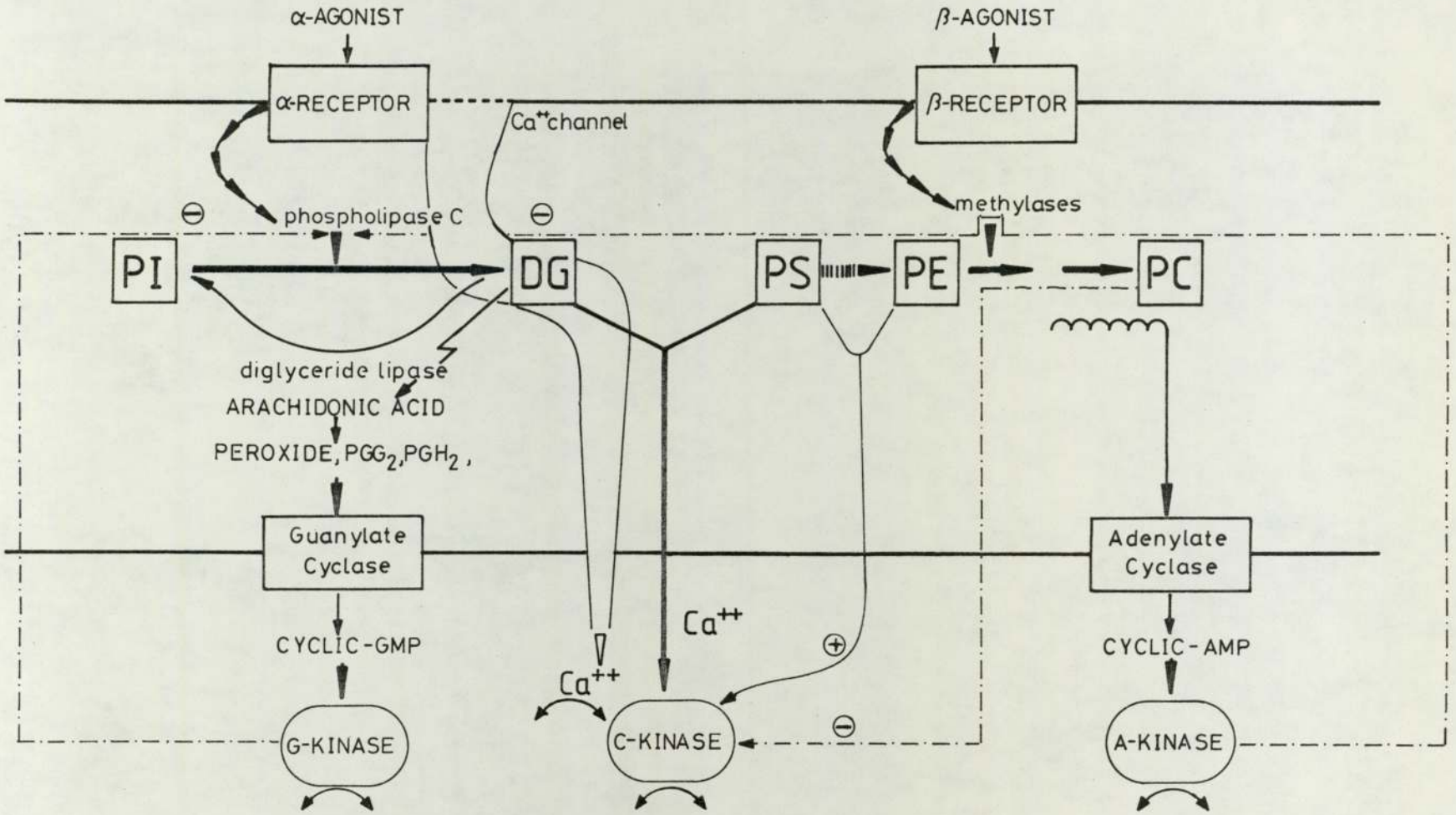


Diagram 10 Schematic Outline of Possible Mechanisms of Transmembrane Control Including Two Major Receptor Functions.

Key: PI, Phosphatidylinositol: D.G., diacylglycerol: PS, phosphatidylserine: PE, phosphatidylethanolamine: PC, phosphatidylcholine: PGG2, prostaglandin G_2: PGH_2, prostaglandin H_2.

1.2.3d Intracellular Regulation of Cyclic Nucleotide Action

The extent of protein phosphorylation induced by the cyclic nucelotide kinase enzymes will be limited by dephosphorylation mechanisms and by reduction of cyclic nucleotide concentrations. This may occur by inhibition of cyclase enzyme activity, by cyclic nucleotide extrusion or by their catabolism.

In the previous section it was seen that a c-AMP induced phosphoprotein may inhibit alpha-receptor-stimulated processes by preventing phospholipid turnover (Takai et al., 1982), thereby depressing intracellular cGMP formation and subsequent G-kinase induced phosphorylation. It is interesting therefore that the cellular actions provoked by many alpha stimulants are closely associated with decreased cAMP levels (Levitzki, 1981). This reduction is mediated via the inhibition of adenylate cyclase by a direct plasma membrane related event rather than by a second messenger system. The transduction of the inhibitory signal from alpha-receptors to adenylate cyclase involves a process in which GTP and sodium ions may play essential roles (Jakobs, Aktories, Lasch, Saur & Schultz, 1980). Alpha-receptor binding may mediate the association of GTP and Ni (Rodbell, 1980) which reduces the affinity of the beta-receptor for its agonists and thus inhibits adenylate cyclase (Lefkowitz, Caron, Michel & Stadel, 1982). In other systems however, alpha-receptor occupancy and adenylate cyclase inhibition appears to require high concentrations of GTP to be effective. Indeed the GTP requirement for alpha-mediated inhibition is higher than that needed for beta-mediated stimulation of adenylate cyclase. In addition, some inhibitory hormonal factors may increase the inherent cellular GTPase activity, leading to trans-

formation to the GDP bound, low activity state of the enzyme (Jakobs, et al., 1980). A similar, but independent inhibition of beta-receptor agonist affinity is exerted by sodium ions (U'Pritchard and Snyder, 1978). Alpha-receptor mediated inhibition of adenylate cyclase is also greatly potentiated by sodium ions (Michel, Hoffman, Lefkowitz, 1980). Since hormone receptor association induces Na^+ influx in many cell types it is most unlikely that the Na^+ requirement of this inhibition of adenylate cyclase is simply casual and not causal. The mechanism(s) underlying the effect of Na^+ however, are unknown.

Cyclic nucleotide extrusion represents a significant contribution to the intracellular homeostasis of these molecules in lower phyla (Konijn, 1972). Mammalian cells also extrude substantial amounts of both cyclic nucleotides against a concentration gradient and for cAMP at least, a specific carrier has been implicated (Clark, Su, Ortmann, Cubbedu, Johnson & Perkins, 1975). Probably the most important physiological regulators of intracellular cyclic nucleotide levels however, are the phosphodiesterases (PDE's) which are enzymes that catalyse the irreversible hydrolysis of the nucleotides to their respective 5'- nucleotide monophosphates (Butcher & Sutherland, 1962; Thompson, Little & Williams, 1973). Several PDE's have been identified which differ in their tissue distribution, kinetic properties, substrate specificities, stabilities and ionic requirements (Weiss & Greenberg, 1978). Most mammalian tissues appear to consistently contain at least three distinct PDE activities which are controlled independently (Strada, 1982; Weiss & Greenberg, 1978; Beavo, Hansen, Harrison, Hurwitz, Martins & Mumby, 1982).

a) Calmodulin - Sensitive PDE

The most intensively studied is the Ca^{++}-CaM-activated PDE which exists in at least three isoenzymic forms, which can be differentiated by molecular weight and substrate specificity (Beavo et al., 1982). The isoenzyme from brain has a higher molecular weight than the heart form but most evidence indicates that both of these enzymes exist as dimeric proteins consisting of two apparently identical subunits (Chau, Huang, Chock, Wang & Sharma, 1982), each of which has a CaM binding site. When all four Ca^{++}-binding sites on the calmodulin molecule are occupied, a hydrophobic region is exposed which allows binding to a metal ion-independent site on PDE (Gopalakrishna & Anderson, 1983; 1982). When a calmodulin - Ca^{++}complex binds to each subunit of the dimer a conformational change is induced (Wolffe, Brostrom, 1979) by which the influence of an integral inhibitory peptide sequence on the catalytic domain of PDE is suppressed and activation of the enzyme ensues (Klee, Crouch & Richman, 1980). The activated enzyme can utilize cAMP or cGMP as substrate but possesses a higher affinity for the latter (Strada, 1982).

Products of phospholipase A_2-catalysed hydrolysis of PI, acidic phospholipids and unsaturated fatty acids mimic the stimulatory effect of CaM on PDE in brain (Gietzen, Sadorf & Bader, 1982) but do not however possess a requirement for Ca^{++} for their action (Gietzen, Xu, Galla & Bader, 1982). Combination of calmodulin and certain PI metabolites (in particular lyso-derivatives of acidic phospholipids) show synergism at suboptimal concentrations (Tai & Tai, 1982). Although the physiological relevance of these observations is still unknown, very recently an alpha-receptor mediated activation of PDE

has been reported (Meeker and Harden, 1982). Thus, it is possible that alpha-receptor dependent phospholipid and fatty acid production (in addition to promoting quanylate cyclase activity) may also have an important role in the stimulation of cAMP hydrolysis. A further Ca^{++}-CaM sensitive PDE with high affinity and specificity for cAMP has been described in developing chick heart muscle, in liver membranes and in bovine lung (Purvis, Olsen & Hansson, 1981; Andrenyak & Epstein, 1982). It does not cross-react with antibody raised against the major heart or brain calmodulin-dependent PDEs; in fact very little is known about the molecular mechanisms of the CaM-induced activation of this isoenzyme.

b) Cyclic GMP - Binding PDE

The second distinct PDE activity also exists in three isoenzymatic forms each of which possesses a high affinity cGMP binding site but has different kinetic, regulatory and physical properties. The c-GMP binding site is distinct from the substrate site which may utilise cAMP or cGMP (Coquil, Franks, Wells, Dupuis & Hamet, 1980; Beavo et al., 1982). Although the probable role of this specific site has been elucidated only in the case of the 'cGMP-stimulated PDE', it is not unreasonable to assume a similar function in the other isoenzymes. Thus, at physiological levels occupation of the cGMP site may induce in a co-operative manner, activation of the catalytic site (Beavo et al., 1982). The inhibition of cAMP hydrolysis by cGMP observed at higher concentrations is probably due simply to competitive inhibition at the catalytic site (Martins, Mumby & Beavo, 1982).

The GMP-stimulated PDE isoenzyme (a single molecular weight polypeptide) has been isolated and purified from bovine adrenal liver

and heart tissue. Kinetic analysis indicates that cGMP at the allosteric site promotes hydrolysis of either cyclic nucleotide but shows a greater positively co-operative effect for cAMP (Martins, Mumby & Beavo, 1982). In adrenal and liver this isoenzyme accounts for greater than 80% of the total cAMP hydrolysis which implies that it is of considerable importance in the regulation of flux and steady state levels of the cyclic nucleotide in these tissues (Beavo et al., 1982). In rod outer segments of the retina, bleaching of rhodopsin facilitates the association of GTP and transducin (composed of two large polypeptide chains and a smaller subunit) which activates a further PDE (Stryer, Hurley & Fung, 1981). Conversion of GTP to GDP catalysed by GTPase (which appears to be an integral part of the transducin protein) limits this reaction (Pober & Bitensky, 1979). The major substrate of this iso-enzyme is cGMP but it will catalyse the hydrolysis of cAMP albeit with a much lower efficiency (Yamazaki, Bartucca, Ting & Bitensky, 1982). In lung and platelets a third isoenzyme which appears to be even more specific for cGMP catabolism (having a very low affinity for cAMP as substrate), has been isolated (Beavo, et al., 1982). In contrast to the other isoenzymes, recent evidence suggests that in lung low concentrations of cGMP do not increase the rate of cAMP metabolism (Hurwitz, Hansen, Harrison, Martins, Mumby & Beavo, 1983). The function of the high-affinity binding site for cGMP in this case thus awaits clarification.

c) Cyclic AMP-Specific PDE

The third form of PDE, with a high affinity for cAMP and little activity towards cGMP is found in most tissues associated with particulate fractions (Beavo, et al., 1982). The kinetic properties of this isoenzyme are suggestive of either multiple substrate

sites having a negatively cooperative interaction,or of multiple enzyme forms that have not been completely separated by the purification procedure (Weiss & Greenberg, 1978). This PDE from membranes of fat and liver cells appears to be activated (by insulin) by a mechanism which apparently involves cAMP-induced-phosphorylation and is inhibited by cGMP (Marchmont & Houslay, 1980). The activity of the cAMP-specific enzyme is also enhanced in several cell types by agents which are known to stimulate adenylate cyclase (Murray & Russell, 1978). The physiological relevance of these findings however, remains to be clarified.

Despite the possibility that the activation of the individual forms of the PDE's might be coupled to other cellular processes the above observations are consistent with the assumption that the major function of these PDE's is to ensure that hormonally-induced elevations in cyclic nucleotides are self regulating and that the steady-state levels of the nucleotides are maintained within the cell.

1.2.4 The Role of Monovalent Cations

In addition to the primary signal transducers (discussed in previous sections of this introduction) it has been suggested that the monovalent cationic environment may also provide a messenger system. Probably the main candidates for roles as regulators are sodium (Na^+) potassium (K^+) and hydrogen (H^+) ions. It is at present unclear as to whether these species act directly to mediate their effects or do so via the modulation of the homeostatic systems of the primary messengers, namely the cyclic nucleotides and the divalent cations.

The sodium ion (as previously discussed) influences the binding of agonists to alpha- and beta-adrenergic receptors (Michell, et al., 1980), and thus indirectly may exert an effect on many cyclic nucleotide dependent mechanisms. Furthermore, in most biological systems, different modalites exist by which Na^+ interferes with Ca^{++} fluxes, and therefore Na^+ may also have profound effects on the widespread calcium sensitive processes within the cell. A decrease in the extracellular Na^+ levels provokes an inward Ca^{++} current across the membrane (Meltas & Nikezic, 1982). Conversely elevated extracellular Na^+ concentrations depress cytosolic Ca^{++} levels by either inhibiting the Ca^{++} uptake process (even if ionophores are employed (Couturier, Deleers & Malaisse, 1980)), or possibly by promoting Ca^{++} extrusion from the cell via the Na^+/Ca^+ - countertransport system predominantly attributed to excitable tissue.

The transmembranal distribution of sodium ions (high outside, low inside), favours the inward movement of the ion. Thus any chemically or electrically provoked alteration of membrane permeability may result in the rapid inflow of Na^+ ions into the cytosolic environment (Lazo, Barros, de la Pena, Ramos, 1981). Na^+ influx may occur via a specific Na^+ channel regulated by cytosolic Ca^{++}-dependent phosphorylation (Shaafi, Molski, Naccache, 1981; Kimmich & Randles, 1982) or via a voltage sensitive Na^+ channel found predominantly if not exclusively in electrically excitable tissue (Catterall, 1982). The channels are glycoproteins containing carboxyl groups within the pore responsible for their ion selectivity (Stefani & Chiarandini, 1982). Since the total number of sodium channels in any cell appears to remain constant the Na^+ ion conductance is dependent on the gating of specific preformed transmembrane channels in a

similar manner to gating in Ca^{++} channels (Ehrenstein, Lecar & Latorre, 1978). The opening of such channels thus results in an increased cytosolic Na^{+} concentration which may also modulate intracellular Ca^{++} levels. Certainly, sodium can promote by Ca^{++} mobilization from organelle-bound pools, such as mitochondria isolated from heart, skeletal muscle, brain and adrenal cortex (Nicholls, 1978).

Paradoxically perhaps both intracellular and extracellular Ca^{++} ions can in turn influence the membrane permeability to Na^{+}. Ca^{++} has a stabilizing effect on the plasmalemma such that its chelation from the extracellular environment induces an increased membrane permeability and cation leak (Shanes, 1958; Quastel, Segal & Lichtman, 1981). Since extracellular Ca^{++} and Na^{+} compete for anionic binding sites on the outer surface of the sarcolemma it is possible that membrane bound Ca^{++} in the proximity of the Na^{++} pore restricts monovalent cation flux into the cell (Hohl, Altschuld and Brierley, 1983). Furthermore intracellular Ca^{++} appears to induce the closure of specific Na^{++} channels by membrane modification, most probably via a Ca^{++} dependent protein kinase (Kimmich & Randles, 1982). It is conceivable that such Na^{+}/Ca^{++} interplay is a subtle but rather complex mechanism which ultimately regulates intracellular Ca^{++} concentration. Thus, it may serve to ensure that at all times there are appropriate amounts of Ca^{++} available for the many Ca^{++} mediated processes.

The K^{+} ion is handled simultaneously by the same membrane pump as the Na^{+} ion and thus K^{+} may similarly have a pertinent role in the regulation of specific cellular events. Indeed intracellular K^{+} ions are required for the maintenance of resting low internal free Ca^{++} levels (Fiskum & Lehninger, 1982) and for the

functioning of several K^+ - dependent cytosolic processes (Kaplan, 1978). There are several specific transmembrane channels for K^+ flux, a) a channel specific for a fast K^+ current involved in the repolarization phase of an action potential which immediately follows the inflow of Na^+, b) a slow channel (to date only demonstrated in frog tonic fibres, c) a Ca^{++} dependent K^+ channel which opens as a consequence of increased intracellular Ca^{++} concentrations and d) an inward channel which is responsible for the resting conductance of a cell and is an integral part of the (Na^+/K^+)-dependent pump (Stefani & Chiarandini, 1982).

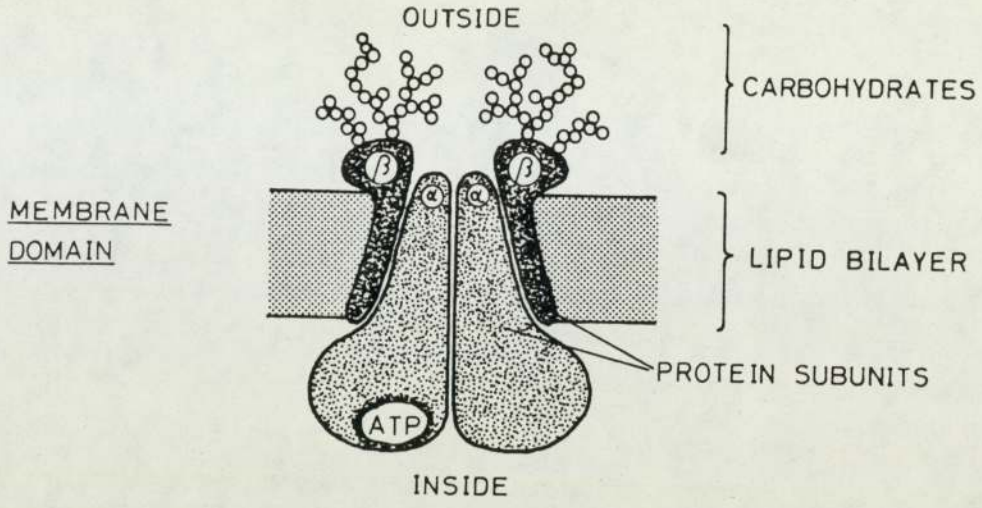
Both potassium and sodium ions in resting cells are unequally distributed across the plasma membrane. The presence of an ATP-dependent pump specific for K^+ and Na^+ thus is required in the membrane of resting cells to maintain a high potassium, low sodium internal environment and conversely a low potassium, high sodium exterior (Racker, 1976). Each functional unit of this (Na^+/K^+)-ATPase consists of two large (alpha) subunits that span the membrane, and two smaller (beta) glycoprotein subunits that are exposed to the outer surface of the membrane. Each enzyme has one (or two) functioning phosphorylation site (Sweadner & Goldin, 1980; Peters, Swarts, de Pont, Schurmans-Stekhoven & Bonting, 1981), three binding sites for Na^+ and two binding sites for K^+ per tetramer (Sweadner & Goldin, 1980). The sites located on the inner surface of the membrane have a high affinity for Na^+ and a low affinity for K^+, whereas the second species of sites present on the external surface bind K^+ with a much greater affinity than Na^+. The precise positions of these binding sites on the subunits are unknown (Sweadner & Goldin, 1980). Although the affinity of the internal sites is about ten fold greater for Na^+

than for K^+, the high intracellular K^+ levels cause some competitive inhibition at each of the three Na^+ sites to ensure that sodium must occupy all of the sites in order to activate catalysis and cationic transport (Lindenmayer, 1976). The enzyme, by the expenditure of metabolic energy drives $3Na^+$ ions out of the cell and simultaneously transports $2K^+$ ions inward probably by a cyclical process. When sodium ions bind to their specific sites, a magnesium requiring phosphorylation of the enzyme is induced and a consequent conformational change permits sodium translocation outwards. Potassium ions bind to the phosphoenzyme and are thus transported in the opposite direction. This phosphorylated intermediate subsquently becomes hydrolysed and the conformation returns to that of the inactive enzyme (Sweadner & Goldin, 1980). A schematic diagram of this model is given in diagram (11).

Other models suggest that a second phosphoenzyme exists as a result of K^+ binding (Lindenmayer & Schwartz, 1975) and that Mg^{++} may bind to this species and therefore also play a role in the dephosphorylation step (Mardh, 1982). The overall reaction may be summarised thus:-

$$E \overset{Na^+}{\rightleftharpoons} E_1.Na^+_3 \overset{ATP \xrightarrow{Mg^{++}} ADP}{\rightleftharpoons} E_1P \overset{K^+}{\rightleftharpoons} E_2P \xrightarrow{(Mg^+?)} E + Pi$$

The extent of pump activity can be modified by two independent processes namely the regulation of the number of pump molecules or the alteration of the sensitivity of the ATPase to both Na^+ and K^+ ions. Although little is known about the metabolism of the pump enzyme, those cells subject to high rates of depolarization or which must secrete sodium are enriched with the pump suggesting that



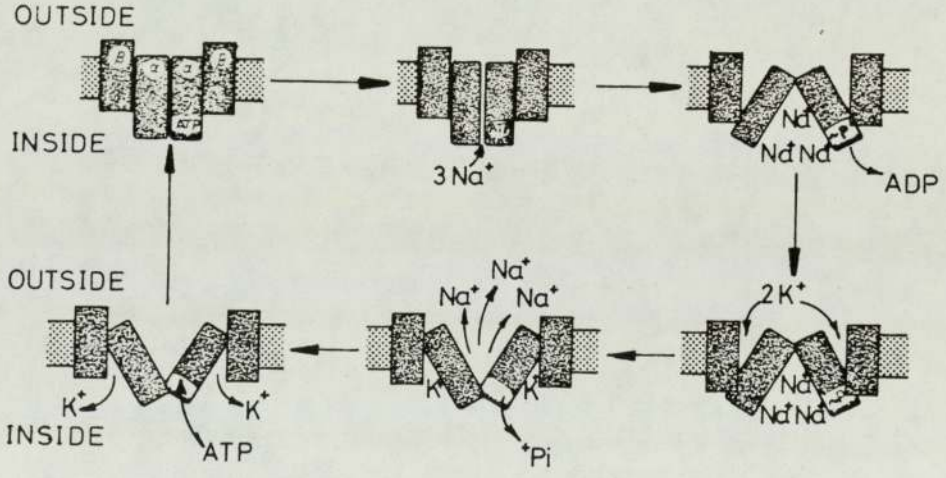


Diagram 11 The Sodium - Potassium Membrane Pump

a) Diagrammatic view of the sodium - potassium - ATPase

b) A model illustrating schematically how active transport might result from cyclic conformational changes of the sodium - potassium ATPase enzyme.

the ions themselves (K^+ and Na^+) may somehow modify the balance between their biosynthesis and degradation (Lindenmayer, 1976). Evidence also supports the existence of a pool of cryptic (Na^+/K^+)-ATPase sites in lymphocyte membranes which may become exposed to Na^+ as a result of some induced conformational change (Hume, Vijakumarek, Schweinberger, Russell & Weiderman, 1978). Alternatively, pump activity may be influenced by the assembly of preformed polypeptide chains to produce an active enzyme in the membrane (Kaplan & Owens, 1980).

The (Na^+/K^+) pump is more active in a high 'fluid' membrane environment than in a 'gel' like state. Thus the ratio of fluid to solid domains in the cell membrane determined by the sterol content will significantly modify the pumping mechanism (Lindenmayer, 1976). The enzyme activity can also be influenced negatively by the presence of calcium ions. Ca^{++} inhibits (Na^+/K^+)-ATPase activity by Ca^{++} - Mg^{++} competition, Ca^{++}.ATP - Mg^{++}.ATP competition or a combination of both mechanisms at the Mg^{++} binding site of the catalytic subunit (Huang and Askari, 1982; Beauge & Campos, 1983). However, since millimolar levels of Ca^{++} were used in these studies, it may not represent a physiological effect. At physiological levels, calcium is able to inhibit the enzyme by competitive inhibition between Na^+ and Ca^{++} at the binding region which appears to raise the sodium threshold for activation of enzyme phosphorylation (Lindenmayer and Schwartz, 1975). Eventually the new threshold for Na^+ may be reached due to passive sodium influx. Pump activation by noradrenaline and other alpha-adrenergic agents may therefore conceivably be achieved by the displacement of an inhibitory Ca^{++} pool located on the internal face of the membrane, in the microenvironment of the pump (Capiod, Berthon, Poggioli, Burgess & Claret, 1982). Thus, Na^+ and K^+

directly or via a complex interplay with Ca^{++} ions may serve as signal transducers.

The electrochemical proton gradient which exists within the physiological system may also serve to trigger cellular responses. Certainly the external pH can indirectly influence the (Na^+/K^+)-ATPase by modifying the ratio of fluid to solid domains in the plasma membrane (Lindenmayer, 1976). More directly, the internal pH can modulate enzyme activity by altering the affinity of the pump for Na^+ and/or K^+ (Bernhardt & Glaser, 1982a, b, c). The E_1 Na^+ - form of the pump is considered to be deprotonated whereas the E_2 K^+ form is protonated probably as a result of the reprotonation of the E_1 Na^+ form (Skou, 1982). Due to its effect on Na^+ and K^+ internal concentrations, pH may thus indirectly influence the cytosolic Ca^{++} concentrations. Furthermore there also appears to be a direct effect exerted by extracellular pH fluctuations on the internal Ca^{++} levels. Isolated mitochondria, endoplasmic reticulum vesicles and plasma membranes are all influenced by the H^+ concentration ($[H^+]$) (Borle, 1981; Haynes, 1982; Ponce-Hornos, Langer & Nudd, 1982; Fiskum & Lehninger, 1980). H^+ is a competitive inhibitor of Ca^{++} uptake by mitochondria; a high pH increases the affinity of the transport system for Ca^{++} and enhances Ca^{++} transport and its sequestration, whereas low pH has the reverse effect (Ponce-Hornos et al., 1982). In sarcoplasmic reticulum and endoplasmic reticulum the effects of pH are the opposite of those observed in mitochondria. A high pH will depress binding and uptake of Ca^{++} and in S.R. vesicles will induce the release of accumulated calcium and thus may act to raise intracellular Ca^{++} levels (Sulakhe, Drummond & Ng, 1973). In support, calcium uptake by muscle sarcolemma vesicles is inhibited by elevating the H^+ ion concentration (Sulakhe et al., 1973).

Transmembranal ion gradients thus, may be considered as a means of transferring information. The messenger systems discussed must not however be thought of as separate or alternative pathways of cell activation, but rather as a complex web of interrelated functions. The systems may operate in some precise type of co-operative or antagonistic manner to couple an extracellular stimulus to a specific cellular response possibly by ultimately modifying the intracellular Ca^{++} level.

Certainly it has been observed that complex interactions between the divalent cations, monovalent cations and cyclic nucleotides provide the necessary coupling between secretagogue-receptor association and secretion in endocrine and exocrine tissue and chemical or electrical stimulation and contraction in smooth, cardiac and skeletal muscle.

1.3 STIMULUS-SECRETION COUPLING

Both vesicular and non-vesicular secretions are regulated by the intracellular levels of cations and cyclic nucleotides. The events involved in some of the secretory processes are summarised below:-

1.3.1 The Parotid Gland (See diagram 12)

(a) Water and Electrolyte Secretion;

key to diagram (12):-

(i) Binding of agonists e.g. substance P, alpha-adrenergic agents and cholinergic agents to membrane receptors (Rudich & Butcher, 1976; Douglas & Poisner, 1962).

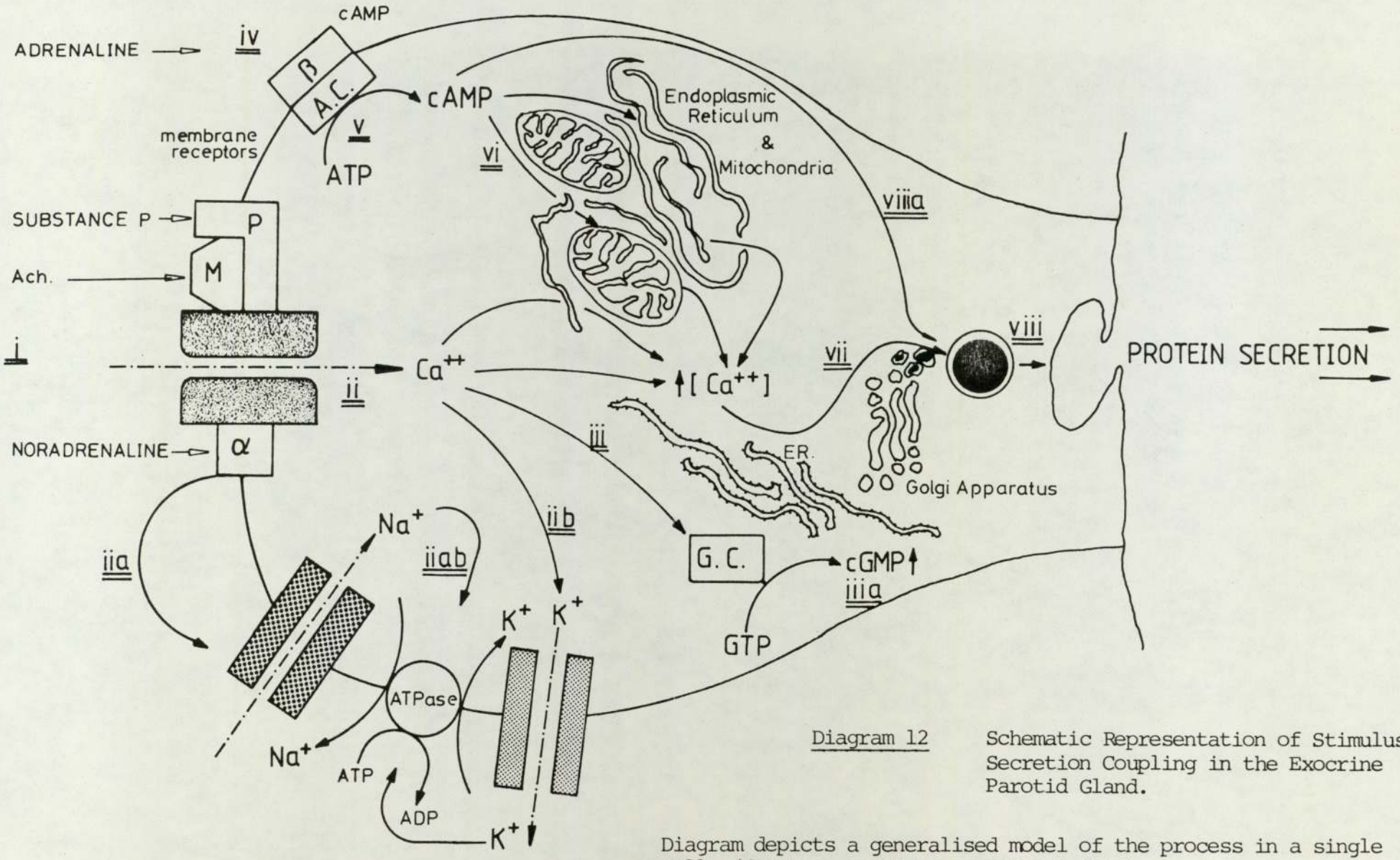


Diagram 12 Schematic Representation of Stimulus-Secretion Coupling in the Exocrine Parotid Gland.

Diagram depicts a generalised model of the process in a single cell: (for key, see text, section 1.3.1.).

(ii) Ca^{++} influx through specific channels (Putney, 1977; Marier & Van de Walle, 1978).

(iia) Binding associated Na^{+} influx, possibly via potential dependent channels (Tangkrisanavininot & Pholpramool, 1979).

(iia,b) Activation of (Na^{+}/K^{+})-ATPase (Putney & Parod, 1978).

(iib) Ca^{++} provoked K^{+} efflux (the major electrolyte secreted) (Putney, 1977).

(iii) Ca^{++} stimulated guanylate cyclase (Rudich & Butcher, 1976)

(iiia) cGMP function unclear

(b) Protein Secretion

(iv) Binding of agonists to beta-receptors, or exogenous cAMP application (Schramm & Selinger, 1975).

(v) Activation of adenylate cyclase

(vi) cAMP-induced mobilization of internal Ca^{++} (Kanagasuntheram & Randle, 1976).

(vii) Ca^{++}-induced release of secretory vesicles from golgi apparatus

(viii) Ca^{++}-promoted fusion of vesicles and secretory apparatus of the plasma membrane and exocytosis (Butcher, 1978).

(viiia) cAMP-potentiates the action of Ca^{++} on amylase secretion (Butcher, 1978).

1.3.2 The Exocrine Pancreas (diagram 13)

Two possible pathways for exocytotic release of enzymes; key to diagram (13):-

(A)(i) Binding of agonists e.g. ACh

(ii) Immediate mobilization of some membrane bound calcium and therefore membrane depolarization (Peterson, 1980)

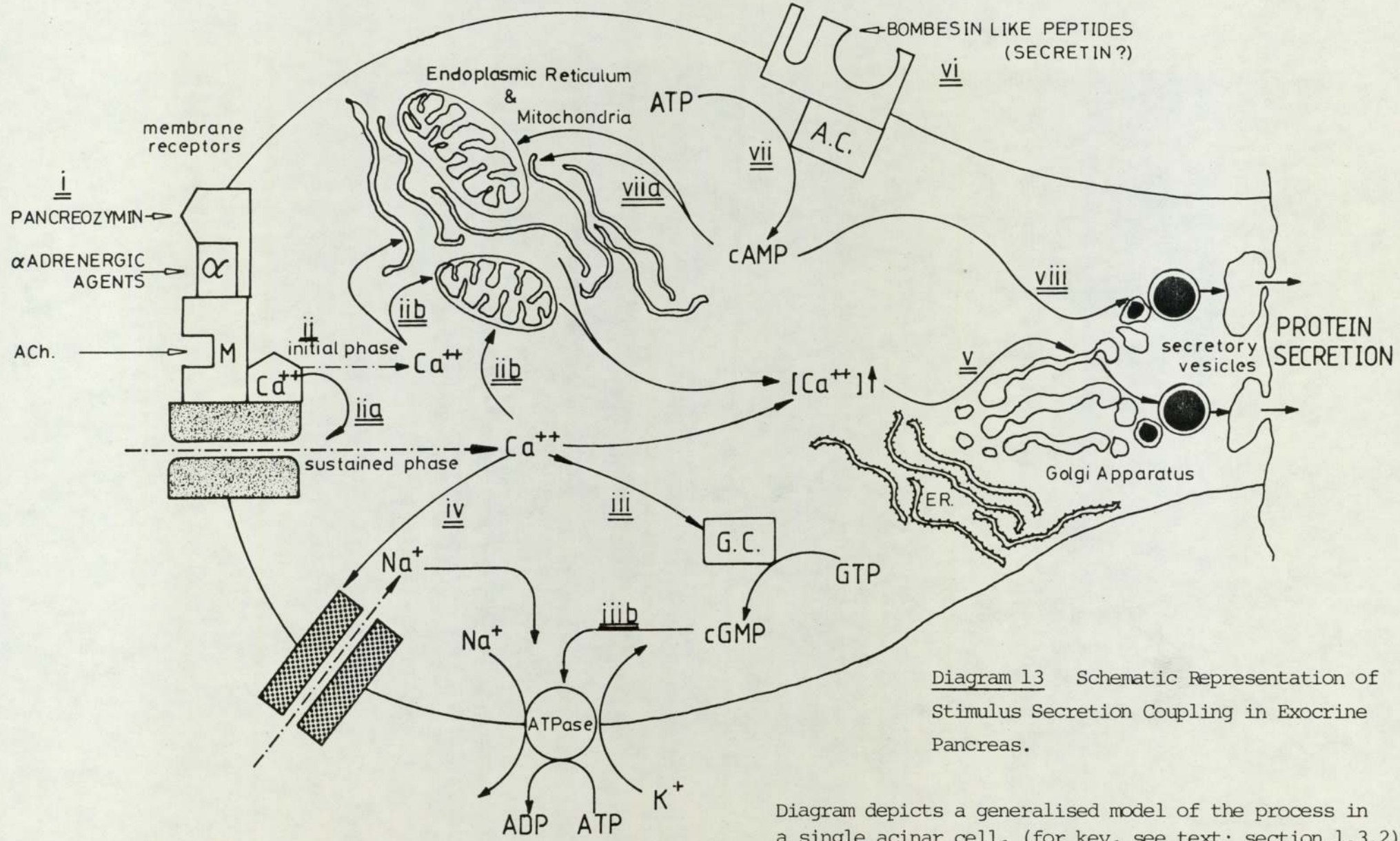


Diagram 13 Schematic Representation of Stimulus Secretion Coupling in Exocrine Pancreas.

Diagram depicts a generalised model of the process in a single acinar cell, (for key, see text: section 1.3.2).

(iia) Increased membrane permeability and Ca^{++} influx (Laugier & Petersen, 1980).

(iib) Mobilization of cellular calcium (Williams, 1980a; Ponnappa Dormer & Williams, 1981).

(iii) Activation of guanylate cyclase (Singh, 1980).

(iiia) Elevated cGMP - function unclear but may have a primary role in protein secretion (Pearson, Davison, Collins & Petersen, 1981).

(iiib) cGMP activated (Na^+/K^+)-ATPase which may be involved in fluid release (Stewart, Sax, Funk & Sen, 1979).

(iv) Calcium induced Na^+ influx which may be directly, or indirectly via the ATPase, involved in fluid secretion (Williams, 1980b)

(v) Ca^{++} promoted fusion of vesicles (from golgi) and secretory apparatus at the membrane then exocytosis (Creutz, Pazoles & Pollard, 1979).

(B)(vi) Binding of agonists (e.g. bombesin)

(vii) Activation of adenylate cyclase (Pearson et al., 1981).

(viia) Elevated cAMP may promote mobilization of sequestered Ca^{++} thus promotion of exocytosis (as in v)

(viii) Direct cAMP-promoted enzyme secretion (Pearson et al., 1981).

(N.B. Fluid secretion and protein secretion are physiologically linked; water and electrolytes must be secreted in sufficient amounts to purge the secreted protein. The release of fluid, rich in Na^+ and bicarbonate ions (in addition to the above) is evoked by secretin, acting via adenylate cyclase (Smith & Case, 1975) and possibly by elevated Ca^{++} (Ueda & Petersen, 1977) as a result of cAMP action (Schulz, Milutinovic & Heil, 1978).

1.4 EXCITATION-CONTRACTION COUPLING

Unlike secretory tissues, which lack direct electrical excitability, muscular tissue is able to respond to both electrical and chemical stimulation. Contraction may be initiated in all three classical muscle types by a rise in cytosolic calcium (Blinks, Pendergast & Allen, 1976; Solaro, Wise, Shiner & Briggs, 1974; Ruegg, 1971). By binding to troponin/tropomyosin units,calcium de-represses the contraction producing interaction between actin and myosin (Ebashi, Nonomura, Toyo-oka & Katayama, 1976). Thus an increased cytosolic calcium level activates the contractile apparatus (Ebashi, 1972). As in the secretory tissue the signal calcium may originate from either the extracellular environment or intracellular calcium stores. In skeletal muscle, the coupling calcium is provided exclusively from internal stores, in particular, the sarcoplasmic reticulum (SR), whilst cardiac and smooth muscles are able to employ both reservoirs (Sandow, Krishna, Pagala & Sphicas, 1975; Ebashi & Endo, 1968; Langer, 1973). The heterogeneous nature of muscle types prohibits somewhat, generalizations concerning the coupling mechanisms. However, as a rule of thumb, the source of signal calcium is dependent on the quantity of cellular sarcoplasmic reticulum (Devine, Somlyo & Somlyo, 1972). Smooth muscles such as guinea pig taenia coli, which lack a well defined S.R., depend almost exclusively upon extracellular calcium and its influx following depolarization (Anderson, Ramon & Snyder, 1971). In such muscle, repeated action potentials may be necessary to activate contraction via Ca^{++} uptake (Casteels & Raeymaekers, 1979). In contrast, those muscles possessing extensive S.R. (including intestinal and arterial smooth muscle and most skeletal muscle) do not exhibit significant transmembrane calcium currents.

(Mangel, Nelson, Connor & Prosser, 1979; Langer, 1976). In this case, the action potential may be transmitted via transverse tubules in the S.R. membrane thereby promoting Ca^{++} efflux via a voltage dependent channel (Miyamoto & Racker, 1981). Alternatively, any Ca^{++} crossing the sarcolemma from the external environment may induce Ca^{++} release from S.R. via a Ca^{++} gated channel (Miyamoto & Racker, 1982). In the extracellular calcium dependent forms of muscle, the relaxation appears to require re-export of the triggering calcium via sodium/calcium exchange (Blaustein, 1977) which itself requires re-establishment of potential gradient (hyperpolarization), (Burgen & Spero, 1968; Tillisch, Ham & Langer, 1979). In the intracellular calcium dependent muscle, the calcium is generally rebound by S.R. via the action of a cyclic AMP sensitive calcium pump (Hurwitz, Fitzpatrick, Debbas, Landon, 1973; Katz, Tada & Kirchberger, 1975). Thus it is evident that muscular contraction is highly dependent on cytosolic calcium originating from extracellular or intracellular calcium stores. The next section is devoted to a discussion of the possible role of such a coupling process (and its complex interplay with the other putative messengers) in mitotic activation.

2.0 STIMULUS-MITOSIS COUPLING

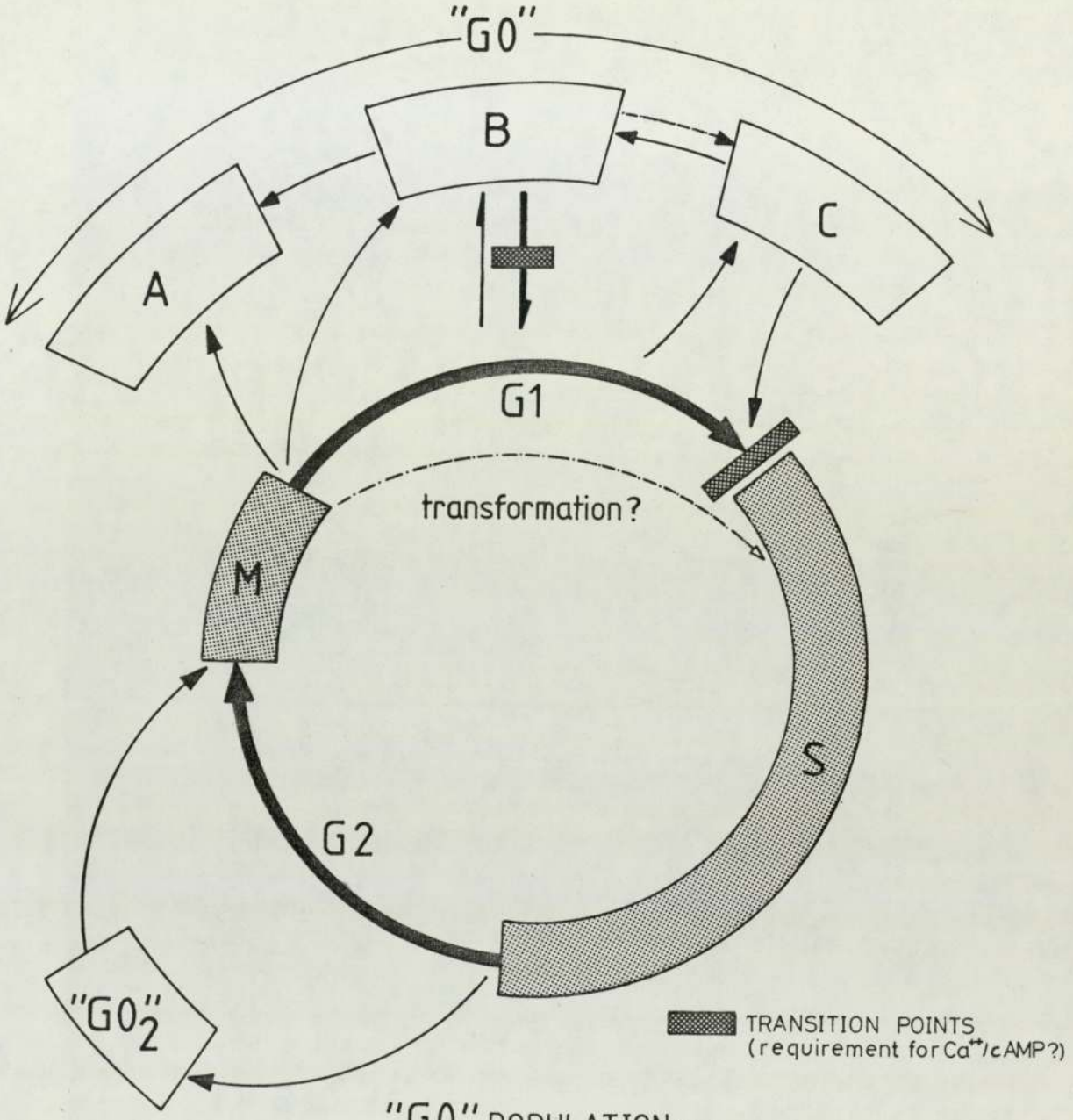
2.1 THE CELL CYCLE

In the strict sense the cell cycle is the interval between the midpoint of mitosis and the midpoint of a subsequent mitosis in one daughter cell or both (Mitchison, 1971). The definition of the cycle however, is traditionally broadened and is thus considered an ordered sequence of interrelated processes distinguishable by physiological and cytological transitions into four clearly recognizable phases of activity. Two well defined events occurring during the cycle, DNA synthesis (S-phase) and mitosis (M-phase) are separated by two gap phases (G1 and G2) (Howard & Pelc, 1953). The G1 phase is the most variable period of the cell cycle, its length largely influencing the rate of cellular proliferation (Hochhauser, Stein & Stein, 1981). In general, slowly growing populations consist of cells with G1 periods longer than those of rapidly dividing cells, and it is thought that some rapidly proliferating cell lines may be totally without a G1 phase (Liskay & Prescott, 1978). In contrast the G2 phase (which follows DNA replication) is short and relatively invariable. During G2 the cell prepares for imminent mitosis by undergoing chromosome condensation and massive microtubule redistribution (Prescott, 1976).

The cell cycle has also been considered as a two phase model (Smith & Martin, 1973). The A phase, of indeterminate length corresponds to early G1 in the classical model, whereas the B phase comprises late G1, S, G2 and M and consists of a predetermined sequence of events each of a relatively constant duration. Thus, during A there is an increase in many replicative enzymes

leading to detectable mRNA, ribosomal RNA and polyamine synthesis. After entry into B, a whole series of DNA replicative enzymes become active, or is synthesised before DNA synthesis and subsequent mitosis and cytokinesis become apparent. Separating the two is a commitment (transition) point governed by random probability and passage through which commits the cell to proliferation. The transition - probability is somehow determined by the environmental conditions and in fibroblasts is a direct function of serum concentration (Brooks, 1981). It is also well established that other cells responding to serum such as lymphocytes do not become committed to DNA synthesis unless the stimulus is present for an extended period which encompasses the time of the transition point (Shields & Smith, 1977). In these cells the continuous presence of mitogen for commitment of a cell to proliferation is not imperative but is required only at two distinct time points (Weber, Skoog, Mattesson & Lindahl-Kiessling, 1974). After the primary signal, cells remain in a 'pre-activated state' for up to three days and then return to an unstimulated state, unless the mitogen is reintroduced. This requirement for two temporally distinct external signals for cell activation in certain cell lines is not strictly compatable with the transition-probability two-phase model which proposes a single site of control. However, an additional 'out of cycle' quiescent state has been proposed from which cells must be signalled to leave before entry to the A-phase (Brooks, 1981). The probability of cells exiting from this quiescent phase (of indeterminate length) increases rapidly following mitogenic stimulation leading to the initiation of an L phase, a lengthy process responsible for the lag observed between leaving 'quiescence' and entering A.

Most other authors prefer to dub any quiescent phase as the "GO" compartment (Patt & Quastler, 1963). "GO" is a broad term encompassing a wide range of metabolic states. One such state includes cells such as neurones, incapable of re-entering the cycle (terminally differentiated cells) whilst a second cohort of "GO" cells are able to rejoin the cycle in response to an appropriate stimulus (e.g. peripheral blood lymphocytes and hepatocytes). These proliferatively inactivated cells have reversibly repressed proliferogenic genes and must be distinguished from a third population residing in "GO". These are the proliferatively quiescent but rapidly recruitable cells, restricted from entering G1 by the temporary lack of, for example, an essential nutrient or growth factor. They retain activated proliferogenic genes and are enzymatically equipped to cycle (Scott, Hoerl, Wille, Florine, Krawisz & Yun, 1982; Epifanova, Setkov, Polunovsky & Terskikh, 1982; Baserga, 1981). If any of the inter-linked processes of G1 fail, the cell may leave the cycle and enter a "GO" state without inactivating its proliferogenic genes. The longer the cell remains in "GO" the more it loses its accummulated components and systems pertinent for proliferogenic activity and the more likely the appropriate genes will become repressed (Whitfield, Boynton, MacManus, Sikorska & Tsang, 1979). In some forms, the classical model includes a second quiescent state, the G2 phase (See diagram 14), in which cells would contain double the normal DNA content and indeed such cells have been identified in certain tissues (Gelfant, 1962). The two "GO" phases however, may not be two distinct entities as suggested here, but rather a single "GO" compartment distributed throughout the whole cycle as proposed by Rotenburg (1982), supporting the idea that the two models discussed, namely the classical cycle and the Smith and Martin cell cycle are not necessarily separate entities as first thought but can easily be superimposed.



"G0" POPULATION:-

A : terminally differentiated cells.
eg. small lymphocytes, erythrocytes

B : proliferatively inactivated cells.
eg. peripheral blood lymphocytes, hepatocytes

C : rapidly recruitable cells.
eg. serum starved cells, density dependent growth inhibited cells, rapidly responding thymic lymphoblasts.

Diagram 14 Diagrammatic Representation of the Cell Cycle
(see text for details: section 2.1)

Thus after the division of a cell each of the daughter cells may become proliferatively inactive, quiescent or immediately embark upon another cycle by moving directly to the transition (or commitment) point within G1 corresponding to the A/B boundary. The transition points of the cell cycle may be surpassed as a result of threshold levels of appropriate chemical factors or other stimuli acting at the relevant positions to control the cells progression. Lack of an essential factor or failure of any process during the cycle recommits the cell to a quiescent phase from which it can later be recalled in response to the appropriate signal.

2.2 SIGNALS FOR CELL PROLIFERATION

A mitogen strictly defined is a factor which acts as a primary signal to induce a cell to undergo proliferation. This definition is generally subdivided into various types of mitogen, those acting upon cells with a temporary nutrient lack causing them to resume cycling, and those capable of activating proliferatively inactivated cells. Some mitogens can act on both of these groups of cells. One much studied model of mitotic arousal from the quiescent state is the peripheral blood lymphocytes. The normal response of quiescent lymphocytes to an appropriate antigen is the division of a restricted clone of cells (Ling & Kay, 1975). The plant derived lectins appear to mimic and amplify this process by acting as polyclonal mitogens stimulating up to 70% of the mitotically competent population (Loeb, 1975). Cell activation by the lectins however is not a single inductive event; their presence is necessary for both initiation of "GO" exit (signal I) and a subsequent event late in G1, i.e. at the

G1/S boundary (Signal II), (Kay, 1970). It appears that signal II may be provided by a lectin-induced factor released from adherent cells (Rosenstreich, 1976). However, if these in vitro studies with plant lectins are to be physiologically relevant, other more natural growth promoters must also be shown to act in a similar manner. Significantly several such physiological regulatory substances have been proposed.

2.2.1 Extracellular Growth Regulatory Substances

The growth of normal cells is controlled by interactions with the extracellular environment (Holley, 1975). In cultured cell lines the external fluid medium contains many protein and polypeptide factors secreted by the cells. Such factors may convey appropriate signals between cells to sustain, stimulate or inhibit cellular proliferation (Nilsen-Hamilton & Hamilton, 1982). It is unclear whether or not all such factors are secreted by cells in vivo, but the detection of at least some of them in animal sera indicate that this may well be the case.

Positive Factors

Growth promoting activity for many cells has been detected in biological fluids. The purification and characterization however, has proved difficult probably because of their very low concentrations. Nevertheless, several positive growth factors have been extensively studied and may be subdivided into those promoting tissue specific proliferation and differentiation and those enhancing mitosis with little or no selectivity. Some may not directly recruit cells into the growth cycle but merely aid the passage through the cycle of already committed cells or postpone their death (Nishawaki, Armelin & Sata, 1975).

Epidermal Growth Factor (EGF) is probably the most studied of the factors so far identified. EGF is an acidic single chain polypeptide of molecular weight 6,045, which was originally shown to stimulate the proliferation of epidermal cells in vivo and in vitro but has since proved to be a potent mitogen for other cell types including human fibroblasts (Gospodarowicz, Greenburg, Bialecki & Zetter, 1978). This factor first isolated from the submaxillary glands of mice, has a similar structure to human urgogastrone which itself plays an important role in the repair process of the stomach wall and both factors bind to the same receptors in various cell types (Schlessinger, Schreiber, Levi, Lax, Libermann & Yarden, 1982). Urgogastrone also appears to exhibit an identical biological activity to EGF and is thus often referred to as human EGF. Virally-transformed cells generally lose their ability to bind EGF due to the autoproduction of an "EGF-like" factor which competes with authentic EGF for the cell surface binding site(s) (Todaro, DeLarco & Cohen, 1976). Such factors often termed "transforming growth factors" TGF (produced by many different tumour cell lines) are thought to permanently stimulate cell proliferation inducing, 'overgrowth' of cells, morphological transformation and other properties correlating with their tumorgenicity (Schlessinger et al., 1982; Matrisian, Pathak and Magun, 1982).

Another extensively studied mitogen is the fibroblastic growth factor (FGF), originally named on the basis of its mitogenic potency for BALB/c 3T3 fibroblasts. However, this basic polypeptide of molecular weight 13,400 has since been observed to stimulate the division of a wide variety of mesoderm-derived cells (Gospodarowicz et al., 1978). Synergism is often observed in the action of pairs

of different growth factors. FGF may act as a recruitment factor in various tissue, by priming a significant portion of a quiescent cell population (GO), to enter a state of 'competence'. Other more selective growth promoting factors may then promote the progression of the cells into the S phase and ultimately into mitosis (Gospodarowicz & Fujii, 1981; Bradshaw & Rubin, 1980).

The platelet-derived growth factor (PDGF) can similarly act as a recruitment factor by a process requiring RNA synthesis (Pledger, Scher, Smith, Goon & Stiles, 1981). In other respects however, it is totally independent of FGF. PDGF is sequestered within the alpha granules of circulating platelets and is released into the serum when the blood clots (Stiles, Pledger, Yucker, Martin & Scher, 1980). It is a heat stable, basic hydrophobic polypeptide with a native molecular weight of approximately 35,000 (Ross, Raines, Glenn, DiCorleto & Vogel, 1981). Little is known regarding the precise action of PDGF, but it is thought that it selectively promotes the rapid synthesis of a family of cytoplasmic proteins within the "GO" cell population, which subsequently incurs a 'competence' for cell division. PDGF, also requires the presence of a further plasma factor (such as EGF) before the cell progresses through the cell cycle (Pledger, Hart, Locatell & Scher, 1981; Pledger et al., 1981a). In addition to hypertrophic and hyperplastic effects associated with growth and repair processes, some polypeptide growth factors have a distinct role as maintenance or survival factors. Most of these such substances are normally present in the mature organism and deprivation causes responsive cells to deteriorate or even die. Indeed antibodies directed against nerve growth factor (NGF) will induce atrophy of neurones in the sympathetic and sensory nervous systems (Bradshaw & Rubin, 1980).

NGF, a polypeptide chain (with similarities to the proinsulin molecule) of molecular weight 13,259 also differs from the other factors discussed, in that its action is restricted to a single tissue type. NGF supports the proliferation and development of neuronal cells only (Levi-Montalcini, 1964; Bradshaw & Rubin, 1980). In the embryo it acts as a trophic stimulator of proliferation of developing neuroblasts and the genesis of axonal processes leading to the ultimate formation of functional synapses. NGF is transported retrogradely via the axon to the cell body in a sequestered form and when released it becomes associated with nuclear membranes and in some way proliferation ensues (Moore, 1980). In the adult animal, the growth factor is pertinent for the maintenance of these neurones (Levi-Montalcini & Angeletti, 1968).

Other less well studied growth factors include those which play an important role in the proliferation and differentiation of haemopoietic cells (e.g. colony stimulating factors (CSF) and a macrophage-granulocyte inducer (MGI) (Dexter, Spooncer, Toksoz & Lajtha, 1980; Lord, Mori & Wright, 1977; Wright & Lord, 1977), or of the immune system. This latter group includes the macrophage-derived interleukin, IL.1 (Duff & Durum, 1983), the lymphocyte derived interleukin, IL.2 (Pauly, Twist, Pirela, Reinertson, Callahan & Russell, 1983), thymocyte specific growth factor (Soder & Ernstrom, 1983), lymphocyte activating factor (LAF) and T-cell growth factor (TCGF) (Smith, Gilbride & Favata, 1980). A factor which is quite distinct from the previously described growth factor has very recently been partially characterized. This mitogen appears to be a peripheral membrane protein of 3T3 cells (releasable by mild proteolysis) which is heat sensitive and has a molecular weight of 150,000 - 200,000. It is possible that the release of this factor is somehow involved

in the transformation of normal cells (Liebermann, 1983). Other polypeptide growth factors have been partially characterised and it is also quite probable that some, as yet unidentified factors, have similarly important roles to play in the mammalian proliferative system (Holley, 1980).

In addition to these putative growth factors, several classical steroid and protein or polypeptide hormones have the capacity to modify cellular proliferation. The proliferogenic propensity of growth hormone (GH) is the resultant of an intermediary series of mitogenic factors derived from GH itself (Salmon & Hosse, 1971). Five such factors are accepted GH mediators, all of which are members of a family of circulating peptides collectively termed, somatomedins (Phillips & Vassilopoulou-Sellin, 1980). These factors are produced in the liver and circulate reversibly bound to large carrier proteins although the specific role of these carriers is unclear. The five factors are two insulin like growth factors (ILA 1 and 2), multiplication stimulating factor (MSA) and somatomedins A and C (Phillips & Vassilopoulou-Sellin, 1980). These compounds all have comparable structures and exhibit parallel growth promoting activities which include enhancement of general cell multiplication and in particular stimulation of cartilage proliferation (Salmon & Hosse, 1971). Moreover, all have anabolic insulin-like actions on fat and muscle which cannot be suppressed by anti-insulin antibodies (Phillips & Vassilopoulou-Sellin 1980). Insulin itself also stimulates a variety of cells to grow (Hollenberg & Cuatrecasas, 1975; Zapl, Rinderknecht, Humbel & Froesch, 1978). Oestradiol and progesterone are essential hormones for continued mitotic activity in secondary sex organ development. Oestradiol exerts some of its mitogenic effects via inducible growth

factors recently termed 'oestromedins' (Sirbasku, 1978, Ikeda, Ltu, Danielpour, Officer, IIo, Leland & Sirbasku, 1982; Leung, Potter & Qureshi, 1981), even it seems in non-secondary sexual tissue (Grossman, Sholiton & Roselle, 1972). Other tropic hormones include ACTH, TSH and FSH/LH. Their mitogenic actions may similarly be via secondary mediators (Nandi, Yang, Richards, Guzman, Rodrigues & Imagawa, 1980). It is apparent thus that circulating factors may contribute to growth and are responsible for many of the growth promoting activities of serum. However it is unclear how (or even if) their circulating levels fluctuate within the plasma in response to physiological demands.

Negative Factors

For technical reasons, growth inhibitors are less well studied than the growth promoting factors. Nevertheless, there is evidence available to suggest that inhibitors are as common as stimulators. There is very little if any, information on normal *in vivo* action of such inhibitory factors but it is possible that they act as differentiation promoters (Holley, 1980). The principal negative factors (studied *in vitro*) are undoubtedly the elusive 'chalones'. According to the proponents of the 'chalone theory' these substances are produced continuously by differentiated cells within an organ or tissue. Reduction in the external chalone concentration should thus automatically lift the mitotic block posed by the inhibitory factor and allow restoration of cell division until the critical level of chalone has again been restored, (Bullough, 1975). A kidney epithelial cell growth inhibitor has been purified and shown to arrest cells in the G1 phase of the cell cycle. Its action is reversible in that its removal allows cells to resume

growth and it appears to be highly specific for kidney epithelial cells (Holley, 1980). Other authors have independently shown that epidermal extract will inhibit cell transit through the cell cycle at several points G1-S, G2-S and G2-M (Elgjo, Clausen & Thorud, 1981). A growth inhibitory activity is also associated with density inhibited fibroblast cultures (Steck, Voss & Wang, 1979). It is possible thus, that the transition of cells from proliferation to rest ("GO") and maintenance there, are both subject to endogenous control carried out by intracellularly produced cytosolic inhibitor(s) whose presence prevents cell cycle progression (Epifanova et al., 1982). Alternatively membrane components may be involved in such growth inhibition. Addition of cell-surface derived fragments (isolated from 3T3 fibroblasts) to growing cells leads to cessation of growth by arresting the cells in the G1 portion of the cell cycle, in a manner which appears identical to that brought about by high cell density (Lieberman, Keller-McGandy, Woolsey & Glaser, 1982). Recently, a specific inhibitory protein has been isolated from such 3T3 plasma membranes but this awaits complete characterization (Peterson, Lerch, Moynahan, Carson & Vale, 1982). The physiological significance of such membrane associated factors however remains unclear. Evidence for the existence of chalones specific for haemopoietic tissue (in particular the lymphoid population) is particularly relevant to studies of lymphocyte proliferation. Some part(s) of the bone marrow cell population readily liberates a chalone (of molecular weight 50,000 to 100,000), capable of limiting the rate of proliferation of cells specifically in the pluripotential stem cell population (Lord, Mori, Wright & Lajtha, 1976). A further specific inhibitor of erythroid multiplication does not block cell proliferation but appears to lengthen the G1

phase of the cycle (Lord, Shah & Lajtha, 1977). The in vivo activity, specificity and non toxicity of this latter red cell extract suggests a physiological role in the regulation of erythropoiesis. Interferon (IFN) first recognised by its efffect on virus - cell interactions has a variety of other effects which include suppression of cell proliferation. It exerts a transient non cytotoxic inhibitory effect on the proliferation of murine pluripotent haemopoietic stem cells (Gidali, Feher & Talas, 1981), probably by promoting the exit of cells from the cycle into a "GO" like state (Leandersson & Lundgren, 1982). IFN also suppresses both mitogen- and antigen-induced leukocyte migration inhibition possibly by acting directly on the granulocytes and/or by influencing the lymphokine production by the lymphocytes (Szigeti, Masucci, Masucci, Klein & Klein and Berthold, 1980). Recently a thymus crude factor (TCF) isolated from bovine thymus tissue has provided a candidate for a specific chalone of thymic lymphocyte proliferation. Attempts directed towards its purification have revealed the presence of at least one extremely potent inhibitory moiety of relatively small molecular weight, in this crude fraction (Rijke, Lempers & Ballieux, 1981). Other negative regulators of proliferation associated specifically with lymphoid tissue include the mitogen suppressive factor (MSF) (Salinas-Carmona, Gery, Russell & Nussenblatt, 1982), low molecular weight factors (Patt, Barrantes, Gleisner, Pickart & Houck, 1981) and the very low density lipoproteins (VLDL) (Yi, Beck & Zucker, 1981). The precise nature and role of such specific chalone(s) for inhibition of lymphoid tissue multiplication however, is still unknown.

The physiological interactions between the inhibitory factors and the stimulatory growth factors are similarly very unclear.

The local concentration of specific factors may be regulated by sequestration and transportation to appropriate sites, or alternatively they may be present at all times in an inactive form which can somehow selectively be activated. Thus, a finely controlled balance of the local levels of positive and negative factors in the extracellular environment may work in a precise manner to provide the specific signals which govern the regulatory processes controlling cell cycle progression.

2.3 SIGNAL TRANSMISSION

2.3.1 Membrane Effects

It is clear that various extracellular regulatory factors, diverse in their nature, are able to maintain a proper balance between cell growth and a quiescent state. The ability of a cell to respond to a particular effector depends upon the presence of specific cell surface receptors. Indeed in most cases the cellular proliferogenic response is primarily the result of mitogen-receptor association at the plasma membrane. The structural heterogeneity of the membrane in its lateral plane may be particularly relevant to the triggering of cell activation and associated membrane enzyme functions. Membrane proteins may be distributed unevenly over the cell surface into specific high affinity receptor domains in such a way that enzymes affected by mitogens are situated in close proximity to the relevant receptors (Resch, Loracher, Mahler, Stoeck & Rode, 1978).

Mitogenesis is frequently associated with a clustering of receptors, often termed capping; for example, lectins will induce capping in lymphocytes (Bourguignon & Hsing, 1983) and EGF acts similarly in epidermal or fibroblastic cells (Das, Pittenger & Bishayee, 1981).

The process of capping which probably results from cross-linking between multivalent ligands and receptors (Bourguignon & Hsing, 1983; Phelps, Williamson & Schlegel, 1982; Cherenkevich, Vanderkooi & Deutsch, 1982), requires energy and involves cytoskeletal elements associated with the internal surface of the membrane (Rao, 1982; Cherenkevich et al., 1982). It is not a property unique to lymphocytes and has been observed in many other cell types (Loor, 1977), though its precise function remains unclear. A co-operative (whether it be positive or negative) interaction between occupied and unoccupied receptor sites may be attributed to this micro-redistribution of the receptors (Jacobs & Cuatrecasas, 1976). Alternatively, capping may be a pre-requisite to internalization of the ligand-receptor complex which itself could be pertinent for mediation of the ligand's effect, or simply be a form of down regulation serving to maintain appropriate responsiveness to the ligand (Branca & Baglioni, 1982; Chang & Polakis, 1978; Das & Fox, 1978). It is evident that many mitogens are able to elicit their own characteristic pattern of membrane changes by mechanisms which in addition to the above processes includes the acquisition of new receptor specificities (up regulation), (Speckart, Boldt, Ryerson, 1978). Thus, during mitogenesis the surface receptor repertoire is altered radically and the receptor expression may therefore regulate the developmental processes of the cell. The 'theory of cytodifferentiation' envisages cells in a semi-differentiated state which respond to external signals that direct further stages of differentiation by the acquisition of specific receptors for anticipated future signals. This should include the expression of receptors for positive and negative factors whose concentrations in the micro-environment will presumably vary appropriately with physiological demand. Concomitantly the cells would lose the

majority of those receptors which are no longer required (Brunner, 1977). Progression through the cell cycle may be considered a comparable process to differentiation in which the quiescent cells develop the ability to respond to mitogenic signals. In such a model the chronic expression of specific mitogen receptors, or the failure to express receptors pertinent for feedback control systems, could lead to the uncontrolled growth exhibited by malignant cells.

2.3.2 Cytoplasmic Metabolism

When quiescent cells are stimulated to proliferate by any extracellular growth factors there are rapid increases in the transport of a variety of substances which include phosphate, uridine, certain amino acids, glucose and other hexoses (Pardee, Dubrow, Hamlin, Kletzien, 1978). These fluxes are not a result of a general increase in membrane permeability but are due to modifications in the activities of specific transport systems (Hochhauser et al., 1981). Similar increases in specific transport rates have also been demonstrated in transformed cells (White & Christensen, 1982). There appears however to be no direct causal relationship between these transport changes and the triggering event for cell proliferation and indeed some growth factors may stimulate division without immediately affecting these transport processes (Rudland & de Asua, 1979). It is nevertheless, very unlikely that such specific transport changes are simply casual events and thus are probably essential for continued proliferation. The depressed transport associated with cell quiescence may therefore be a feedback-type adaptation in response to the reduced nutrient requirement for maintenance of basal metabolic activity (Pardee et al., 1978).

2.3.3 Intracellular Messengers

Alterations in intracellular metabolism associated with cellular proliferation occur in response to primary agents acting without necessarily crossing the membrane. The metabolic response evoked therefore requires that some intracellular messenger system transmits the signal, initially generated by interaction of mitogen with its specific cell surface receptor, to the cytoplasm. The putative intracellular messengers discussed in section one may similarly serve to couple mitogenic stimuli and the proliferogenic response.

2.3.3a The Divalent Cations

Both calcium and magnesium ions have been implicated in stimulus-mitosis coupling.

Calcium Ions

In addition to its role in the activation of various other cellular processes such as secretion and contraction (as previously mentioned), it is very likely that calcium is also involved in the regulation of cell growth. Certainly the removal of extracellular calcium or the impairment of its influx by low density lipoproteins inhibits the division of fibroblasts and peripheral blood lymphocytes (Boynton, Whitfield, Isaacs & Tremblay, 1977; Hesketh, Smith, Houslay, Warren & Metcalfe, 1977; Hui & Harmony, 1980a). In contrast, raising the extracellular calcium concentration can overcome the inhibition of growth induced by serum starvation (Dulbecco & Elkington, 1975), and enhanced calcium influx in general seems to accompany the arousal from quiescence in several cell types including eggs, T and B lymphocytes and A-431 cells (Jaffe, 1980; Freedman, Raff & Gomperts, 1975; Freedman, 1980;

Deutsch & Price, 1982a; Larner, Rebhun, Larner & Oran, 1980; Sawyer & Cohen, 1981). Opinion however,seems divided as to whether this calcium influx occurs during an early or late portion of the G1 phase of the cell cycle (Bard, Colwill, L'Anglais & Kaplan, 1978; Engstrom, 1981; Whitfield, 1982). In fact some authors have suggested that a biphasic increase in calcium influx may occur (Hazelton, Mitchell & Tupper, 1979; Eilam & Szydel, 1981) which would be consistent with a calcium-triggered re-entry into the cycle from GO, followed by a calcium dependent passage through the G1/S boundary.

A recent report suggests that Ca^{++} influx after lectin treatment may be regulated by early membrane phospholipid methylation, (Toyoshima, Hirata, Axelrod, Beppu, Osawa & Waxdal, 1982). Furthermore, EGF promotes PI turnover and enhances Ca^{++} uptake in A-431 cells (Sawyer & Cohen, 1981) whereas low density lipoproteins inhibit PHA-enhanced turnover of PI and Ca^{++} accumulation (Hui & Harmony, 1980b). However, in such cases, Ca^{++} uptake appears to preceed the phospholipid metabolism which is compatible with the proposed inciting role for Ca^{++} in the proliferative process. Although some groups have failed to detect an enhanced Ca^{++} uptake it is possible that the signal calcium may originate from intracellular pools or as a result of the inhibition of the normal activity of plasma membrane Ca^{++} efflux pumps. Alternatively, calcium transmembrane inflow may be very small and therefore experimentally undetectable in some systems. Indeed, a direct calcium detection technique has recently revealed a very small increase in free Ca^{++} level in the cytoplasm of lectin-stimulated lymphocytes (Tsien, Pozzan & Rink, 1982a). Thus, small alterations in the intracellular calcium concentration must be able to initiate key physiological processes. Certainly,

changes of very modest proportions in cytosolic calcium will accelerate mitochondrial pyruvate oxidation (Hume, Vijakumar, Schweinberger, Russell & Weidemann, 1978) and promote degranulation of mast cells (Grossmann & Diamant, 1978). It is thought that there is a range of free calcium ion concentrations as yet undefined, but probably lying within the range 10nm - 10μm, which determines that cells recruited from "GO" remain in the cell cycle. If or when intracellular calcium concentration exceeds the upper limit, a reversible block of progress through the cycle may occur and cell growth is inhibited (Metcalfe, Pozzan, Smith and Hesketh, 1980; Lichtman, Segel, Lichtman, 1983; Nykyforiak, Young & Phillips, 1980). Provided the intracellular calcium levels have not risen to such excessive concentrations as to cause such cycle exit or cytotoxicity, the next step in the activation sequence may be the association with calmodulin (CaM) which is itself synthesised during the late G1 or early S phase of the cycle (Means, Chafouleas, Bolton, Hidaka & Boyd, 1981). Indeed, a doubling in calmodulin concentration has been detected at this stage (Whitfield, 1982; Durham & Walton, 1982; Lichtman et al., 1983). The length of G1 in fact, seems to be inversely proportional to the CaM concentration. (Means et al., 1981) and thus, it can be assumed that this protein is synthesised by the rapidly recruitable cell population prior to their exit from "GO".

The activated calmodulin (Ca^{++}-CaM) may subsequently activate a kinase to promote the phosphorylation of a proliferation-related protein PRP (Malkinson, Wang & Foker, 1978) or histone (Iwasa, Iwasa, Higashi, Matsui & Miyamoto, 1981), both of which could be of significance in commiting a cell to DNA synthesis. Certainly, a PRP of M.W. 120,000 140,000 daltons becomes phosphorylated at about

the time of commitment to DNA synthesis. It is also phosphorylated in tumour cells and other rapidly proliferating adult and foetal tissues (Malkinson, et al., 1978). It is also possible that Ca^{++}-CaM plays an important role in the cell division (M) phase of the cycle (Sasaki, Hiroyoshi & Hidaka, 1982).

In some cell lines which initially exhibit calcium-dependent growth regulation their progress towards neoplasticity is associated with a gradual loss of sensitivity to this ion, so that cancer cells may proliferate quite normally in media containing very little calcium (Boynton et al., 1977; Durham & Walton, 1982). This may result from the loss or bypass of some Ca^{++}-dependent and possibly anchorage coupled mechanism required for the regulation of cell multiplication (Whitfield, Boynton, MacManus, Rixon, Sikorska, Tsang & Walker, 1980). The observed increase in CaM (MacManus, Braceland, Rixon, Whitfield & Morris, 1981) in conjunction with the de novo synthesis of a tumour specific calcium binding protein oncomodulin, following neoplastic transformation may be responsible for the ability of cancer cells to become independent of extracellular calcium (MacManus, 1982). Oncomodulin is smaller than CaM (Mwt. 11,500) and has only two Ca^{++} binding sites per molecule but may be activated by abnormally low intracellular calcium concentrations and therefore can usurp the role which calmodulin normally occupies (MacManus, 1982).

Magnesium Ions

Magnesium has a wide role as a cofactor in many intracellular transphosphorylations and macromolecular syntheses (King & Carlson, 1981; Mardh, 1982; Cech et al., 1981). Protein synthesis for example, is very sensitive to small changes in intracellular Mg^{++} concentration,

whereas very high levels of the cation will inhibit the process (Rubin, Terasaki & Sanui, 1979). The intracellular regulation of magnesium availability thus, may represent a plausible mechanism for the control of the co-ordinated array of events associated with the initiation of DNA synthesis and cell division. Indeed, it has even been suggested that the effect of extracellular calcium on cell proliferation is achieved indirectly by ultimately modifying the intracellular Mg^{++} activity (Rubin, 1977). Ca^{++} may undergo a rapid exchange with membrane bound Mg^{++} at the cell surface thereby liberating free intracellular Mg^{++} to modulate the biochemical processes which regulate cell multiplication. The effects of Ca^{++} deprivation on normal cell growth may similarly result directly from competition between Ca^{++} and Mg^{++} for common intracellular binding sites. Ca^{++} deprivation could inhibit cell proliferation through loss or redistribution of cell Ca^{++} such that Mg^{++} occupies those sites occupied formerly by Ca^{++}, hence reducing the availability of Mg^{++} for critical proliferative processes (Rubin, 1975). Moreover, recent experiments employing a specific cell line (BALB/c3T3 cells), have demonstrated changes in Mg^{++} cellular content which parallel increases in the rates of DNA synthesis induced by various mitogenic stimuli. In contrast, a decrease in intracellular Ca^{++} accompanied the proliferogenic response (Sanui & Rubin, 1982). Other authors have demonstrated that both Mg^{++} and Ca^{++} may be essential in the regulation of cellular proliferation acting in a sequential rather than a competitive manner. Serum growth factors are thought to primarily utilise a calcium dependent mechanism followed by a magnesium-dependent event which initiates DNA synthesis and cell division (McKeehan & McKeehan, 1980; McKeehan and Ham, 1978). It

is evident that the cellular content of Mg^{++} in transformed and nontransformed lines differs. The transformed cells have higher Mg^{++} levels than their normal counterparts which correlates with their higher rate of DNA synthesis (Rubin, 1982). Furthermore, reduction of the extracellular concentration of Mg^{++} causes the highly transformed cells to assume the shape and growth behaviour of non-transformed cells (Rubin, Vidair & Sanui, 1981; Rubin, 1981, 1982). Mg^{++} deprivation, however, also increases the cellular Ca^{++} content of the cell by a process which is independent of protein synthesis (Vidair & Rubin, 1982), although the physiological significance of this latter effect in reducing the rate of DNA synthesis is unknown. It is consistent however, with the premise that calcium content of the cell is sensitive to changes in magnesium level. It is possible that Mg^{++} is not actually a specific proliferogenic signal but is required, throughout the whole of the division cycle, as a permissive factor which modifies for example, calcium-calmodulin induced activation of magnesium-dependent enzymes (Whitfield, 1982; Hazelton, Mitchell & Tupper, 1979). Likewise, where supranormal magnesium levels promote cell proliferation it may not necessarily imply a direct initiating role for Mg^{++} but simply Ca^{++} displacement from endoplasmic reticulum (Chiesi and Inesi, 1981) or inhibition of Ca^{++} uptake into or stimulation of Ca^{++} extrusion from mitochondria (Nicholls, 1978; Akerman, 1980; Fiskum & Lehninger, 1980). If this is the case, then it is the raised intracellular calcium ion concentration which serves as the primary mitogenic trigger.

It is therefore uncertain as to whether or not a single cation activation 'axis' (be it calcium or magnesium) exists in the normal cell but it is clear that both of the ions are important at

some level of the cell proliferation process. It may be that enzymes involved in DNA synthesis and subsequent mitosis show variable activity depending on the magnesium/calcium ratio in their local environment. Thus, the mediator of macromolecular rearrangements during mitogenesis could be the changing intracellular ratio of the two main cellular divalent cations (i.e. free Ca^{++} : free Mg^{++}) (Staron & Jerzmanowski, 1981).

2.3.3b The Cyclic Nucleotides

Cyclic nucleotides have also been implicated as initiators of the proliferogenic response although their precise roles are the subject of much controversy.

Cyclic AMP

Two lines of experimental evidence have led to the belief by many authors that cAMP acts primarily as a negative modulator of cell proliferation.

Firstly, many cells entering the quiescent cell population "GO", as induced by nutrient depletion have an elevated intracellular cAMP concentration (Pastan, Johnson & Anderson, 1975; Friedman, Johnson & Zeilig, 1976; Moens, Vokaer & Kram, 1975). In a similar manner, application of exogenous cyclic AMP, its analogues or PDE-inhibitors, inhibit the growth of a variety of cell types including murine fibroblasts (Froelich & Rachmeler, 1974), normal human lymphoid cells (Millis, Forrest & Pious, 1974; Parker, 1976), lymphoma cells (Coffino, Gray & Tomkins, 1975), Lewis lung carcinoma cells (Bertram, Bertram & Janik, 1982), fibrosarcoma (Elgebaly, Kunkel, Lovett & Varani, 1982) and Chinese hamster V79-1A cells (Kovar, 1982), although the concentrations of exogenous cAMP or derivatives employed in

these studies were frequently in the millimolar range and therefore unphysiological. Analysis of intracellular cAMP has also revealed lower levels in growing compared to quiescent fibroblastic cells from human or murine tissue (Pastan et al., 1975; Marks & Grimm, 1972; Voorhees, Duell, Bass, Powell & Harrell, 1972). In fact, resting mammalian fibroblasts can be stimulated to divide by the application of a specific cAMP-PDE (Lawrence, Dezelee & Jullien, 1977). 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 (Seifert & Rudland, 1974; Pastan et al., 1975). Similarly, in other cell types the cellular cAMP concentration is inversely related to the mitotic rate induced by many mitogenic stimuli such as fibroblastic growth factor, insulin, phorbol esters and trypsin (Rudland, Gospadorowicz & Seifert, 1974; Goldberg, Haddox, Dunham, Lopez, & Hadden, 1974; Rochette-Elgy & Castagna, 1979).

Secondly, in vitro studies have generally revealed that neoplastic cells have a lower cAMP content than normal cells (Elgebaly et al., 1982; Hunt & Martin, 1979). The amount of cAMP bears no relationship to the stages of the cell cycle (Pastan et al., 1975) but is inversely correlated with the degree of malignant potential (Elgebaly et al., 1982). However, measurements of cAMP levels in tumours in vivo have proved less consistent, mainly due to technical problems. Nevertheless, some authors report low cAMP content in various tumour types including Morris hepatoma (Hickie, Thompson, Strada, Couture-Murillo, Morris & Robison, 1977), adenocarcinoma of the human colon (Derubertis, Chayoth & Field, 1976) and leukaemic lymphocytes (Monahan, Marchand, Fritz & Abell, 1975).

In contrast other results have failed to substantiate the notion that a decrease in cAMP concentration triggers the initiation of proliferation. There is no apparent change in intracellular cyclic AMP in sea urchin or amphibian eggs from fertilization through to cleavage (Pays de Schutter, Kram, Hubert & Brachet, 1975; Rebhun, 1977) or in the response of lymphocytes to mitogens (Coffey, Hadden, Lopez & Hadden, 1978; Anderson, Gallo, Wilson, Lovelace & Pastan, 1979). Moreover, the prevention of the early detected cAMP drop by application of exogeneous cyclic cAMP or PDE inhibitors does not prevent the normal mitotic response to serum in parotid gland cells (Tsang, Rixon & Whitfield, 1980) or 3T3 fibroblasts (Rechler, Bruni, Podskalny, Warner & Carchman, 1977). Similarly, a mutant cell line, lacking cAMP-protein kinase shows normal cell cycle characteristics, suggesting that cAMP concentration fluctuations are certainly not universally pertinent for the timing of the cell cycle or for the survival of cells in culture (Coffino, Gray, Tomkins, 1975). Despite such observations, other studies have implicated an increase in intracellular cAMP content as initiator of the proliferogenic process. A positive role for the nucleotide alone or synergistically with growth stimulating factors has been demonstrated in 3T3 fibroblasts (Rozengurt & Courtenay-Luck, 1982; Rozengurt, 1981; Rozengurt, Legg, Strang & Courtenay-Luck, 1981, Pruss & Herschman, 1979), rat Schwann cells (Raff, Hornby-Smith & Brockes, 1978), hepatocytes (Short, Tsukada, Rudert & Lieberman, 1975), rat pancreatic islet cells (Pruss & Herschman, 1979), bone marrow and thymus cells (Rixon, Whitfield & MacManus, 1970) and human and rat epidermal keratinocytes (Kuroki, Ito, Hosomi, Munakata, Uchida & Nagai, 1982). Intracellular cAMP concentration analyses during mitogenesis have revealed cAMP surges correlating with increased

growth rates in many cells, including lymphocytes (Parker, Sullivan & Wedner, 1974; Wang, Sheppard & Foker, 1978), hepatocytes (Tsang et al., 1980; MacManus, Boynton & Whitfield, 1978; Whitfield, 1979), Chinese hamster ovary cells (CHO) (Sheppard & Prescott, 1972) and 3T3 fibroblasts (MacManus et al., 1978). Furthermore, during hepatic carcinogenesis, basal adenylate cyclase activity is raised concomitantly with an increased sensitivity and greater response to beta-adrenergic agonists, probably due to increased numbers of specific receptors (Boyd, Louis & Martin, 1974; Christofferson & Berg, 1975).

In normal cells opinion seems divided as to whether the transient cAMP elevation occurs during an early or late portion of the G1 phase of the cycle. Since the cAMP surge evoked immediately after lectin treatment of lymphocytes is still detected at lectin concentrations that inhibit cell division or with non mitogenic lectins (Parker, 1978), the physiological significance of this primary elevation is unclear. The initiation of 3T3 fibroblast proliferation is also independent of any early cAMP surge (Boynton, Whitfield, Isaacs & Tremblay, 1978). Some authors have detected a biphasic increase in cAMP levels corresponding to both the "GO"/G1 and G1/S boundaries in regenerating liver (Whitfield, 1979; Whitfield et al., 1979). However, in agreement with others the initial surge may simply be a side effect since its abolition with dL propanolol does not seem to influence either the later transient cAMP increase or the initiation of DNA synthesis (Whitfield, 1979). On the other hand, prevention or facilitation of the later surge abolishes or enhances DNA synthesis respectively (Whitfield, et al., 1979). It is unlikely therefore that an elevation of intracellular cAMP concentration signals "GO" exit but

its role at the G1/S boundary remains to be clarified. It is possible that cells rely on temporally distinct fluctuations in cell cAMP content (first a decrease followed by an increase) to direct their progression through the cycle (Sheppard & Prescott, 1972; Seifert and Rudland, 1974).

Despite the uncertainties revolving around the precise role of cAMP or calcium ions in cellular proliferation it is clear that both in some way, may be involved in growth regulation, at least in some of the cells studied. Indeed, calcium and the cyclic nucleotide may act in concert in the sequence of events leading to cell division (Rochette, Egly & Kempf, 1981).

If it is accepted that cAMP may act as a positive trigger for DNA synthesis the next step in the activation process is presumably the association of the cyclic nucleotide with its protein kinase. Certainly, the cAMP surge during the late G1 in some cells is accompanied by a brief rise in type II A-kinase (Costa, Gerner & Russell, 1976; Boynton, Whitfield, MacManus, Armato, Tsang & Jones, 1981). The exogenous addition of type II A-kinase or of a specific polypeptide inhibitor of the catalytic subunit of the holoenzyme, to a cell culture in fact promotes or prevents the DNA synthetic response as expected (Boynton & Whitfield, 1980; Boynton, et al., 1981). The means by which the active catalytic subunits from the protein kinase triggers transition over the G1/S boundary is unknown but because cAMP and A-kinase molecules are too large to rapidly penetrate the plasma membrane, the DNA synthetic response might originate at the cell surface. Cyclic AMP, calmodulin and catalytic subunits of types I and II A-kinase each will stimulate the phosphorylation of six distinct membrane proteins in calcium deprived T51B hepatocytes

(Boynton, Kleine, Durkin, Whitfield & Jones, 1982). Other authors have also detected a similar cell cycle associated modulation of cAMP-dependent plasma membrane phosphorylation (Scott, Boman, Swartzendruber, Zschunke & Hoerl, 1981). The phosphorylation product(s) is thought in some way to initiate DNA synthesis and subsequently ensure progression through the cell division cycle. It is possible that the membrane phosphorylation(s) are related to the Ca^{++} gating mechanism previously discussed (Braunwald, 1982). It may be pertinent for the opening of specific transmembrane calcium channels and thus be a prelude to increased cytosolic calcium levels which might then serve as the ultimate mitogenic signal.

Cyclic GMP

The role of cyclic GMP in the regulation of the cell cycle is a similarly controversial area of study. Several lines of evidence have implicated this cyclic nucleotide as a negative regulator of cell proliferation. Quiescent cells have been shown to have elevated cGMP levels (Miller, Lovelace, Gallo & Pastan, 1975) whilst many transformed cells show greatly diminished guanylate cyclase activity (Nesbitt, Russell, Miller & Pastan, 1975) and thus have very low cytosolic cGMP levels. Addition of serum to 3T3 fibroblasts seems to induce a prompt fall in cellular cGMP content and promote cell growth (MacManus et al., 1978; Miller et al., 1975) although some authors have failed to detect any cGMP change at all following mitogenic activation (Weber & Goldberg, 1976; Parker, 1978). It is possible however, that redistribution of intracellular cyclic nucleotide may provide for specific local actions on proliferogenic processes (Whitfield, 1979).

In contrast, many authors accept that cGMP has a positive role in cell growth. A rise in lymphocytes cGMP content has been observed following their recruitment from quiescence (Hadden, Hadden Haddox & Goldberg, 1972; Coffey, Hadden, Lopez, Hadden, 1978; Whitfield, MacManus, Boynton, Gillan & Isaacs, 1974) by the activation of both membrane and soluble forms of the specific cyclase (Coffey, Hadden & Hadden, 1981). The rise appears to be associated with subsequent division since both, the cGMP surges and mitosis are mitogen concentration-dependent, calcium dependent and both can be provoked by A23187-induced ionophoresis (Coffey et al., 1978; Hadden et al., 1977). A similar increase in cGMP is apparent in other mitogen treated cells including BALB/C 3T3 foetal mouse cells (Seifert & Rudland, 1974), foetal rat hepatocytes (George, Rodgers & White, 1978) and Chinese hamster ovary cells (Millis, Forrest & Pious, 1974). Moreover, at least in some studies tumour cGMP values have been high (Hickie, et al., 1977; Derubertis et al., 1976) and exogenous cGMP, its derivatives or cGMP-elevating agonists (such as acetylcholine) all will promote a mitogenic response in several cells (Hadden, Coffey, Ananthakrishnan & Hadden, 1979; Whitfield et al., 1974; Seifert & Rudland, 1974; De Asua, Clingan & Rudland, 1975). Indeed, such exogenous applications can reverse the inhibitory actions of cAMP on PHA responses of B and T lymphocytes (Diamantstein & Ulmer, 1975).

Cyclic GMP appears to be involved, via its protein kinase, in the phosphorylation of specific non-histone chromatin proteins which occurs in association with a marked early increase in RNA synthesis following mitogenic activation of lymphocytes (Johnson, Hadden Karn & Alfrey, 1975 Johnson & Hadden, 1975; Hadden & Coffey,

1982). The level of cGMP dependent protein kinase in the rat liver nucleus also increases in response to partial hepatectomy (Tse, Mackensie & Donnelly, 1981) and indeed cGMP will stimulate RNA polymerase activity in isolated nuclei from quiescent cells (Ananthakrishnan, Coffey & Hadden, 1981). The process appears to require calcium ions suggesting that nuclear activation and subsequent cell division may involve some interplay between Ca^{++} and cGMP.

Clearly, the specific roles of the cyclic nucleotides in the initiation of cellular proliferation are unknown. The apparent interactions between the two cyclic nucleotides and divalent cations in similar and different cell types, only add to the complexity and difficulty of interpretation of the current experimental evidence. In addition to the interrelations discussed elsewhere, it is known that Ca^{++} will stimulate guanylate cyclase activity and since cGMP itself when added exogenously will promote an immediate cAMP surge in thymic lymphocytes (Whitfield, 1979) the individual role of each of the messengers in the cell activation process is clearly obscured.

With regard to the cyclic nucleotides however, it is possible that each cell can use precise, temporally distinct fluctuations in the local intracellular concentrations of the molecules or in their ratio, as 'cues' serving to regulate cell cycle progression. Other cellular components (such as the cationic environment) may initiate or modulate such fluctuations to correspondingly, control the ultimate cellular response.

It must also be remembered that there are cues required for progression through other phases of the cell cycle which may also involve cyclic nucleotides and divalent cations.

2.3.3c The Monovalent Cations

The monovalent cations have also been considered as putative initiators of cell proliferation.

Sodium and Potassium Ions

Increased activity of the (Na^+/K^+)- dependent ATPase has been shown to be positively associated with cell growth. Indeed, cell lines transformed by virus or tumour promoting agents exhibit greatly increased active Na^+ and K^+ transport, whereas the pump activity diminishes as normal cells become confluent and undergo contact inhibition (Kaplan, 1978). Furthermore, the (Na^+/K^+) pump is rapidly activated in response to mitogenic stimulation of a variety of cell types including lymphocytes (Quastel & Kaplan, 1970; Averdunk & Lauf, 1975; Kaplan & Owens, 1982), neuroblastoma cells (Moolenaar, Mummery, van der Saag & de Laat, 1981), mammary epithelial cells (Shen, Hamamoto, Bern & Steinhardt, 1978) and fibroblasts (Smith, 1977; Tupper, Zorgniotti & Mills, 1977). Lectins will also increase the (Na^+/K^+)- ATPase of membrane fragments isolated from both resting and stimulated human lymphocytes (Averdunk, 1972). Ouabain is a cardiac steroid which is a specific and competitive inhibitor of the K^+ site of the (Na^+/K^+)-ATPase and blocks nucleic acid synthesis in stimulated lymphocytes (Szamel, Somogyi, Csukas & Solymosy, 1980) or fibroblasts (Tupper et al., 1977; Rozengurt & Heppel, 1975). Low (i.e. not resulting in complete inhibition) concentrations of ouabain cause Friend erythroleukemic cells to undergo differentiation to non-proliferating, haemoglobin synthesizing cells (Bernstein, Hunt, Critchley & Mak, 1976) and thus a four-phase (Na^+/K^+)-ATPase 'switching' mechanism has been proposed (Kaplan & Owens, 1982). Each of the settings represents one of four possible states of the cell:-

1. OFF (metabolic activity ceases, cell remains viable).

2. LOW (signals terminal differentiation).

3. NORMAL (resting cell with basal RNA and protein and no DNA synthesis).

4. HIGH (signals activation of proliferation).

(From Kaplan & Owens, 1982).

The mechanism(s) of activation of the pump however, is still uncertain. It is possible that its stimulation involves either direct interaction between the loaded mitogen receptor and enzyme or occurs via local or generalised perturbations of membrane structure. There is evidence for the existence of a pool of cryptic (Na^+/K^+)-ATPase sites in the plasma membrane of lymphocytes, which remain inaccessible to the monovalent cations until exposed by specific conformational changes evoked by lectin binding (Dornand, Reminiac & Mani, 1978). Significantly, the binding of labelled ouabain to membranes, also increases in mitogen - stimulated cells, (Kaplan, 1978). Alternatively, at least part of the increased pump activity, might arise from the assembly of preformed polypeptide chains to form the active enzyme in the membrane (Quastel & Kaplan, 1970; Kaplan & Owens, 1980).

Although it is clear that increased active monovalent cation transport via the (Na^+/K^+)-specific membrane pump is somehow associated with the proliferative response, the precise function of this activity is unknown. The most obvious proposal is that an increased intracellular concentration of potassium ions or an elevated potassium: sodium ratio constitutes a growth signal in the cytoplasmic environment. Indeed, some normal cells appear to grow very poorly in low K^+ medium whereas

tumour lines are insensitive to such conditions (Jayme, Adelberg & Slayman, 1981; Lubin, 1980). Likewise, quiescent epithelial cells and density inhibited 3T3 fibroblasts have reduced intracellular K^+ levels (Cameron, Pool & Smith, 1980; Adam, Kleuser, Seher & Ullrich, 1982; Frantz, Nathan & Scher, 1981). Furthermore, application of valinomycin, a specific ionophore for K^+, transiently increases passive ion efflux out of L cells and concomitantly inhibits growth (Adam et al., 1982). If this K^+ efflux is prevented by furosemide no such inhibition occurs (Jayme et al., 1981). Reseeding of density inhibited 3T3 fibroblasts (Adam et al., 1982) or addition of serum growth factors to these cells (Frantz et al., 1981) promotes their proliferation and is also associated with a raised cellular potassium content probably as a result of K^+ influx. Certainly mitogenic stimulation of 3T3 fibroblasts by various agents (Lopez-Rivas, Adelberg & Rozengurt, 1982), epithelial cells by oestradiol (Cameron et al., 1980), hepatocytes by partial - hepatectomy (Wondergem, 1982) and human lymphocytes by lectins (Quastel & Kaplan, 1970; Averdunk & Lauf, 1975) is accompanied by enhanced K^+ influx.

High levels of cytosolic K^+ are essential for polypeptide chain elongation during protein synthesis and low levels inhibit this process (Cahn & Lubin, 1978). Since the initiation of DNA synthesis relies on the accummulation of a specific protein(s) (Brooks, 1977), the role of K^+ in cell cycle progression is probably related to its effect on protein synthesis. In fibroblasts the dependence of DNA synthesis on the cell potassium content, seems to be associated with early G1 of the cycle rather than the G1/S boundary (Lopez-Rivas et al., 1982). This suggests that active K^+ uptake, evoked by a variety of agents, is required to sustain protein synthesis at a level pert-

inent for optimal rate of entry into the S phase rather than being directly involved in the initiation of DNA synthesis. However, other authors have shown that in neuroblastoma cells there is a direct correlation between a rapid increased K^+ influx and transition from the G1 to S phase (Mummery, Boonstra, Van der Saag & de Laat, 1982a). In spite of the evidence supporting a positive role for the K^+ ion in the proliferogenic response, K^+ recapture may simply be a mechanism serving to compensate for K^+ loss perhaps induced by an earlier Ca^{++} influx seen immediately after mitogen application. Elevation of intracellular calcium certainly provokes potassium egress in both electrically excitable and non-excitable tissues (Hofmeier & Lux, 1979; Isenberg, 1975; Lew & Ferrera, 1976). Other authors, suggest that K^+ influx via pump activation precedes, but is balanced by passive efflux which thus maintains a constant cellular K^+ content (Kaplan, 1978; Segal & Lichtman, 1977). To reset the balanced K^+ transport by a higher pump setting following mitogenic stimulation therefore appears wasteful in terms of energy usage. However, pump activation would diminish the pool of peripheral ATP for which membrane bound (Na^+/K^+)-ATPase and adenylate cyclase compete, (Kaplan & Owens, 1980). This would reduce intracellular cAMP levels which itself is thought by some authors to provoke the mitogenic response (Seifert & Rudland, 1974; Pastan et al., 1975; Rochette-Egly & Castagna, 1979). Indeed, ouabain application appears to promote increased cAMP formation as this theory predicts (Lelievre, Paraf, Charlemagne & Sheppard, 1977). In contrast to the proposed direct or indirect effects of (Na^+/K^+)-ATPase on mitotic activity, many authors support the contention that it is actually an influx of Na^+ which stimulates the ATPase and thus that pump activation may simply be a secondary

effect, a consequence of Na^+ association with its specific cytosolic orientated pump binding sites (Smith & Rozengurt, 1978 a, b; Mendoza, Wigglesworth, Pohjanpelto & Rozengurt, 1980; Moolenaar, Mummery, van der Saag & de Laat, 1981). In this way, a transient influx of Na^+ might act as the primary proliferogenic trigger.

Although some authors have failed to detect any changes in cell Na^+ content following growth stimulation by serum (Rubin et al. 1979; Sanui & Rubin, 1982b), others have observed an increased sodium uptake in a variety of cells induced to divide by different stimuli. Such cells include regenerating liver and nerve tissue (Koch & Leffert, 1979; Cone, 1980), newly fertilized eggs (Epel, 1980) and neuroblastoma cells or fibroblasts in response to several mitogenic agents (Moolenaar et al., 1981; Rozengurt & Mendoza, 1980; Villereal, 1981a; Owen & Villereal, 1982 a, b). Significantly, such influxes of Na^+ are rapid transient events whose rates are directly proportional to mitogenic dose (Koch & Leffert, 1979; Villereal, 1981a; Owen & Villereal, 1983). Removal of extracellular sodium ions, or blockade of these sodium influxes by amiloride inhibits stimulated DNA synthesis in most cell types studied (Deutsch, Price and Johansson, 1981; Deutsch & Price, 1982b; Moolenaar, et al., 1981; Rozengurt & Mendoza, 1980; Koch & Leffert, 1979). Moreover, although there are some conflicting results, many rapidly dividing tumour cells appear to have a higher intracellular sodium ion content than their normal counterparts, again supporting the positive role of Na^+ in cell growth (Moyer, Moyer & Waite, 1982; Banerjee & Bosmann, 1976; Bader, Okazaki & Brown, 1981). In normal cells, throughout the cell cycle the rate of sodium uptake appears constant except for a rapid decrease after mitosis and a large transient increase immediately preceeding DNA synthesis (Mummery

et al., 1982a, b). Thus, during late G1, sodium ions seem to enter the cytosol from the extracellular environment via a specific amiloride sensitive channel in a linear manner which is dependent on the transmembranal Na^+ gradient (Villereal, 1981a). The precise mechanism by which serum activ ates this transport system however is still unclear.

Recently, it has been shown that the effect of serum on Na^+ flux can be mimicked by the divalent cation ionophore A23187 (Villereal, 1981b) which increases cytosolic Ca^{++} concentrations in many cell types (Thomas, 1982; Duffus & Patterson, 1974; Lichtman et al., 1983). Since such Na^+ influx is also inhibited by amiloride (Villereal, 1981b), serum-induced Na^+ uptake may be mediated via an elevation in intracellular Ca^{++} concentration and presumably the formation of the calcium-calmodulin complex. Certainly, various calmodulin inhibitors are known to abolish serum-stimulated Na^+ flux in a dose dependent manner (Owen & Villereal, 1982a). The serum-enhanced sodium movement however, does not seem to be dependent on an influx of calcium ions from the external medium since Na^+ transport provoked by serum will occur even in the total absence of extracellular Ca^{++} (Villereal 1982). Thus, serum may activate the Na^+ channel by primarily promoting Ca^{++} mobilization from intracellular stores. The compound TMB-8 (8-N,N-diethyl amino)-octyl-3,4,5 - trimethoxy-benzoate hydrochloride), an intracellularly acting Ca^{++} antagonist, which supposedly blocks Ca^{++} release from cytosolic stores (Malagodi & Chiou, 1974) inhibits the stimulated Na^+ transport (Owen & Villereal, 1982b), suggesting that Ca^{++} derived from some intracellular source indeed acts as the second messenger in this process. The precise mechanism via which Ca^{++}-CaM opens Na^+ gates however is unknown.

There is much evidence therefore, that Na^+ plays a positive role in cell proliferation but the question of how sodium influxes are linked to the initiation of DNA synthesis remains unanswered. The consequences of its actions on the (Na^+/K^+) pump and therefore on K^+ and cAMP levels have been discussed previously. In addition to playing a role in the early activation of the membrane pump, the increased cellular Na^+ concentration may directly mediate the derepression of some mitogenesis-specific gene(s) so that DNA synthesis and ultimately mitosis ensues. In support of this theory, rapid elicitation of specific patterns of gene expression by isolated chromosomes has been observed in direct response to sodium concentration fluctuations in the incubation medium (Kroeger, 1966). It is anticipated that Na^+ influx is accompanied by a decrease in membrane potential (depolarization) and thus, it is possible that this might serve to initiate mitogenesis. However, measurements of membrane potential during the cell cycle have indicated that the transient Na^+ influx has no electrophysiological correlate and thus must occur electroneutrally (Deutsch, & Price, 1982b; Boonstra, Mummery, Tertoolen, van der Saag & de Laat, 1981). Amiloride-sensitive Na^+ fluxes may be accompanied by transport of Ca^{++} through a surface membrane Na^+/Ca^{++} exchange system which would tend to restore intracellular calcium content following mitogenic activation (Villereal, 1981b). Paradoxically, a similar Na^+/Ca^{++} antiporter has also been identified in the mitochondrial membrane of some cell types (Fiskum & Lehninger, 1980) suggesting that at least in some cells Na^+ may act ultimately to promote cell division by raising the cytosolic calcium content. It is

generally accepted however, that serum stimulated Na^+ transport is electroneutrally coupled to a simultaneous efflux of protons out of the cell and it is possible that it is this phenomenon which provides the proliferogenic signal (Moolenaar et al., 1981; Leffert & Koch, 1982).

Hydrogen Ions

Enhanced Na^+-coupled proton efflux observed in various cell types simultaneously raises the intracellular pH (Moolenaar et al., 1981; Moolenaar, Tsien, van der Saag & de Laat, 1983). Such intracellular alkalinization frequently accompanies high rates of cellular proliferation or the initiation of DNA synthesis. In particular an internal pH rise is a key event in the activation process of fertilized sea urchin eggs (Johnson, Epel & Paul, 1976) and many mitogenic agents rapidly activate H^+ efflux, again implicating cytoplasmic pH elevation as a possible mediator of the growth response (Moolenaar, et al., 1981; Horne, Norman, Schwartz & Simons, 1981). Furthermore, rapidly proliferating lymphocytes generally have a more alkaline intracellular pH than quiescent lymphocytes (Gerson & Kiefer, 1982). There is in fact, a highly significant correlation between the rate of DNA synthesis and the degree of alkalinization of mitogen-stimulated lymphocytes (Gerson & Kiefer, 1982; 1983). It is also significant thus, that cells grown in an external medium of high pH are less likely to undergo contact inhibition than those in lower pH medium, and that confluent cells in the lower pH are somewhat less sensitive to growth stimulation than are confluent cells raised in a more alkaline environment (Froehlich & Anastassiades, 1974). Therefore, an early rise in intracellular pH appears to be a common response of metabolically quiescent cells to various mitogenic stimuli, although its specific mechanism of

action remains unclear. There is an excellent correlation between pH and the rate of protein synthesis (Winkler, 1982) and significantly an increase of only 0.1 pH unit will stimulate certain enzymes' activities by up to 20-fold (Trivedi & Danforth, 1966). Indeed, many growth related enzymes are activated by such alkalinization (Wang, Marquardt, & Foker, 1976; Gillies, 1982). This alkaline shift may also have profound effects on cytoskeletal regulation; raised pH will similarly promote the disassembly of microtubules which itself is an event pertinent for DNA synthesis to ensue (Crossin & Carney, 1981).

In conclusion, it is possible that growth factors and other mitogenic agents may then initiate a change in intracellular Ca^{++} levels which in turn triggers Na^{+} uptake and cells thus might ultimately utilize the Na^{+}/H^{+} exchanger as a signal tranducer to shift pH to levels which will somehow promote the proliferogenic responses(s).

2.4 THE CONTROL OF THYMIC LYMPHOCYTE PROLIFERATION

Studies on the control of cell proliferation have, in a sense been compromised by the search for a common mechanism of action for the many growth promoting hormones and agents that can arouse a variety of cell types from quiescence. Divalent and monovalent cations and the cyclic nucleotides have all been implicated as the primary intracellular mediator of the proliferogenic response (as discussed in the previous sections). Examination of the cell cycle (see diagram 14) and its associated dormant cells which can be recruited into the division cycle when the need arises, make it highly unlikely that all mitogenic agents act in an identical fashion in all cell

types. For example, hepatocytes and peripheral blood lymphocytes may remain permanently quiescent in the intact animal but can be stimulated to divide by partial hepatectomy and antigens or plant lectins. The sequence of events which eventually brings such cells into DNA synthesis is complex and protracted (MacManus, Franks, Youdale & Braceland, 1972; Rixon & Whitfield, 1982; Anathakrishnan, Coffey & Hadden, 1981). On the other hand, dormant bone marrow cells can be rapidly recruited into the cell cycle and into DNA synthesis in vivo and in vitro. Presumably upon the assumption of a dormant state, certain preludes to DNA synthesis have already been accomplished in these cells (or at least certain proliferogenic genes remain active), so that they remain poised upon the brink of DNA synthesis and can be induced to cycle very rapidly upon receipt of an appropriate signal (Whitfield et al., 1979; Perris, Whitfield & Rixon, 1967; Edwards, Rimmer, Atkinson & Perris, 1981). This situation may be analagous to the resumption of growth in serum-deprived, or density dependent inhibited cultured cell lines such as fibroblasts, HeLa cells and Chinese hamster ovary cells (Otten, Johnson & Pastan, 1972; Oey, Vogel & Pollack, 1974; Moens, Vokaer & Kram, 1975; Seifert & Rudland, 1974; Russell & Stambrook, 1975; Dulbecco & Elkington, 1975; Eilam & Szdel, 1981). Furthermore, since these various cell types possess receptors which bind to mitogenic agents of vastly different size and structure it is unlikely that a single common mediator will be found. However, whether cells are more or less permanently quiescent, or are only temporarily prevented from cycling because of nutrient deprivation, it is a general assumption that there is a boundary or control point close to the G1/S boundary of the cell cycle through which all normal cells must obligatorily pass.

The purpose of this study is to investigate various mitogenic stimuli and the events which couple their initial association with specific receptors to the ultimate mitotic division. It will attempt to demonstrate the role of putative mediators and any interplay amongst them during the activation process. The experimental system employed has been that of readily cultured lymphocytes isolated from the rat thymus (Whitfield, Brohee and Youdale, 1964; Atkinson, Cade & Perris, 1983). The thymus lymphoid population is broadly classifiable into three categories; firstly the proliferatively inactivated small lymphocytes which comprise some 75% of the total thymic lymphoid pool. These are the end product of intrathymic division and differentiation of appropriate stem cells (Craddock, Nakai, Fukuta & Vansleyer, 1964). Despite this high proportion of non dividing cells the entire murine lymphoid compartment is renewed within four days (Metcalfe & Wiadrowski, 1966). This self-renewal is due to the second class of the lymphoid population, the actively cycling large and medium lymphoblasts. These stem cells represent a mere 10-15% of the total number of cells. The remaining 10-15% are the rapidly responding lymphocytes, which cannot exogenously initiate DNA synthesis but are enzymatically equipped to do so very shortly after exposure to mitogenic agents. These cells are thought to occupy a "GO" compartment at, or very close to the G1/S boundary of the division cycle and have a total cycle time of 5 - 7 hours (Whitfield, et al., 1979; Edwards, Rimmer, Atkinson & Perris, 1981).

Short term cultures of the thymus thus have the distinct disadvantage that only approximately ten per cent of the cells are mitotically stimulatable. Nevertheless, they have the important advantage that the cells respond in culture in an almost identical fashion to their behaviour in the intact animal (Perris, 1971)

implying that such studies are physiologically significant. The same may not be said of many cell lines used in mitotic investigations which are highly adapted to tissue culture conditions (Chlapowski, Kelly & Butcher, 1975).

A surge of mitosis can be induced in native thymus tissue by artificially increasing plasma calcium or magnesium concentrations (Perris, Whitfield & Rixon, 1967). Indeed, natural hypercalcaemic episodes during times of rapid body growth, or after antigenic stimuli likewise stimulate proliferation in the thymus (Perris, Whitfield & Tolg, 1968; Hunt & Perris, 1973; Edwards, Rimmer, Atkinson & Perris, 1981). Elevating the calcium or the magnesium concentration in the culture medium effectively mimics these in vivo events. Surprisingly, the effect of plasma [Ca^{++}] elevation in the in vivo studies appear to be confined to the male rate (Dawson & Perris, 1972). This is due to the presence of the anti-proliferative sex steroid, oestradiol in the female (Smith, Gurson, Riddell, & Perris, 1975; Morgan & Perris, 1974). During the oestrous cycle decline in oestradiol, the calcium stimulatable response may reappear, as a marked increase in both plasma calcium and thymus activity is evident (Smith et al., 1975). High concentrations of beta-oestradiol, likewise prevent the mitotic response of cultured thymocytes to elevated extracellular calcium concentration (Morgan & Perris, 1974). In a similar manner, magnesium-dependent mitosis is abolished by high levels of testosterone in vitro (Morgan & Perris, 1974). In addition to the mitogenic propensities of raised calcium and magnesium ion concentrations a whole series of stimulatory compounds (including

many hormones) have been identified. These mitogenic stimuli show the same capacity to initiate the proliferation of the 'quiescent' population both in the whole animal and in short term culture (Morgan, Hall & Perris, 1975).

In vitro studies employing thymus tissue have thus far provided a great insight into the mitotic activity of the thymic lymphocyte. Table (ii) lists many of the agents shown to initiate their recruitment in vitro. It has been found that these mitogens can easily be divided into two distinct categories. Type 1 mitogenic agents almost exclusively are effective in the 10^{-4} to 10^{-7} M range, are magnesium-dependent and inhibitable by testosterone. Type 2 mitogens on the other hand appear to function in the 10^{-9} to 10^{-13} M range, show an obligatory requirement for calcium ions and are oestradiol blockable (Morgan, Hall & Perris, 1975). Interestingly, some of the type 1 and type 2 mitogens are physiological antagonists which in other tissues are thought to exert their effects via the contrasting actions of the secondary messengers, cyclic-AMP and cyclic-GMP. Indeed, exogenous additions of either cyclic nucleotide have proved to promote cellular proliferation but in a somewhat unexpected biphasic manner (Morgan, Hall & Perris, 1977). At concentrations which were thought to approximate those prevailing naturally within the cellular system, cAMP (at 10^{-7}M) could be classed as a type 1 mitogen whereas cGMP (at 10^{-11}M) was a class 2 mitogen. However, at unphysiological levels of cAMP and cGMP, (i.e. 10^{-12} and 10^{-6} M respectively) their ionic dependencies were reversed as were their sensitivities to steroid blockade (Perris & Morgan, 1976). It has been suggested that type 1 mitogens bind to their surface receptors and consequently stimulate adenylate cyclase thus

Table (ii) Mitogenic Compounds for Thymic Lymphocytes

Type 1 Mitogens	Effective Concentrations	Type 2 Mitogens	Effective Concentrations
Glucagon	10^{-4}M	Insulin	10^{-10} M
Adrenaline	5×10^{-6} M	*Acetylcholine	10^{-11} M
*Acetylcholine	5×10^{-5} M	Noradrenaline	10^{-12} M
Isoprenaline	10^{-6} M	Histamine	10^{-13} M
Dopamine	10^{-6} M	Parathyroid Hormone	10^{-13} M
Cyclic AMP	10^{-7} M	Cyclic AMP	10^{-12} M
Cyclic GMP	10^{-6} M	Cyclic GMP	10^{-11} M

When thymic lymphocytes are incubated in basal conditions (0.6 mM, Ca^{++}, 1 mM Mg^{++}) in the presence of the above compounds at the concentrations listed significant stimulation of mitosis can be observed. These mitogenic responses are not a result of non-specific membrane perturbations for the actions of many of these agents can be specifically blocked by pharmacological antagonists which leave the action of the other mitogens unimpaired. Considering type 1 mitogens specific inhibition of the mitogenicity could be provided by propanolol (for adrenaline and isoprenaline), hexamethonium (for acetycholine) and pimozide (for dopamine). Specific inhibition of type 2 mitogens can be achieved by atropine (for acetycholine), phentolamine (for noradrenaline) and mepyramine (for histamine).

* All experiments involving acetylcholine included 10^{-9} M eserine to prevent degradation.

raising the intracellular cAMP level. The magnesium dependent event appears to be after cAMP formation since mitogenic agents that are adenylate cyclase activators still require the presence of extracellular Mg^{++} to exert their effects (Perris & Morgan, 1975). The precise interplay between the divalent cation and cyclic nucleotide however, remains to be clarified. On the other hand, type 2 mitogens may promote cyclic cGMP formation as a prelude to some calcium dependent process. However, detection of any increase in endogenous cGMP after the exogenous application of any type 2 mitogens has thus far proved unsuccessful. Moreover, acetylcholine appears only to activate guanylate cyclase at non-mitogenic concentrations (MacManus, Boynton, Whitfield, Gillan & Isaacs, 1975). Since high concentration cGMP ($10^{-6}M$) can inhibit thymocyte PDE thereby elevating cAMP within the cell, it is possible that high cGMP acts mitogenically by ultimately raising intracellular cAMP (Whitfield, MacManus, Franks, Gillan & Youdale, 1971; Morgan et al., 1977). This would conveniently explain its magnesium dependence. If low cAMP ($10^{-12}M$) could similarly enhance cGMP formation this may account for its calcium dependency.

Thus, it is clear that mitogens of thymic lymphocytes act via one of two discrete axes which are regulated by calcium and magnesium respectively. In turn these axes can be influenced in a precise manner by the sex steroids. Furthermore, there is evidently some interplay between the putative mediatiors of the mitogenic response. Therefore, the current study is designed to try and clarify certain aspects of stimulus-mitosis coupling in rat thymic lymphocytes aiming towards a more complete understanding of this complex phenomenon.

3.0 METHODS AND MATERIALS

The experimental techniques employed in this study reflect necessarily, the nature of the area of investigation namely the effect and mechanism of action of various mitogenic stimuli. The inter-relationships between hormones, ionic environment and mitotic activity in the rat thymic lymphocyte have been specifically studied. Thus, methods for the estimation of mitotic indices, for measurement of cationic fluxes using radioisotopes and for determination of cytosolic calcium levels employing a specific intracellular fluorescent indicator are described in this section.

3.1 GENERAL CONSIDERATIONS

3.1.1 Experimental Animals

Male albino rats of the Wistar strain (Bantin & Kingman Ltd) and weighing approximately 200g have been used throughout, thus avoiding fluctuations in basal mitotic activity due to age-related thymic involution and oestrous cycle endocrine periodicity (Perris, 1971; Smith, Gurson, Riddell & Perris, 1975). The animals were maintained under constant laboratory conditions, which included a standard 41B diet (Pilsbury's Ltd) and tap water ad libitum.

Animals were routinely sacrificed between 08.30 and 09.30 hours, thereby minimizing changes due to inherent circadian rhythm in the rat thymocyte mitotic activity (Hunt & Perris, 1974). Despite such precautions some seasonal drift in basal activity could be observed.

3.1.2 Anaesthesia

The animals were sacrificed using diethyl ether. Ether is commonly employed as it possesses advantages over injected agents, in that for example, barbiturates have a reported antimitotic action (Baserga & Weiss, 1967). Ether is also rapidly inducted and eliminated from the system and has no short term effects on total red and white blood cell levels or plasma corticosteroid concentration (Besch & Chou, 1971).

3.2 MITOTIC ACTIVITY IN THE THYMOCYTE

This investigative study has been limited to measurements carried out on cultured thymic lymphocytes isolated from male rats.

3.2.1 Cultured Thymocytes

Unlike the majority of continuously cultured cells the mitotic behaviour of thymic lymphocytes in short term suspension cultures, follows closely that observed in the intact animal (Perris, 1971). Comparable basal proliferative activities result in the regular appearance of mitotic cells *in vivo* and in culture (Hunt, 1974). Moreover, a series of mitogenic agents exhibit the same capacity to promote the entry of a normally quiescent cell population into the cell cycle *in vivo* and *in vitro* (Morgan & Perris, 1974). Such parallel activity thus provides an almost unique insight into mitotic events which are closely related to proliferative responses within the native tissue.

The technique of short term (6 hours) suspension culturing of thymic lymphocytes employed here, is ideally suited to in vitro investigations of mitotic activity. Many potential problems of tissue culture have been easily overcome;

1. The ready availability of a responsive, naturally quiescent cell population avoids any need to induce synchrony or quiescence by employing 'artificial' procedures.
2. The short term incubation avoids the necessity for serum supplementation and thus prevents any interference resulting from complex interactions which may occur between added mitogens and serum factors. Furthermore, the manipulation of ionic or hormonal composition of the extracellular environment becomes greatly simplified.
3. The thymocytes are not adherent and consequently the maximum cell surface area is exposed to added mitogens.
4. The short period precludes the possibility of density-dependent growth inhibition and nutrient depletion. Constant agitation (cells in culture tubes are rolled for 6 hours) also counteracts the local accumulation of potentially toxic metabolites.
5. The rapid and simple preparation of the suspensions avoids the metabolic duresses incurred during the preparation of cell cultures from already established cell lines.

3.2.1a Preparation of Thymocyte Cultures

The cell suspensions were prepared using a modification of the technique developed by Whitfield, MacManus, Boynton, Gillan and Isaacs (1974). Thymus glands were rapidly excised from lightly anaesthetised rats employing a weak ether/air mixture. The entire organ in each case was rapidly rinsed in three changes of culture medium to remove superficial debris and red blood cells. The thymic lymphocytes were released from the reticulum by thorough mincing of the gland in culture medium. Cell aggregates and reticulum fragments were subsequently removed by filtration through moistened four-ply cheesecloth and the cell concentration was adjusted to approximately 5×10^7 cells ml^{-1}. One millilitre (1 ml) aliquots were distributed amongst the sterile plastic culture tubes (Sterilin Limited), and incubated at 37°C in a rotating roller drum assembly for 6 hours.

Although thymocytes appear to proliferate normally in a simple salts solution a more complete pre-prepared culture medium was employed throughout. The medium 199 (Wellcome Ltd.) was manufactured nominally free of added calcium and magnesium ions, having no detectable calcium present (detectable ion limit being 3×10^{-5} M). EGTA buffers were not used as this chelator may perturb plasma membrane function or chelate other vital ions. Where required, calcium and/or magnesium ions were added as ten microlitre (10 µl) aliquots from concentrated stock solutions of their respective chloride and sulphate salts to give basal concentrations of 0.6 mM Ca^{++} and 1mM Mg^{++}, chosen to mimic the ambient levels of the free ions in normal rat blood (Perris, 1971). Similarly, addition of putative mitogenic or antimitogenic agents was performed as desired in (10 µl aliquots) from stock solutions made up in 0.9% saline or 0.9% NaCl-Ethanol. Equal volumes of the diluent

were also added to control cultures. Since the mitotic activity is markedly pH sensitive (Eagle, 1973) precise buffering is essential, particularly in long term cultures. For these short period cultures Hepes buffer (20 mM), (Flow Lab. Ltd.) and $NaHCo_3$ (3 mM) provided adequate buffering in the stoppered plastic tubes maintaining a pH of approximately 7.2 throughout. Where appropriate, the metaphase arresting agent colchicine (Ciba Ltd.) was employed at a final concentration of 0.6×10^{-4}M, this being the minimum required to provoke spindle disruption without influencing the mitotic activity (Whitfield, et al., 1974). Moreoever, colchicine at this concentration does not influence glucose, leucine (Morgan, 1976) or calcium (Cade, unpublished data) transport across the plasma membrane.

A small reduction (approx. 2-4%) in the viability of the total cell population was observed over the 6 hour incubation period (as assessed by Trypan blue exclusion). The technique however is considered satisfactory since the drop is accounted for by a reduction in the small lymphocyte population which is mitotically inert.

3.2.1b Estimation of Mitotic Activity

Since only a relatively small part of the thymocyte population is mitotically active, assessment of proliferative activity by measurement of an increase in total cell numbers, during the 6 hour incubation period, is precluded. The general approach then, was to prepare smears, stain them and score for the percentage of nuclear cells that have been arrested in the metaphase stage of mitosis after prior treatment with colchicine.

Following a 6 hour incubation period at 37°C, two drops of each culture were removed and placed on microscope slides each with one drop of a binding agent (calf serum). The two were smeared together and dried in a warm air stream. Each slide was subsequently fixed in neutral phosphate-buffered formalin and stained with Delafields Haematoxylin according to established procedures (Whitfield Brohee & Youdale, 1964). Permanent slides were prepared by mounting in DPX and these were subsequently scored.

Colchicine arrests a great variety of cell-types in their progression through mitosis at the metaphase stage (Eigsti & Dustin, 1949). Thymic lymphocytes progress essentially linearly into mitosis and are arrested in a quasi-metaphase configuration where they accumulate. Thus, by scoring the percentage of nucleated cells in colchicine metaphase (C-metaphase) one can compare the effect of various potential mitogens upon the mitotic activity of the cells. One thousand cells from duplicate cultures were examined under oil emersion at 1250X magnification by two independent counters, to reduce subjective error such as mistaken identification of the quasi-metaphase configurations or the scoring thereof. Previous studies have demonstrated that trained observers rarely differ by more than 0.5% using this technique. Where differences occurred between counters, the slides were always rescored until agreement was obtained. Using this technique with the divalent cationic environment adjusted to reflect ambient levels of rat blood (0.6 mM Ca^{++}; 1 mM Mg^{++}) approximately 3.5% of the cells accumulated in C-metaphase after 6 hours incubation. Elevation of the concentration of either cation (to 1.8 mM Ca^{++} or 2.5 mM Mg^{++}) provoked approximately 6% to enter mitosis. (See Figure 1). These were used as internal controls in each of the

experiments carried out in this study but generally only the basal level of mitotic activity is depicted in the figures.

The use of this somewhat subjective method, to measure such small changes in mitotic activity is potentially innaccurate but has however, proven superior to alternative techniques. Probably the simplest direct method of scoring mitotic activity is that of measurements of the progressive increase in absolute cell numbers. However, as mentioned previously, over this short 6 hour period insufficient numbers of cells would divide to make such results valid. Other direct methods include the scoring of the mitotic index (MI), a measurement of the percentage cells undergoing mitosis, in the entire population. This method supposes that if a given treatment recruits new cells then the proportion of cells in distinct phases of the cell cycle would increase correspondingly and thus an elevated mitotic index would be scored. However, an increased MI could alternatively be a consequence of a) the prolongation of the mitotic period alone, b) of the shortening of the overall cycling time without a corresponding decrease in the mitotic phase or even possibly c) of the destruction (or extrusion) of large numbers of mitotically incompetent non-cycling cells hence reducing population size. Furthermore, since thymocytes are recruited in a semi-synchronous fashion the transient appearance of cells in mitosis may easily be missed and the values obtained may thus be inaccurate.

The laborious and somewhat subjective nature of such direct techniques has encouraged the development of certain indirect methods for the estimation of cellular mitotic activity. Most of these methods are based on the assumption that DNA synthesis is directly correlated with mitotic activity. The proportion of cells synthesizing

DNA can be measured by the autoradiographic analysis of the incorporation of the radiolabled precursor ^{3}-HThymidine (^{3}H.TdR). However, DNA synthesis by non-mitotic cells, tracer reutilization or changes in the length of the cell cycle may spuriously alter the number of labelled cells. Some problems can be overcome by scoring the percentage of cells which are both labelled and in recognisable mitotic configuration but again this makes the technique both subjective and laborious. The radiometric quantification of labelled precursor incorporation into newly synthesised DNA has proved a successful method of measuring proliferative activity, in many different cell types. However, in thymic lymphocytes, proliferation does not correlate with isotope incorporation (Atkinson, unpublished data; Youdale & MacManus, 1975). In fact, ^{3}H-thymidine incorporation appears to decrease after mitogenic stimulation probably resulting from the presence of endogenous thymidylate synthetase and thymidine kinase (which produce de novo thymidine) in the rat thymocyte. The endogeneous thymidine thus, dilutes out the isotopic pool and specific activity within the cell.

The colchicine-metaphase (C-metaphase) technique therefore in addition to being convenient and simple to perform appears to be the most suitable method for use in this study.

3.3 MEASUREMENT OF INTRACELLULAR CALCIUM LEVELS

There is increasing evidence consistent with a role for Ca^{++} as a transducing mediator in the proliferative response of many cells. These responses appear to be dependent in the most part, on extracellular calcium and thus, presumably on transmembrane ion

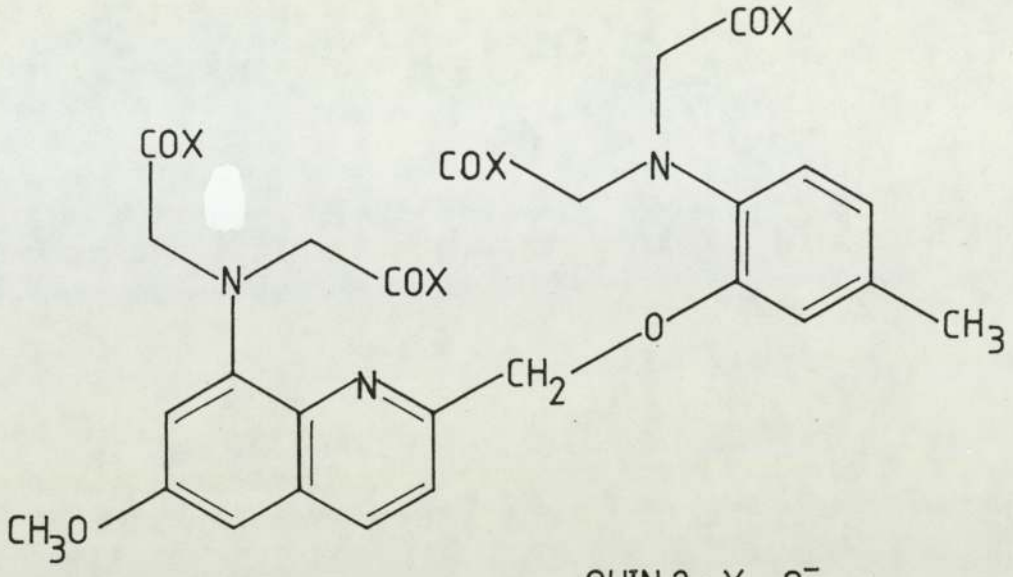
distribution. The measurement of increasing concentrations of free calcium ions inside cells is therefore vital in the investigation of cellular mechanisms involved in stimulus-mitosis coupling. The measurement of total calcium content appears not to be directly relevant to cellular Ca^{++} metabolism since 90-95% of the cell calcium is bound to intracellular ligands and furthermore calcium redistribution may occur during measurement procedures (Borle, 1981). Ion selective microelectrodes give continuous readouts with a fast enough response to detect most transient increases in ionic levels (Tsien & Rink, 1980). Their signals cannot be contaminated by bound or sequestered ions and do not intrinsically introduce significant buffering or perturbation of Ca^{++} levels which they are measuring. The use of such electrodes is however impractical with the thymocyte of this study. The electrodes have proved to be much too large to penetrate the membrane without causing significant damage. Even if the size problem could be overcome there still persists an inherent sampling problem. Microelectrodes detect only the chemical environment at its tip whose location is generally unknown and moreoever such a one point sample may be totally unrepresentative of other localities within a particular cell or of other cells. Ca^{++}-induced fluorescence of specific chromophobe dyes although commonly used to measure intracellular Ca^{++} levels in many cellular systems (e.g. Palade & Vergara, 1982; La & Shimomura, 1982) has also previously proved inappropriate for thymocytes. Their small size has always precluded the micro-injection of such fluorescent dyes, but very recently a novel method of introducing fluorescent probes into the cytosolic environment has been established. The technique is particularly applicable to small mammalian

cells in suspension and thus to the thymic lymphocytes of this study.

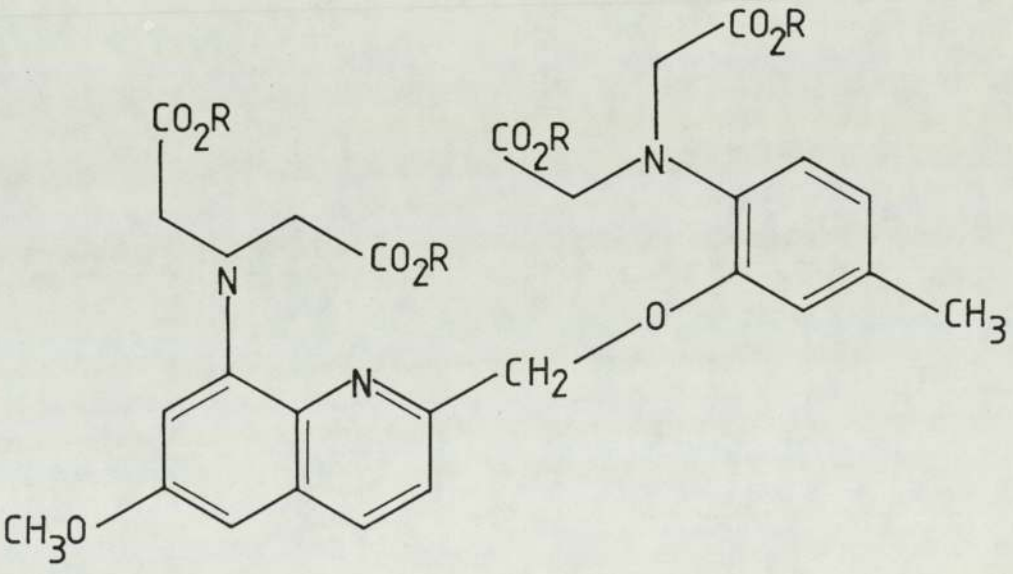
3.3.1 Measurement of Intracellular Ca^{++} by a Fluorescent Indicator

A novel family of calcium chelators has been prepared with high affinity for Ca^{++}, low affinity for Mg^{++} and H^{+}, large absorbance and fluorescence changes as a consequence of Ca^{++} binding and little or no detectable binding to membranes (Tsien, 1980). The fluorrescent quinoline Ca^{++}-indicator termed 'Quin 2' has been used in this study. This tetracarboxylate anion however, is hydrophilic and thus totally membrane impermeant. A non-disruptive technique for loading the indicator has therefore been devised. 'Quin 2' is made temporarily membrane permeable by masking the four carboxylates with esterifying groups (See diagram 15). This uncharged derivative now readily diffuses into the cells where cytoplasmic esterases hydrolyse the ester groups regenerating and trapping the parent tetranion in the cytosol (Tsien, 1981). Quin 2 tetraacetoxymethyl ester, (Quin 2/AM) supplied as a freeze dried oil (Amersham International plc) was dissolved in dry dimethyl sulphoxide (DMSO) and desiccated at $-20^{o}C$ between use.

Quin 2 binds Ca^{++} with a 1:1 stoichiometry and an effective dissociation constant of 115 nM in a cationic environment chosen to mimic the cell cytoplasm. Its fluorescent signal increases when the Ca^{++} free form is converted to the Ca^{++} saturated form. Intracellular Quin 2 is not sequestered inside cellular organelles, the loading level does not appear to perturb the steady state intracellular calcium concentration and it is without serious toxic effects although at very high levels some lowering of cellular ATP has been observed



QUIN 2 : X = O^-



QUIN 2/AM : R=$-CH_2-O-C(=O)-CH_3$

Diagram 15 Structure of Fluorescent Quinoline Calcium Indicator, Quin 2 and its Acetoxymethyl Ester, Quin 2/AM.

(Tsien, Pozzan & Rink, 1982a; b). Similarly intracellular pH has little effect on the fluorescent signal (Tsien et al., 1982a). Thus, it appears that this is a simple but very effective method for monitoring changes in intracellular free Ca^{++} levels, ($[Ca^{++}]i$).

For this investigation thymic lymphocytes were isolated, as previously described and adjusted to a final concentration of 2 x 10^8 cells ml^{-1}. Loading of the cells with Quin 2 and the fluoressence measurements were performed, employing a modification of the technique developed by Tsien and colleagues (1982a;b). Quin 2 acetyoxymethyl ester (Quin 2/AM) was added to each cell suspension at a final concentration of 0.2 mM (shown to give optimal fluroescence signals with mitogens of rat thymocytes without showing cytotoxicity) and incubated in stoppered plastic tubes in the roller drum assembly at 37°C for 20 minutes. The suspension was subsequently diluted ten fold and incubated for a further 60 minutes (the final concentration of DMSO from the Quin 2 stock did not exceed 0.02% vol/vol). After loading, cells were centrifuged (at 1,000g for 3 minutes) and resuspended in fresh media at approximately 2 x 10^7 cells ml^{-1}. The cell suspensions were rolled in the roller drum assembly at room temperature until measurements were to be made (Cells have been shown to lose less than 5% of their dye each hour (Tsien et al., 1982a)). The fluorescence measurements were recorded with an Aminco-Bowman Spectrophotofluorimeter (American Instrument Company Ltd). Standard monochromator settings were 339 nM and 492 nM, excitation and emission wavelengths respectively. Wavelengths longer than 339 nM have been shown to decrease the sensitivity to calcium binding whereas shorter wavelengths promote cell autofluorescence.

Fluorescence was determined in a 2 ml cell suspension containing 10^7 cells ml^{-1}. One millilitre of cells was removed from the cell suspension which had been kept at room temperature (21 - 25°C) and was centrifuged rapidly for a few seconds in a MSE Micro Centaur (MSE Scientific Instruments). The cells whose viability was maintained at greater than 90% were resuspended in 2 mls of a physiological saline and transferred to a square cuvette for fluorescence measurements. This latter step minimizes any carryover of Quin 2. The simplified saline was employed in this study because medium 199 contains substances (such as phenol red) which may filter out excitation and emission energies and also which may contribute their own fluorescence. This physiological saline consisted of (mM):NaCl, 145; KCl, 5; Na_2HPO_4, 1; glucose, 5; Hepes buffer, 10; and was adjusted to approx. pH 7.4. Mg^{++} and Ca^{++} were added from stock solutions of their respective sulphate and chloride salts as required.

The fluorescence was continuously monitored using a flat bed recorder (J.J. 'XY' plotter). Gaps on the traces denote additions to, or stirring of, the cell suspension within the cuvette. Additions of saline and DMSO were employed as controls. Unfortunately the cuvette could not be thermostatted at 37°C but measurements have shown that the cell suspension in the cuvette during fluorescence determinations rapidly reached and was maintained at a constant 30°C (30 ± 1°C). The suspensions in the cuvette were therefore allowed a 10-15 minute period to reach this temperature before basal fluorescence was recorded. The putative mitogenic agents were subsequently added as 20 microlitre aliquots and any change in fluorescence monitored.

For calibration, two arbitrary reference points were chosen, the fluorescence of quin 2 within the resting cell (i.e. in basal intracellular [Ca^{++}]) and its fluorescence in the extracellular environment (i.e. at 100% Ca^{++} - saturation). This latter signal was achieved by releasing the indicator from cells with Triton X-100 at the end of each experiment. Quin 2 was shown to be fully saturated in 0. 6mM Ca^{++} (the extracellular Ca^{++} level used in these studies) since an increase of Ca^{++} above this value failed to alter the fluorescence signal. Thus, assuming that most resting thymic lymphocytes maintain a constant basal [Ca]i any change in intracellular Ca^{++} level following mitogen addition may be represented by:- $\triangle I = \frac{(Im - Ib)}{(Is - Ib)}$, where, Im denotes the intensity of fluorescence after mitogen application, Ib the intensity observed in resting cells and Is the fluorescence at saturation of quin 2 (at 0.6mM). Autofluorescence measured from unloaded cells at the same density was negligable under the conditions of this experiment.

Quin 2 thus has distinct advantages as an indicator of [Ca^{++}]i over other techniques, which include a simple non disruptive loading procedure, a 1:1 stoichiometry which simplifies calibration and a high sensitivity at low resting calcium levels. Quin 2 however, is still in its early stages of development and there are aspects which are to be improved. For example modification of the dye structure to increase slightly the dissociation constant would make it more accurate in the quantification of stimulated [Ca]i levels. Also lower loadings would suffice if the dye worked at longer wavelengths thus exciting less cell autofluorescence. Independent studies have reported that Quin 2 is a mitogenic agent in its own

right at concentrations required for the measurements of intracellular Ca^{++} concentration (Hesketh, Smith & Metcalfe, 1982). This has not been verified in the thymocyte culture system. There are therefore some uncertainties in the interpretation of quin 2 data. It is also unclear whether or not the $[Ca^{++}]i$ indicated by quin 2 fluorescence is the same as in resting thymic lymphocytes before loading or whether or not there is any metabolic stimulation by quin 2 which induces a change in the calcium level. It is similarly unknown whether the changes in $[Ca^{++}]i$ observed by mitogen application to quin 2 loaded cells are comparable to those which occur in cells unperturbed by the presence of the dye. It should be noted thus, that because there are still such uncertainties these preliminary quin 2 studies have been employed in combination with other measurements obtained from an indirect technique which quantified the transient transmembrane fluxes of radiolabelled calcium molecules during mitogenic stimulation.

3.3.2 Radioisotopes for Measurement of Ionic Translocation

Radioisotopes can be used in biological experiments principally because living cells do not discriminate between isotopes of a particular element. The effective tracer thus,

1) must have the same thermodynamic properties as the molecule it replaces.
2) must not alter the system chemically, nor must its transport cause a shift in the centre of mass of the system (i.e. tracer/substituent mass ratio must be close to unity).

3.3.2a Calcium Uptake

To reveal the proposed mitogen-induced transmembrane Ca^{++} fluxes, the beta-emitting isotope calcium-45 (Amersham International p.l.c.) was employed to represent calcium. Being the isotopic form of the natural molecule it satisfies both of the above criteria. When added at a concentration of 1 μCi ml^{-1} the calcium content of the tracer (with specific activities between 10 and 40 m Ci mg^{-1}) is at most 0.1 μg Ca^{++} ml^{-1}. Since in basal conditions the Ca^{++} concentration is equivalent to 0.6 mM (24 μg ml^{-1}) the weight of $^{45}Ca^{++}$ present in any 1 millilitre cell culture can be considered negligible.

The beta radiation from this calcium isotope was detected and measured using liquid scintillation spectroscopy in a Packard tri-carb counter, model No. 2660. An internal standard was used to correct for decay and the efficiency of the beta-detectors corrected using an on-line data handling system. It proved necessary to modify the cultures to enable measurement of tracer redistribution. To give maximal $^{45}Ca^{++}$-uptake the cell concentration was adjusted to 10^8 cells ml^{-1}, above which over crowding appears to decrease measurable activity (Freedman, 1979). This optimal cell concentration and the use of 2 ml cultures where required (to allow removal of sufficient aliquots to give triplicate results) did not influence the mitotic capacity of the cells (results not shown). At least a thirty minute preincubation period, allowing recovery from the preparative procedures, was employed for all isotope uptake measurements. Preliminary experiments showed that the basal uptake profile does not differ significantly with differing preincubation times (from 30 minutes to 360 minutes) providing that the isolation and preincubation occurs in the presence of normal

calcium levels. The problem of efficient removal of unincorporated isotope in the extracellular environment without any exchange of tracer between the cells and any washing medium was surmounted by employing a modification of a centrifugal technique developed by Freedman, Gamperts & Raff, (1975).

After the preincubation, aliquots from cell suspensions were added to prewarmed tubes containing the isotope and desired ionic and hormonal concentrations. At the appropriate time(s) thereafter, '200 microlitre' aliquots were removed in triplicate and centrifuged rapidly in pre-prepared microfuge tubes. The cell associated $^{45}Ca^{++}$ was distinguished from the unincorporated extracellular tracer by spinning through a water impermeable oil barrier. The polypropylene microcentrifuge tubes (Beckman PRO-22) were prepared by spinning two density gradients achieved with 0.05 ml 98% formic acid and 0.1 ml oil (Silicone oil MS 550) in each microfuge tube. At each desired time point the 200 µl aliquots of cell suspension were layered above the oil, forming a three tier gradient, and rapidly centrifuged at 8500g for two minutes in a Beckman microcentrifuge model B (Beckman Limited). The isotope laden cells thus pass through the oil layer into the formic acid where they are subsequently lysed releasing the isotope to be counted. The incubation time for each tube was taken from the time of addition of cells to the isotope to the beginning of the centrifuge run; the microfuge achieves maximum force within five seconds. After centifugation the contents of the tubes were frozen by immersion in liquid nitrogen and the bottom (approximately 1 cm) containing the lysed cells was amputated by a lateral cut through the oil layer and placed in scintillation vials for counting. The inherent properties of the oil present potential

errors to this technique. The oil used must necessarily be chemically inert, highly water impermeable and allow maximal cell passage with minimal supernatant carryover. Preliminary experiments revealed that a minimum of 0.075 ml oil was required to prevent the mixing of the cell suspension and acid during the aliquoting procedure. Thus a standard oil volume of 0.1 ml was adopted throughout. Silicone oil (MS 550) proved to be suitably water resistant as demonstrated by employing numerous aqueous dyes and an aqueous $Na^{125}I$ solution. Controls (blanks) were run throughout each experiment by centrifuging 200 µl of the cell free isotope (1µCi ml^{-1}) through the oil to monitor any inherent changes in the physical properties of the oil during the course of the experiment. This was generally found to be zero but where necessary the mean was systematically subtracted from each $^{45}Ca^{++}$ uptake value. The use of ^{51}Cr labelled cells also revealed that this oil allowed the passage of a majority of cells into the formic acid (Atkinson, upublished data). As cells traverse the oil layer it is likely that extracellular fluid will be trapped within the interstitial spaces carrying isotope into the formic acid layer or alternatively some activity may be lost during the cells passage through the oil generating a radioisotopic gradient in the hydrophobic region. These problems would obviously pose considerable inaccuracies in distinguishing incorporated from supernatant isotope. The use of labelled ^{3}H inulin as a marker of entrained water space provided a tool for the quantification of isotopic carryover. Under basal conditions carryover of extracellular fluid represented 0.13% $\pm$ 0.03 (n=35) of total supernatant activity. Mitogens used during this investigation

do not appear to significantly influence this carryover and thus the value was subtracted from each uptake value observed. The isotopic content throughout the oil layer has been previously determined using two additional oils (one more and the other less dense than MS 550) to separate the original oil from both supernatant and formic acid activity. Employing this procedure it has been shown that there is no exchange of tracer within the silicone oil layer (Atkinson, unpublished data). When chilled $^{45}Ca^{++}$ was added to incubated cells at 4°C and immediately centrifuged a relatively large uptake of activity was consistently observed accounting for approximately 66% of the $^{45}Ca^{++}$ uptake determined. Over a 20 minute period at 4°C this value marginally increased to 71% $\pm$ 3 (n=20) at 20 minutes (in general the time chosen for $^{45}Ca^{++}$ uptake measurements as discussed in results section). The 71% most likely represents rapid exchange of labelled material with cations adsorbed to the cell surface and is therefore probably not related to transmembrane transport. The value was employed to correct each of the $^{45}Ca^{++}$ uptake measurements to allow determination of absolute calcium influx.

The calcium taken up into the cells can be derived therefore from the following equation; all the values substituted, already being pre-corrected for counting efficiency, and background activity

$$\%Ca^{++}\ \text{uptake at time } t = \left[\frac{(\text{Sample d.p.m.} - \text{Blank d.p.m.})}{\text{Total d.p.m.}} - \text{carry over} \right] - \text{Membrane Associated } Ca^{++} \text{ at time } t\ (\%) \quad \ldots.(1)$$

Total represents the counts of the whole tube and blank is a measurement of any passage of cell free isotope through the hydrophobic layer. Membrane associated Ca^{++} is the isotope attachment at 4°C.

To calculate the actual weight of Ca^{++} translocated from the equation the total Ca^{++} concentration of the culture must be considered. Thus, the weight taken up per 10^8 cells is calculated from the product of calculated % Ca^{++} (from (1)) and the respective extracellular Ca^{++} contents employed in the experiment i.e. 24 µg for basal conditions and 72 µg for high Ca^{++} conditions.

The uptake of $^{45}Ca^{++}$ by isolated rat thymic lymphocytes may be a function of two independent variables namely the rate of exchange across the membrane (influx and efflux) and the size of the compartment with which it exchanges. It is possible that the uptake profile may be misinterpreted since an increase in cell volume and thus $^{45}Ca^{++}$ may occur without an absolute increase in intracellular Ca^{++} concentration. A series of experiments performed in this laboratory (Atkinson, unpublished data) have provided evidence that there is no detectable change in cell volume during thymocyte activation.

3.3.2b Sodium Efflux Studies

In an attempt to assess the activity of the (Na^+/K^+)-membrane pump and thus give an indication of the intracellular sodium levels, the efflux of radiolabelled ^{22}Na from preloaded thymic lymphocytes was monitored. ^{22}Na (Amersham) of specific activity 0.265µCi ng^{-1} was added to the standard cultures to give

a final concentration of 1.2 μCi ml^{-1}, (the additional Na^+ added to the medium in radioisotopic form did not influence the osmotic potential of the extracellular environment), and incubated at 37°C for 3 to 4 hours. Preliminary experiments showed that after such time the uptake of ^{22}Na had reached a plateau.

The cultures were subsequently centrifuged for 3 to 4 minutes (1,000 g), the supernatant decanted and the cell pellet re-suspended in prewarmed medium. This procedure was repeated three times to minimise carryover of extracellular, non cell-associated ^{22}Na.

Sodium efflux was assessed in the presence or absence of mitogen required at 37°C, over a twenty minute period by measuring the cell associated radioactive sodium. For this purpose a Tracerlab gamma set 500 counter was employed, pre-calibrated for the sodium photopeak. An internal standard was used to correct for decay. Cells were rapidly separated from the culture medium via the centrifugation technique of Freedman et al., 1975) as previously discussed. Appropriate corrections were made as regards carryover when calculating cell associated $^{22}Na^+$.

3.4 STATISTICAL ANALYSIS

Groups of data have been compared by the use of Student's (unpaired) 't' test; values of 'P' less than 0.05 ($P < 0.05$) were considered to indicate significance. In each of the figures (of section 4.0), values have been expressed as the means $\pm$ the standard error of the mean (S.E.M.); n represents the number of separate experiments in each group. Asterisk(s) denote the degree of significance * $P<0.05$; ** $P<0.01$; *** $P<0.001$ in all figures and tables.

4.0 EXPERIMENTAL RESULTS

In accordance with previous studies (see section 2.4) rat thymic lymphocytes continued to enter mitosis and accumulate as recognisable mitotic configurations when suspended in colchicine supplemented Medium 199. With the calcium and magnesium ion concentrations adjusted to reflect the ambient levels of normal rat blood (0.6 mM Ca^{++}; 1.0 mM Mg^{++}) approximately 3.5% of the cells entered mitosis over the six hour incubation period (figure 1). This basal proliferative activity was not influenced by the removal of either, or both, of the divalent cations from the external medium (results not shown) suggesting that neither cation is essential for the progression through the cycle of cells already committed to divide in the native tissue, prior to its removal from the animal. When the concentrations of the extracellular calcium or magnesium ions were elevated to 1.8 mM or 2.5 mM respectively an additional cohort of cells served to raise the proportion of mitotic cells to 6%. Thus, both of these divalent cations at high concentrations are capable of rapidly recruiting thymic cells from a normally quiescent cell population "GO". Consistent with the in vivo observations that castration and hence removal of circulating sex steriods increases thymus size, whilst their administration produces marked thymic atrophy (Scheiff & Haumont, 1979; Ho & Hoshino, 1963), high concentrations (0.1 $\mu g\ ml^{-1}$) of beta-oestradiol and testosterone were found to influence the mitogenic response. Oestradiol was found to inhibit mitosis provoked by high Ca^{++}, whereas testosterone abolished the high Mg^{++}-induced mitotic stimulation (figure 1).

The elevation in the extracellular divalent cation concentration employed here to promote mitosis would most likely temporarily

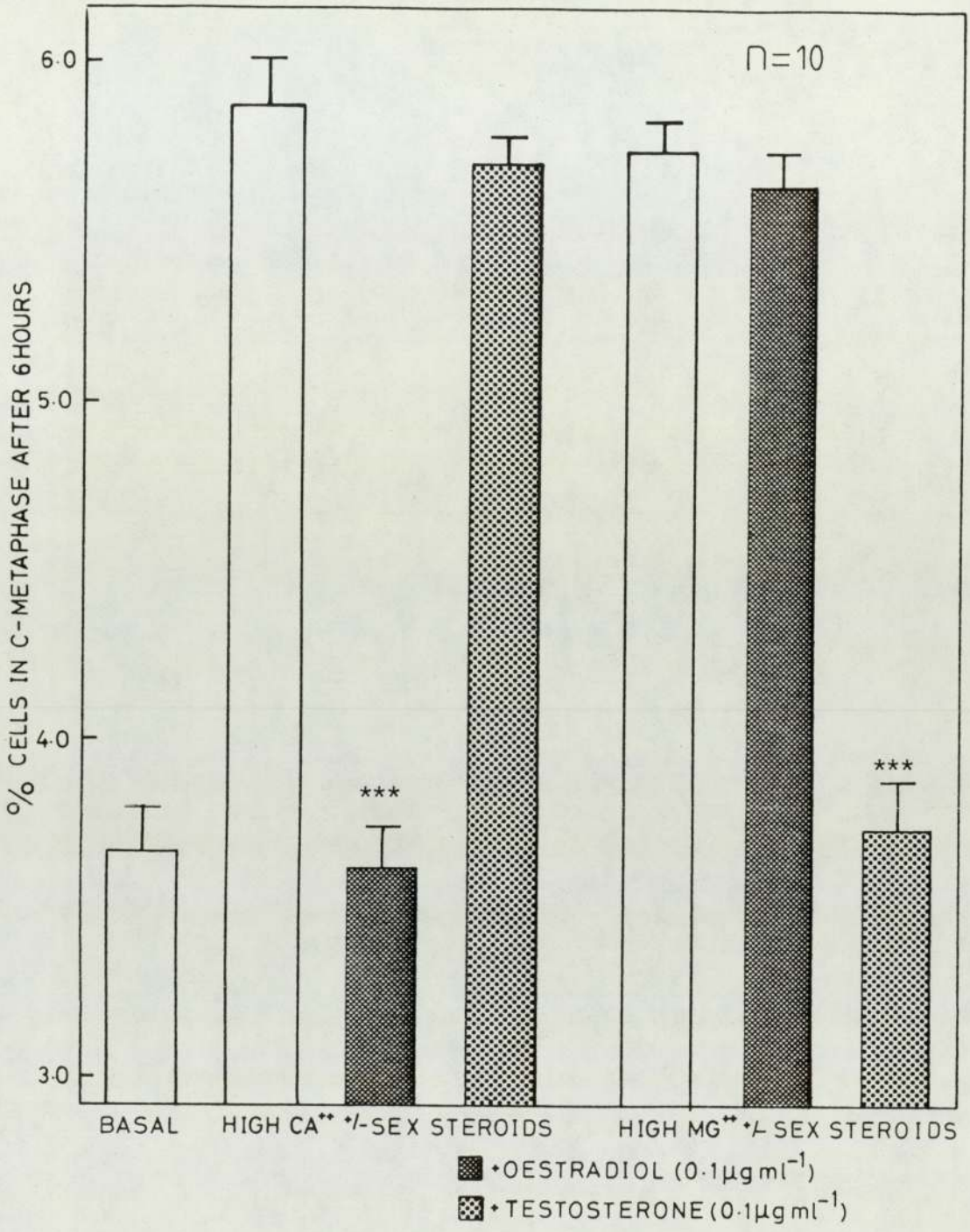


Figure 1 Effects of Sex Steroids on Calcium- and Magnesium-Induced Mitogenesis.

The basal medium contains 0.6 mM Ca^{++} and 1.0 mM Mg^{++}. High Ca^{++} and High Mg^{++} indicate where these concentrations were increased to 1.8 mM and 2.5 mM respectively. Columns represent mean values $\pm$ S.E.M., and n = the number of separate experiments in each group. Values significantly different from those of corresponding steroid free cultures are indicated by asterisk(s).

overwhelm internal calcium and magnesium homeostatic processes. A transient rise in intracellular calcium for example, might therefore be expected to initiate the mitogenic response in thymic lymphocytes. Such an alteration in cytosolic Ca^{++} concentration ($[Ca^{++}]i$) certainly appears to be a common factor in stimulus-response coupling of secretory and contractile tissues (sections 1.3 and 1.4) and indeed has been implicated in the mitotic recruitment of various cell types (sections 2.3.3a). Calcium influx may thus mediate stimulus-mitosis coupling of the cells of this study.

Verapamil (a papavarine derivative) is a calcium antagonist with the ability to block calcium specific channels (Fleckenstein, 1977; Flaim & Craven, 1981; Van Neuten & Vanhoutte, 1981; Janis, 1981; Lee and Tsien, 1983). If calcium movement into thymocytes is indeed a mitogenic trigger, then high Ca^{++}-induced mitosis should be compromised by the addition of verapamil to the culture medium. The mitogenic response normally produced by 1.8 mM Ca^{++} was indeed blocked by the antagonist in a dose-dependent manner (figure 2). Maximal inhibition was achieved at 5×10^{-6} gml^{-1}. Concentrations above 5×10^{-5} gml^{-1} proved highly cytotoxic. Thus, Ca^{++} influx appears to be a prerequisite to Ca^{++}-induced mitotic response in thymocytes. Verapamil at 5×10^{-6} gml^{-1} did not influence basal mitotic activity as would be expected and significantly did not abolish magnesium-stimulated proliferation (figure 3). Magnesium thus does not appear to promote mitosis via enhanced calcium influx and furthermore any signal magnesium movement does not occur through a calcium channel. Such conclusions however are only valid if as assumed verapamil does prevent transmembranal movement of calcium into thymic lymphocytes stimulated to divide. To verify these

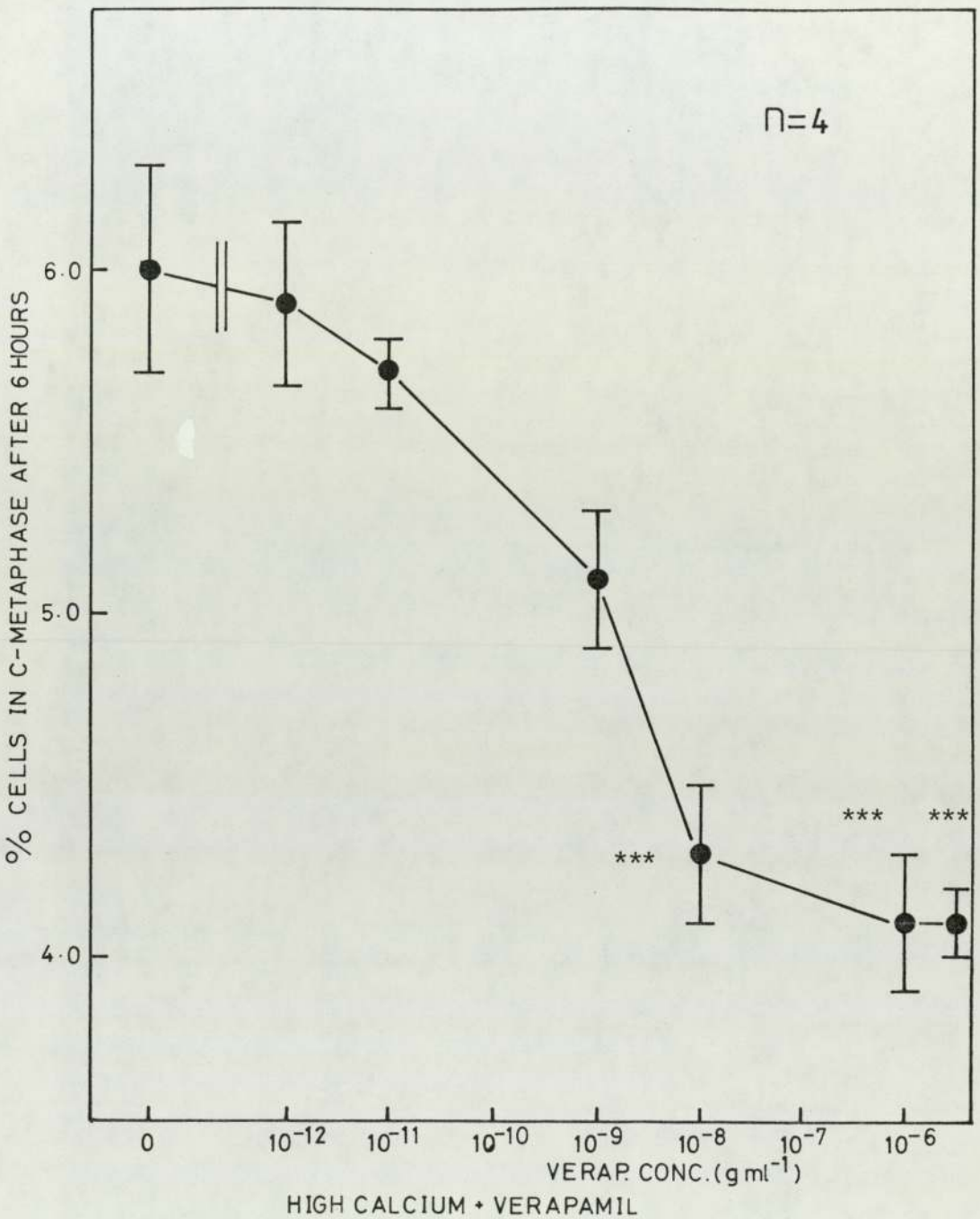


Figure 2. Effect of Verapamil Concentration on High Ca^{++}-Induced Mitogenesis.

High Ca^{++} represents calcium concentration in culture medium of 1.8 mM. Values significantly different from those of cultures incubated free of verapamil are indicated by asterisk(s). Other details as in Figure 1, see section 3.4.

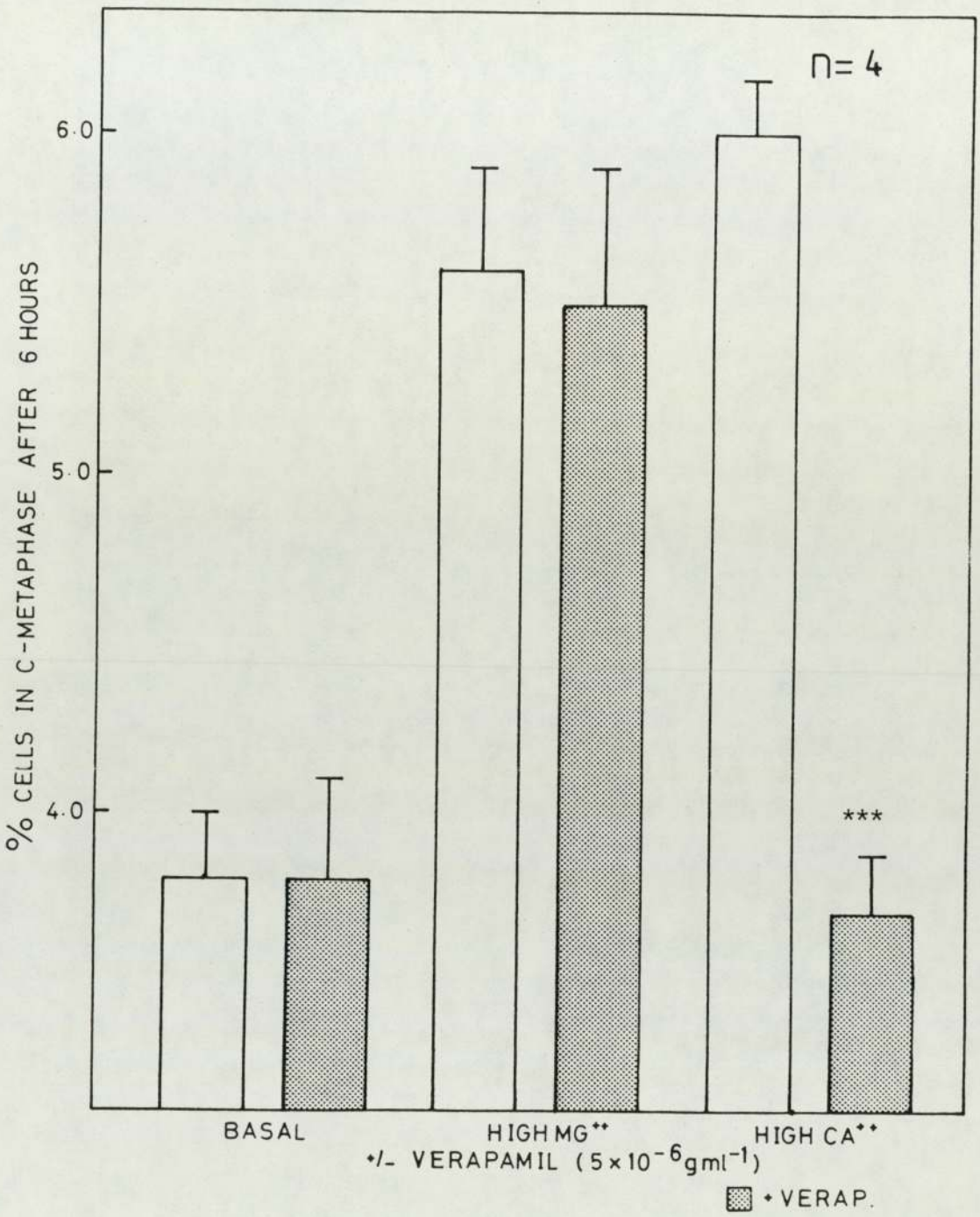


Figure 3. Effect of Verapamil on Basal, High Ca^{++}-Induced and High Mg^{++}-Induced Mitotic Activity.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}; High Ca^{++} and High Mg^{++} indicate where these concentrations were raised to 1.8 mM and 2.5 mM respectively. Concentration of verapamil employed was $5 \times 10^{-6} gml^{-1}$. Values significantly different from those of corresponding cultures incubated free of the calcium antagonist are indicated by asterisk(s).

assumptions the radioisotope of calcium (^{45}Ca) was employed to monitor calcium transport in thymocytes at rest and during their recruitment from "GO" and the subsequent effects of the Ca^{++} antagonist on such movement (see section 3 for details of methodology).

Thymocytes have been shown to undergo DNA synthesis in less than one hour after mitogenic-stimulation (Morgan, 1976; Whitfield, MacManus, Boynton, Gillan & Isaacs, 1974). The crucial period of time which elapses between mitogen application and commitment to the division cycle obviously lies within this period. To determine the extent of such an 'activation period', experiments were conducted where the cells were systematically removed from the high calcium environment and resuspended in basal conditions at different times during the incubation. At six hours the mitotic cells were assessed in the usual way. The high Ca^{++} level was required only for the first twenty minutes of incubation, after which time the thymic lymphocytes became irreversibly committed to proliferation (figure 4). Therefore, any calcium movement pertinent to the mitogenic-triggering process must be completed during this period. Freedman (1979), using mouse lymphocytes, has also shown that the maximum Ca^{++} uptake during mitogenic activation occurred at between 6 and 30 minutes. Consequently, all ion-flux investigations have been limited to the first twenty minutes after mitogen addition.

Under basal conditions (0.6 mM Ca^{++}) the uptake of calcium (more properly termed cell associated calcium) seemed to indicate the existence of a relatively large, rapidly (within 5 minutes) equilibrating reservoir which is probably analogous to the glycocalyx or membrane bound pool (figure 5). Once this extracellular compartment

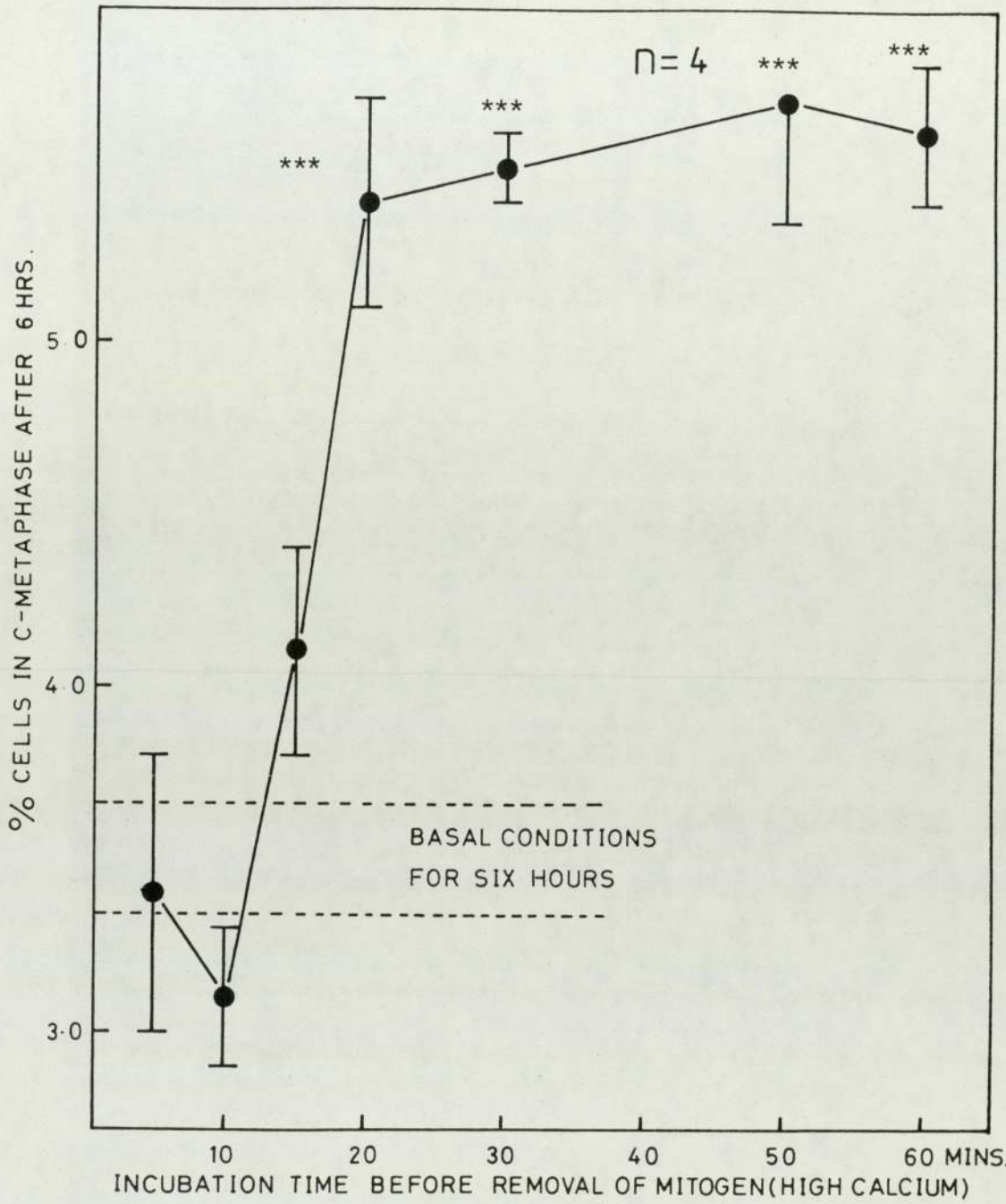


Figure 4 Determination of the 'Activation Period' for Mitogens of the Thymic Lymphocyte.

Mitogen employed was 1.8 mM Ca^{++}. Cells were removed from the high Ca^{++} environment and resuspended in basal conditions at times indicated, for the remainder of 6 hours incubation period. Values significantly different from those of cultures incubated in basal conditions for 6 hours, are indicated by asterisk(s).

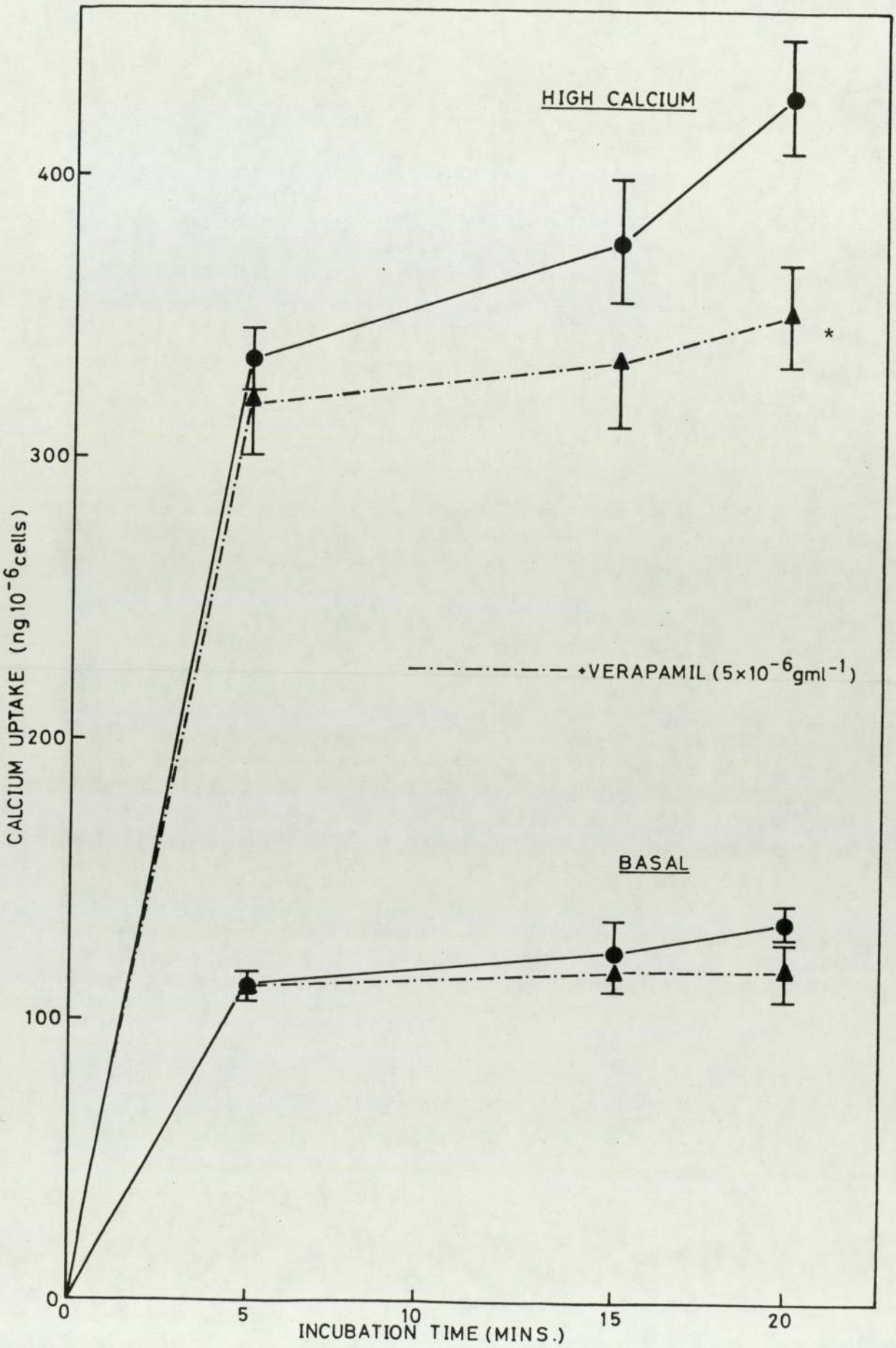


Figure 5 Effect of Verapamil on $^{45}Ca^{++}$ Uptake into Resting and Mitogen-Stimulated Thymocytes.

became saturated the relatively linear uptake observed presumably corresponds to transmembrane flux. Elevation of the extracellular Ca^{++} concentration to 1.8 mM would be expected to throw the calcium homeostatic system out of equilibrium. Indeed, the rapidly saturated pool dramatically expanded (approximately three fold) probably a result of simple electrostatic attraction between Ca^{++} and the cell surface. The rate of transmembrane flux similarly increased above the basal rate (figure 5). Experiments were executed to determine the actual size of the membrane associated pool as compared to the transmembrane movement. $^{45}Ca^{++}$ uptake measured at 4°C presumably represents the rapid exchange of labelled material with cations adsorbed to the cell surface and is therefore unrelated to the transmembrane flux. The membrane pool was almost saturated at 30 seconds and at this time represented a large percentage of the total cell associated calcium (approximately 66%). After 20 minutes the value rose to 71%. This percentage was unaffected by verapamil addition which suggested that indeed it was unrelated to transmembrane movement. Similarly it was not influenced by the presence of various mitogenic stimuli. After subtraction of membrane associated Ca^{++}, the mean rate of Ca^{++} uptake over 20 minutes in basal conditions was found to be 2.0 ng/min/10^6 cells. The increasing calcium uptake over this time period suggests that the simultaneous efflux, which under "steady state" conditions would be equal and opposite, is occurring from a pool which is not fully saturated with radioisotopic calcium. The effect of verapamil on this basal and stimulated $^{45}Ca^{++}$ influx was subsequently determined. The mean rate of Ca^{++} uptake in basal conditions was marginally, but not significantly ($P > 0.05$), depressed by verapamil at 5×10^{-6} gml^{-1}. In

contrast, verapamil significantly ($P < 0.05$) depressed calcium influx when cells were exposed to high extracellular calcium concentrations (table 1). This is consistent with the view of others that Ca^{++} antagonists inhibit the activation of, or block flux through, activated calcium channels rather than influencing membrane permeability at rest (Rosenberger, Ticku & Triggle, 1979).

Table 1 The Effect of Verapamil on the Mean Rate of $^{45}Ca^{++}$ Uptake in Resting and Mitogen-Stimulated Lymphocytes

Treatment: Verapamil Concentration ($g ml^{-1}$)	Medium Cation Concentration (mM): Calcium	Magnesium	Mean Rate of Ca^{++} Uptake over 20 Minutes ± S.E.M. ($ng\ min^{-1}$ 10^{-6} Cells)
0	0.6	1.0	*** 2.0 ± 0.3 (n=4)
5×10^{-6}	0.6	1.0	*** 1.1 ± 0.5 (n=4)
0	1.8	1.0	6.3 ± 0.3 (n=5)
5×10^{-6}	1.8	1.0	*** 2.2 ± 0.1 (n=5)

Significantly different from high Ca^{++} cultures *** $P < 0.001$

Therefore the probable trigger for mitosis (in the case of high $[Ca^{++}]o$ at least) is a rise in the cytosolic calcium concentration. Agents which modify Ca^{++}-dependent mitogenesis may thus act by influencing calcium uptake into the cells.

The capacity of oestradiol (at 0.1 $\mu g\ ml^{-1}$) to inhibit calcium induced mitogenesis may thus stem from its ability to prevent Ca^{++} influx. Ca^{++} movement was therefore monitored in resting and stimulated cells in the presence of 0.1 μgml^{-1} beta-oestradiol to test this hypothesis. When unstimulated cells were incubated with this anti-mitotic concentration of the sex steroid there was not a significant alteration in Ca^{++} entry over the initial twenty minute period (figure 6). Surprisingly however, oestradiol did not prevent Ca^{++} influx into the mitotically stimulated cells. In fact the steroid appeared to potentiate the cation uptake. In high Ca^{++} conditions the Ca^{++} uptake at 20 minutes was significantly higher ($p < 0.05$) in the presence of oestradiol. After subtraction of membrane bound Ca^{++}, this effect represented an increase in the mean rate of Ca^{++} influx from 5.8 $ngml^{-1}10^{-6}$ cells to 10.1 $ngml^{-1}10^{-6}$ cells (table 2).

Table 2 The Effect of High Oestradiol Concentration on Basal and Stimulated Rates of Ca^{++} Influx Into Rat Thymic Lymphocytes.

Treatment Oestradiol Concentration (μgml^{-1})	Medium Cation Concentration (mM) Calcium	Magnesium	Mean Rate of Ca^{++} Uptake Over 20 mins $\pm$ S.E.M. ($ngmin^{-1}\ 10^{-6}$ cells)
0	0.6	1.0	2.3 $\pm$ 0.3 (n = 4)
0.1	0.6	1.0	1.78 $\pm$ 0.3 (n = 4)
0	1.8	1.0	5.8 $\pm$ 0.8 (n = 5)
0.1	1.8	1.0	*10.1 $\pm$ 1.0 (n = 5)

Significantly different from corresponding cultures incubated in the absence of oestradiol * $P < 0.05$.

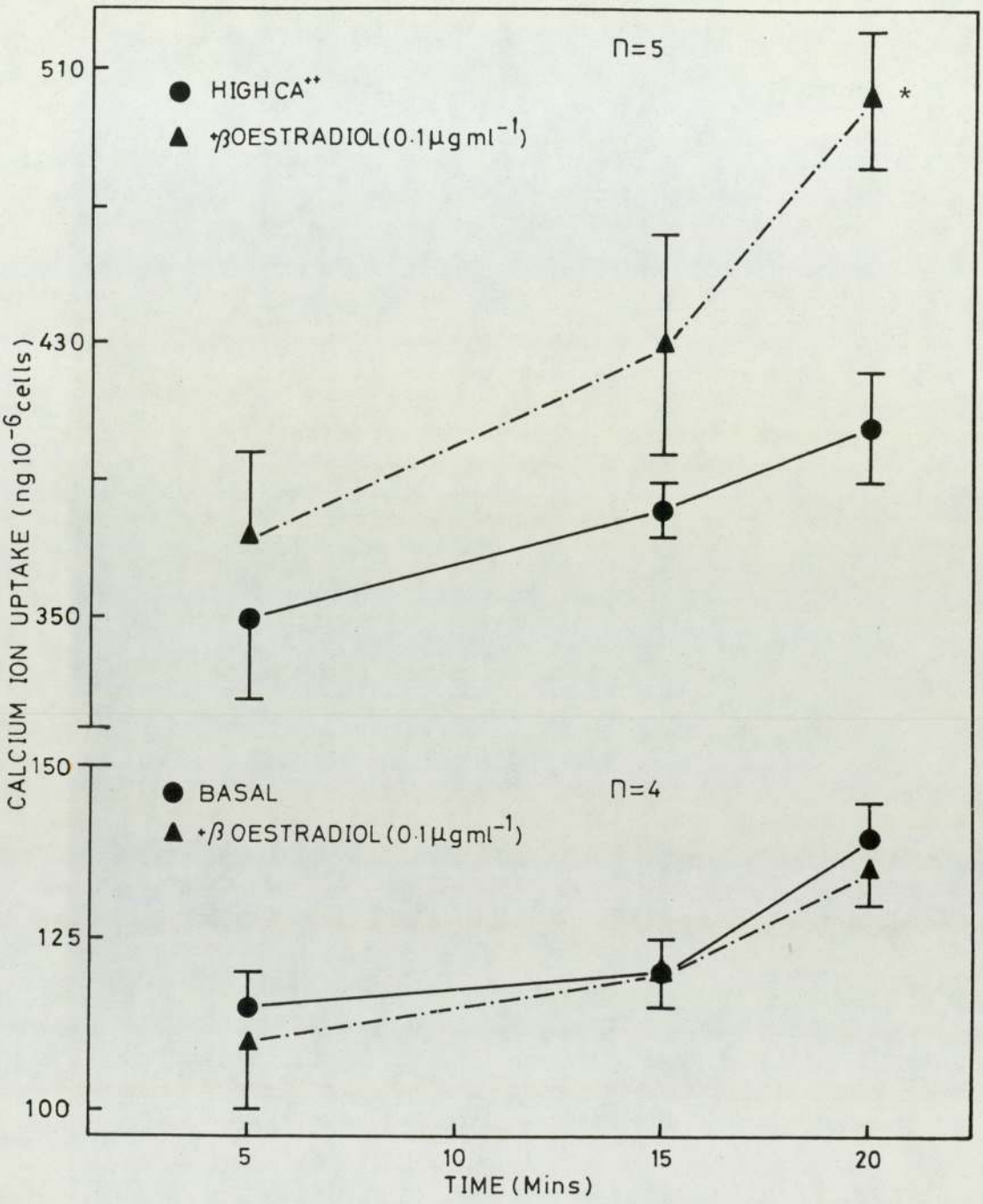


Figure 6 Effect of Beta-Oestradiol (0.1 $\mu g ml^{-1}$) on $^{45}Ca^{++}$ Uptake Into Rat Thymic Lymphocytes.

Uptakes were measured in both basal and high Ca^{++} media (0.6 mM and 1.8 mM Ca^{++} respectively). Values significantly different from those of corresponding cultures incubated free of the sex steroid, are indicated by asterisk(s).

This effect however, may actually represent an indirect effect occurring via activation of the Ca^{++} extrusion pumps at the membrane and efflux of unlabelled calcium ions. Since in this case, Ca^{++} influx would increase to compensate for the excess Ca^{++} loss, (thus maintaining intracellular calcium at the levels of stimulated cells) it is necessary to measure cytosolic concentrations of the ion during the time of action of oestradiol. For this purpose Quin 2 (see section 3.3.1) was employed. The ionophore A-23187 which is known to stimulate mitosis and to raise the cytosolic calcium content by the promotion of Ca^{++} specific ionophoresis (Coffey, et al., 1978; Hadden et al., 1977) was used to demonstrate the effectiveness of the procedure. An increase in cytosolic Ca^{++} concentration is represented by the change in fluorescence intensity of Quin 2 (in arbitary units) from the basal levels in the resting cell.

$\triangle I = \frac{(Im - Ib)}{(Is - Ib)}$ (see methods, 3.3.1 for details). For the ionophore, Ca^{++} concentration increased rapidly over a twenty minute period, $\triangle I = 0.59$ (figure 7), whereas the addition of equal volumes of physiological saline and DMSO (0.02% vol/vol) did not influence the $[Ca^{++}]i$ over similar 20 minute periods (figure 7) as was expected. Elevation of the extracellular Ca^{++} concentration caused an increase in $[Ca^{++}]i$ to a level which was less than that provoked by A-23187 which may have been expected, $\triangle I = 0.31$ (figure 7, table 8). The cells retained their ability to respond to elevated extracellular calcium conditions even after four hours incubation at room temperature (figure 7, table 8). Cell viability was also maintained at greater than 90% during this period. In high Ca^{++} conditions cells continued to accumulate calcium ($\triangle I = 0.56$ at 44 minutes). Significantly, oestradiol at the inhibitory dose promoted a further

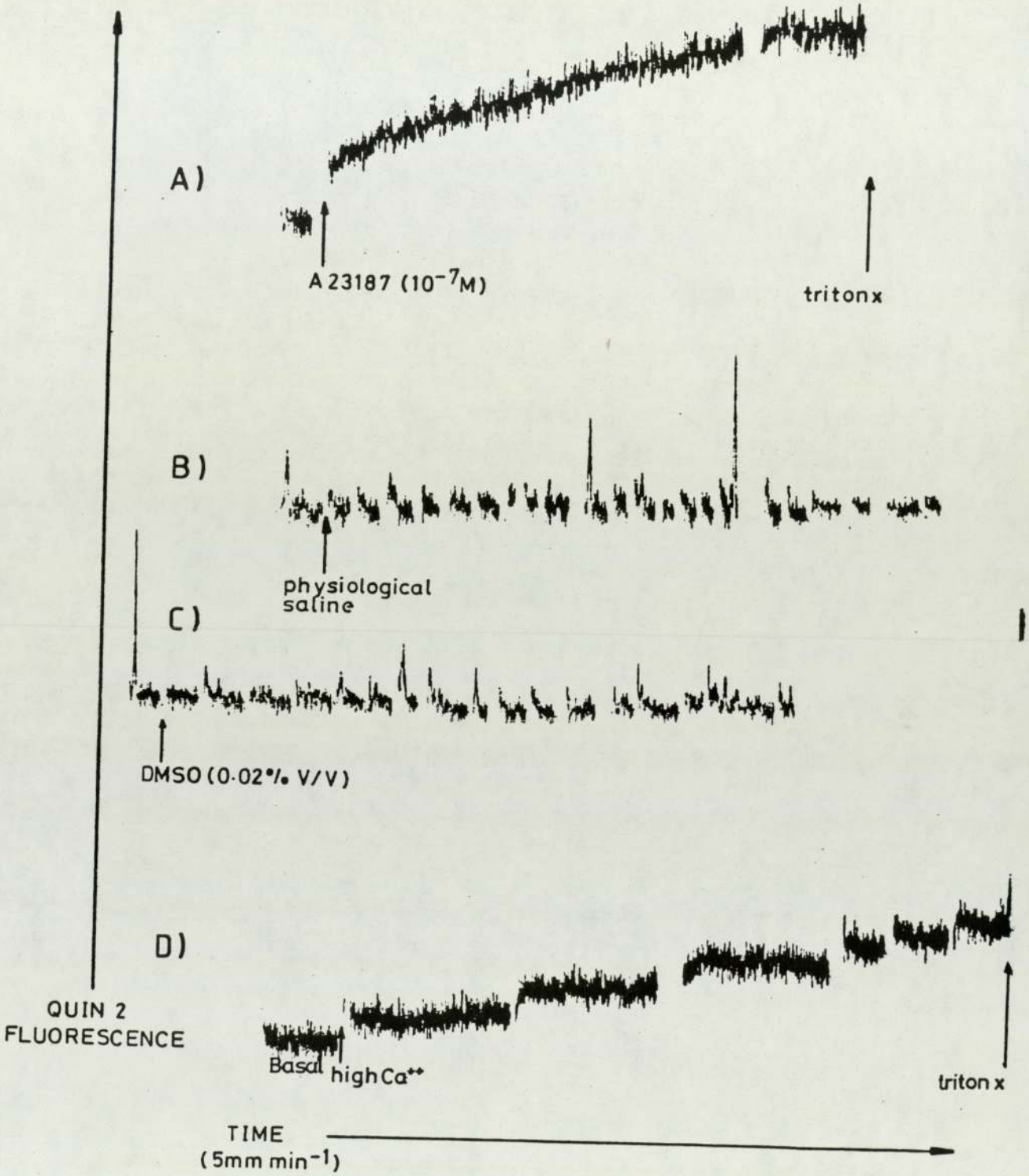


Figure 7 Records of Intracellular Calcium Concentration as Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.

a) Effect of ionophore A23187 on $[Ca^{++}]_i$
b) Effect of 0.9% saline on $[Ca^{++}]_i$
c) Effect of DMSO on $[Ca^{++}]_i$
d) Effect of high Ca^{++} on $[Ca^{++}]_i$

elevation in cytosolic calcium content ($\triangle I = 0.76$ after 20 minutes) as seen on trace in Appendix I). This is consistent with the Ca^{++} uptake profile achieved with the radioisotope ^{45}Ca. Since oestradiol (at high concentrations) influenced the high $[Ca^{++}]o$-induced mitosis and calcium uptake, it is possible that under basal conditions, oestradiol (at some other concentration) may raise intracellular $[Ca^{++}]$ to levels which should initiate a mitogenic response. Accordingly, Ca^{++} uptake was monitored in the presence of a whole range of oestradiol concentrations. Indeed, as predicted oestradiol at lower (more physiological concentrations (1 pgm^{-1} and 10 $pgml^{-1}$)) significantly enhanced transmembrane movement of calcium ions into the cytosol ($P< 0.01$ and $P< 0.001$). Higher concentrations failed to influence basal influx rates (figure 8).

When intracellular calcium was monitored using Quin 2, an elevation in calcium concentration was similarly observed with oestradiol at 10 $pgml^{-1}$ (table 3). Removal of extracellular calcium ions did not abolish this response, suggesting that the increased cellular Ca^{++} content may be attributed to mobilization of the ion from internal stores such as mitochondria and endoplasmic reticulum. The fluorescence signal of Quin 2 in cells incubated for short periods ($\sim$30 minutes) in Ca^{++} free saline was only marginally increased over 20 minutes after readmission of external calcium (results not shown). This suggests that the intracellular calcium status of rat thymocytes is not significantly influenced by lack of external calcium for short time periods.

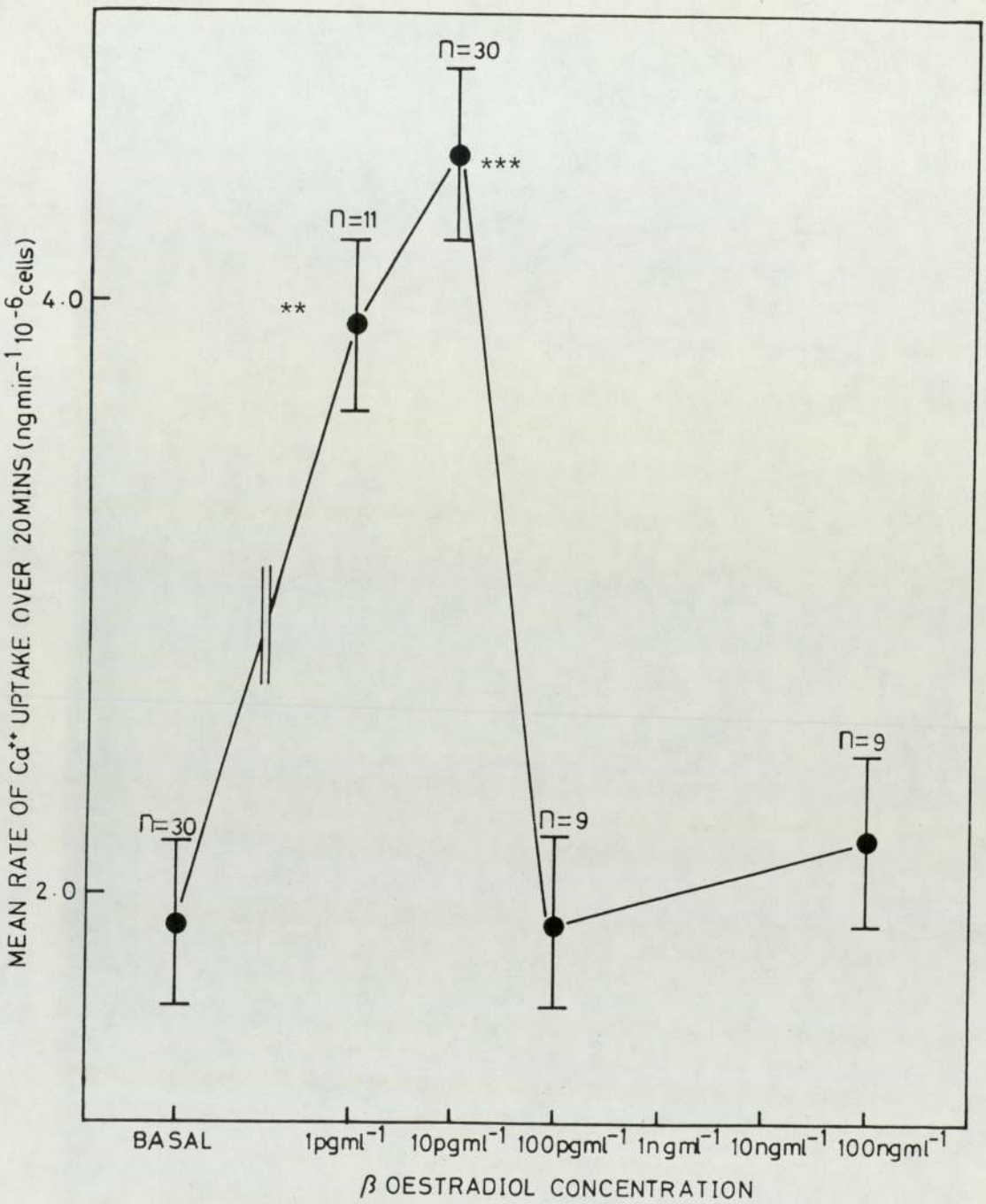


Figure 8 Effect of Beta-Oestradiol Concentration on Calcium Uptake From Basal Media Into Thymic Lymphocytes.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}; values significantly different from those of basal uptake in the absence of any beta-oestradiol are indicated by asterisk(s).

Table 3 Effects of Oestradiol on Intracellular Calcium Concentrations of Thymic Lymphocytes Employing Quin 2, as an Indicator.

Oestradiol Concentration (pg ml^{-1})	Isomer	Medium Cation Concentration (mM) Calcium	Magnesium	Change in Intracellular $[Ca^{++}]$ above basal as indicated by Quin 2 fluorescence after 20 minutes. ($\triangle I$)
10	beta	0.6	1.0	0.38
10	beta	0	1.0	0.26
10	beta	0.6	0	0.07
100	beta	0.6	0	0.35
10	alpha	0.6	1.0	0.25

(for traces see Appendix 1)

It appears then, that oestradiol at low concentrations promotes both transmembranal movement of calcium ions and in some way also induces the release of sequestered calcium from cytosolic stores, serving ultimately to raise the intracellular calcium content of the cells, ($[Ca^{++}]i$). If, as proposed previously, this raised $[Ca^{++}]i$ serves as an important trigger for DNA synthesis and mitosis, the lower oestradiol concentrations should also prove mitogenic. Indeed, oestradiol (at concentrations which induced maximum Ca^{++} influx (1 pg and 10 pg ml^{-1}), stimulated an additional cohort of normally quiescent thymic lymphocytes to embark upon DNA-synthesis and enter mitosis. Other higher concentrations including the normally inhibitory dose of 0.1 $\mu g ml^{-1}$ (100 $ng ml^{-1}$) did not significantly influence basal mitotic activity (figure 9). In fact the stimulation of mitosis by oestradiol shown in figure 9 precisely parallels the stimulation of calcium

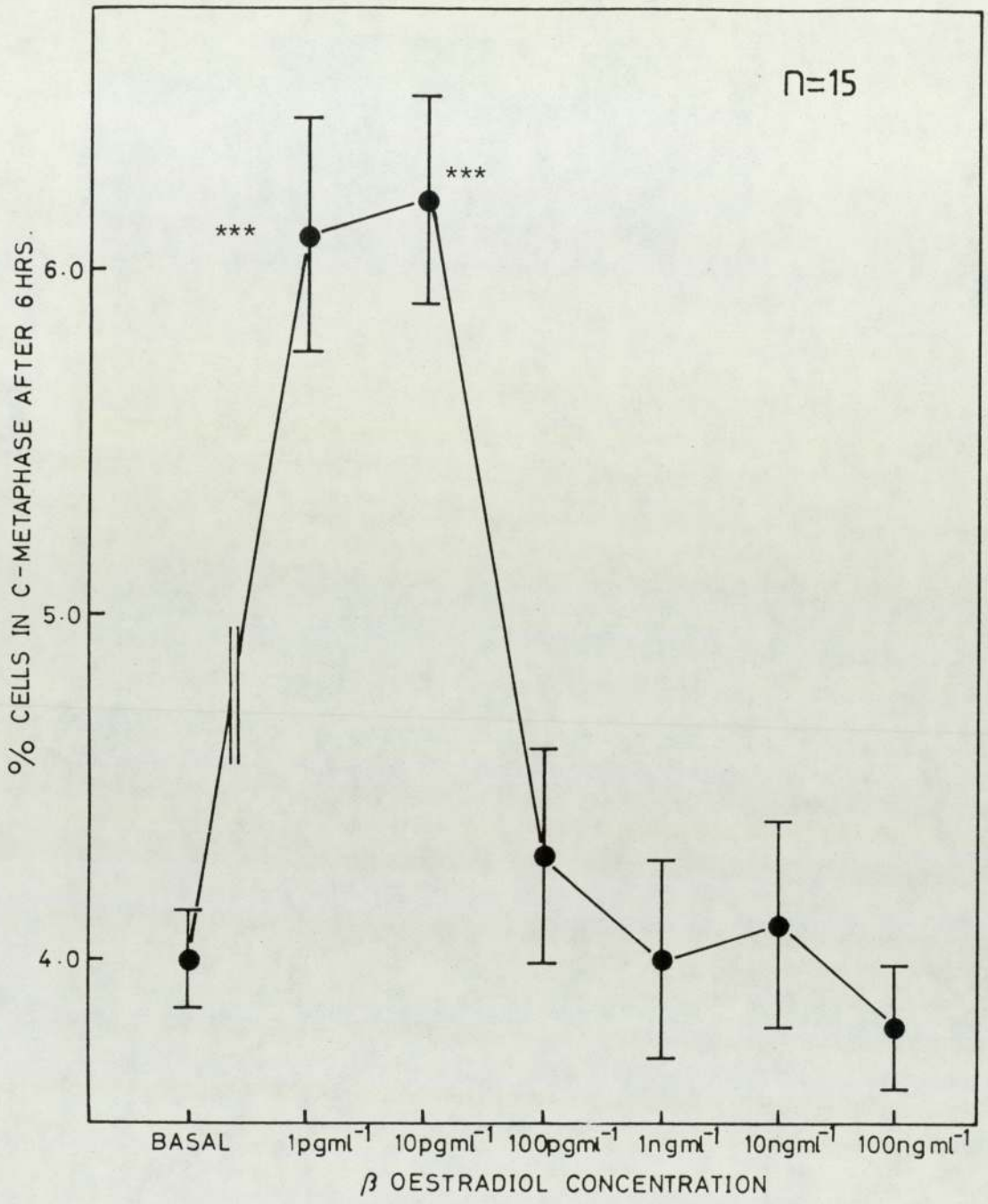


Figure 9 Effect of Beta-Oestradiol Concentration on Mitotic Activity of Thymic Lymphocytes Incubated in Basal Media.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}; values significantly different from basal mitotic activity in the absence of any oestradiol are indicated by asterisk(s).

influx in figure 8. Neither the inhibition of messenger RNA synthesis by actinomycin D (10×10^{-6} gml^{-1}) nor inhibition of protein synthesis by cycloheximide (2×10^{-7}m) prevented the enhancement of calcium influx induced by beta-oestradiol (figure 10). These observations and the rapidity of the response to oestradiol suggests that oestradiol is not exerting its effect via its classical genomic mode of action. Furthermore, alpha-oestradiol, which is physiologically inert in the classical target tissues of uterus and vagina, also enhanced calcium influx (figure 11), raised cytosolic calcium levels (table 3), and promoted mitosis in the thymic lymphocytes, (figure 12). It is not unreasonable therefore to propose that the oestradiol epimers exert an effect at the plasma membrane to enhance calcium influx which in turn triggers DNA synthesis and subsequently mitosis. For the beta isomer at least, oestradiol appears also to be able to exert an effect at the membranes of intracellular organelles serving to release sequestered Ca^{++}. Because of the parallelism existing between the rate of Ca^{++} uptake and mitosis provoked by oestradiol it was most surprising to find that the omission of calcium from the culture medium did not prevent the increase in cytosolic Ca^{++} concentration (table 3) or significantly influence oestradiol-induced mitosis whereas removal of external magnesium completely inhibited the mitogenic response to either epimer (figures 13 and 14).

Furthermore testosterone, which inhibited magnesium dependent mitosis (figure 1) also inhibited the mitogenicity of alpha and beta-oestradiol (figures 15 and 16). A concentration of beta-oestradiol (0.1 μg ml^{-1}) which inhibits mitogenic action of high calcium and calcium-dependent hormones had no effect on the mitogenic action of

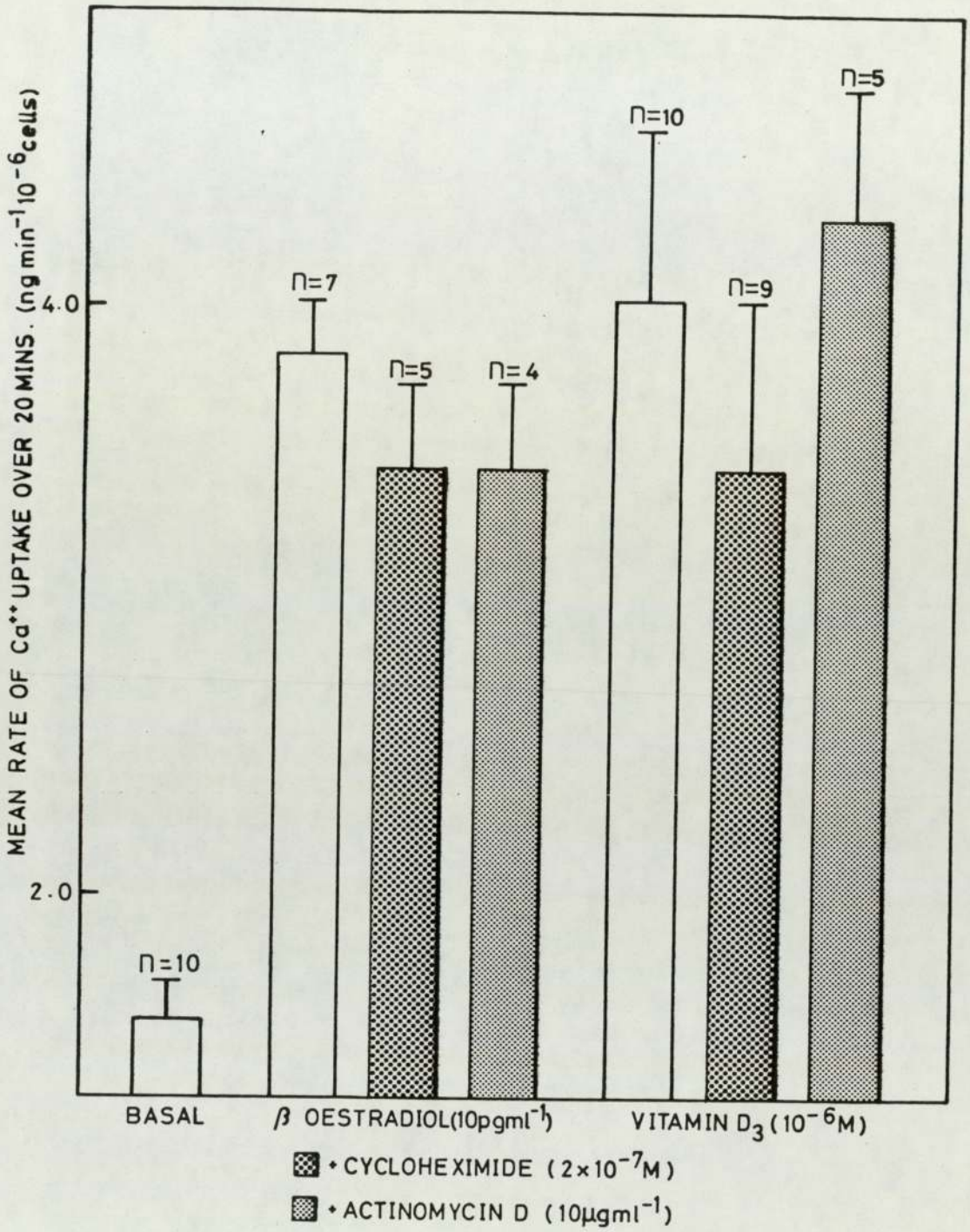


Figure 10 Effect of Cycloheximide and Actinomycin D on Beta-Oestradiol- and Vitamin D_3-Induced Calcium Uptake.

Concentrations of cycloheximide and actinomycin D employed were 2×10^{-7} M and 10^{-5} gml^{-1} respectively. Values significantly different from those of corresponding cultures incubated free of cycloheximide or actinomycin D are indicated by asterisk(s).

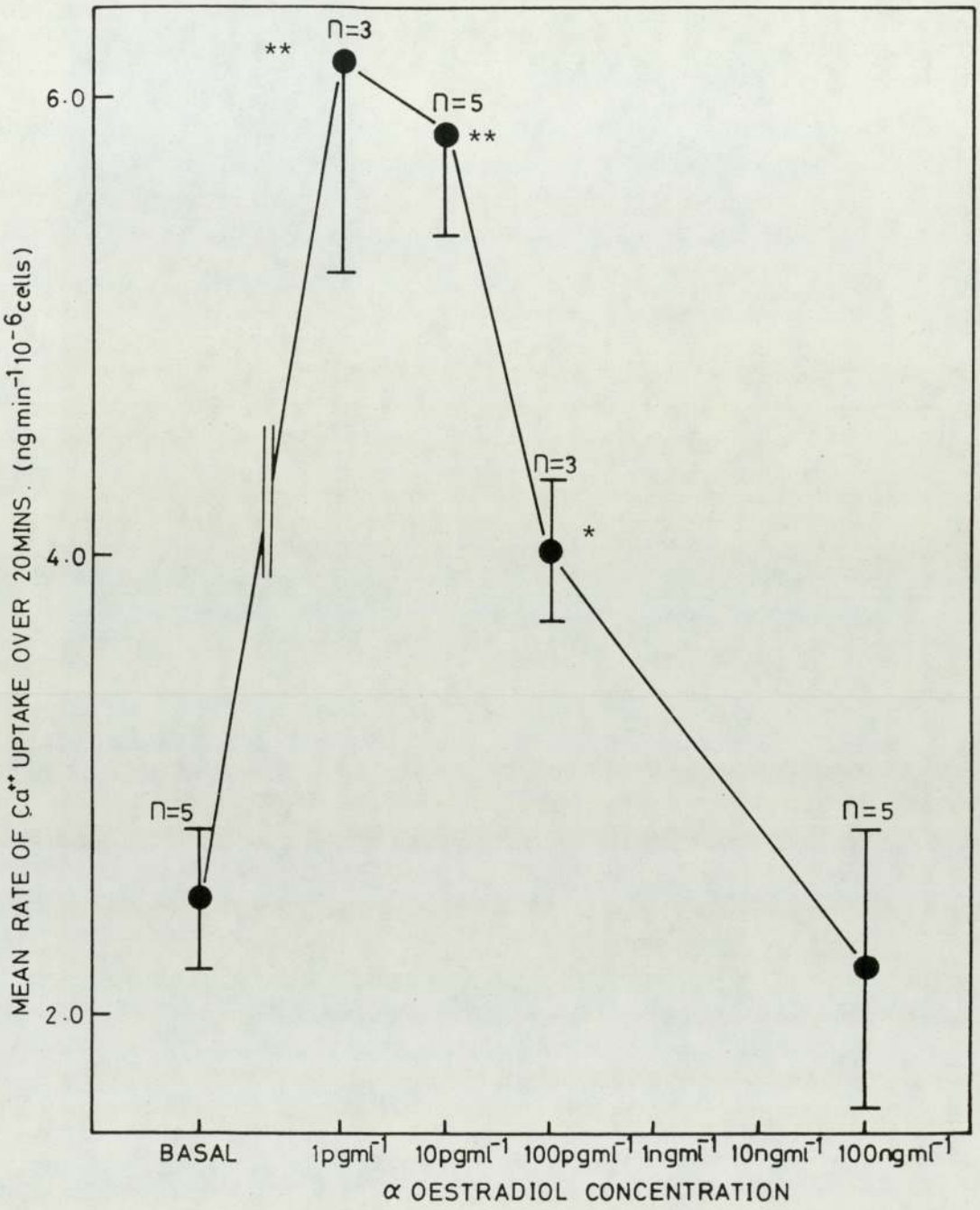


Figure 11 Effect of Alpha-Oestradiol Concentration on $^{45}Ca^{++}$ Uptake from Basal Media Into Rat Thymic Lymphocytes.

Basal media contains 0.6 mM Ca^{++}, and 1.0 mM Mg^{++}. Values significantly different from those of basal uptake in the absence of any alpha-oestradiol are indicated by asterisk(s).

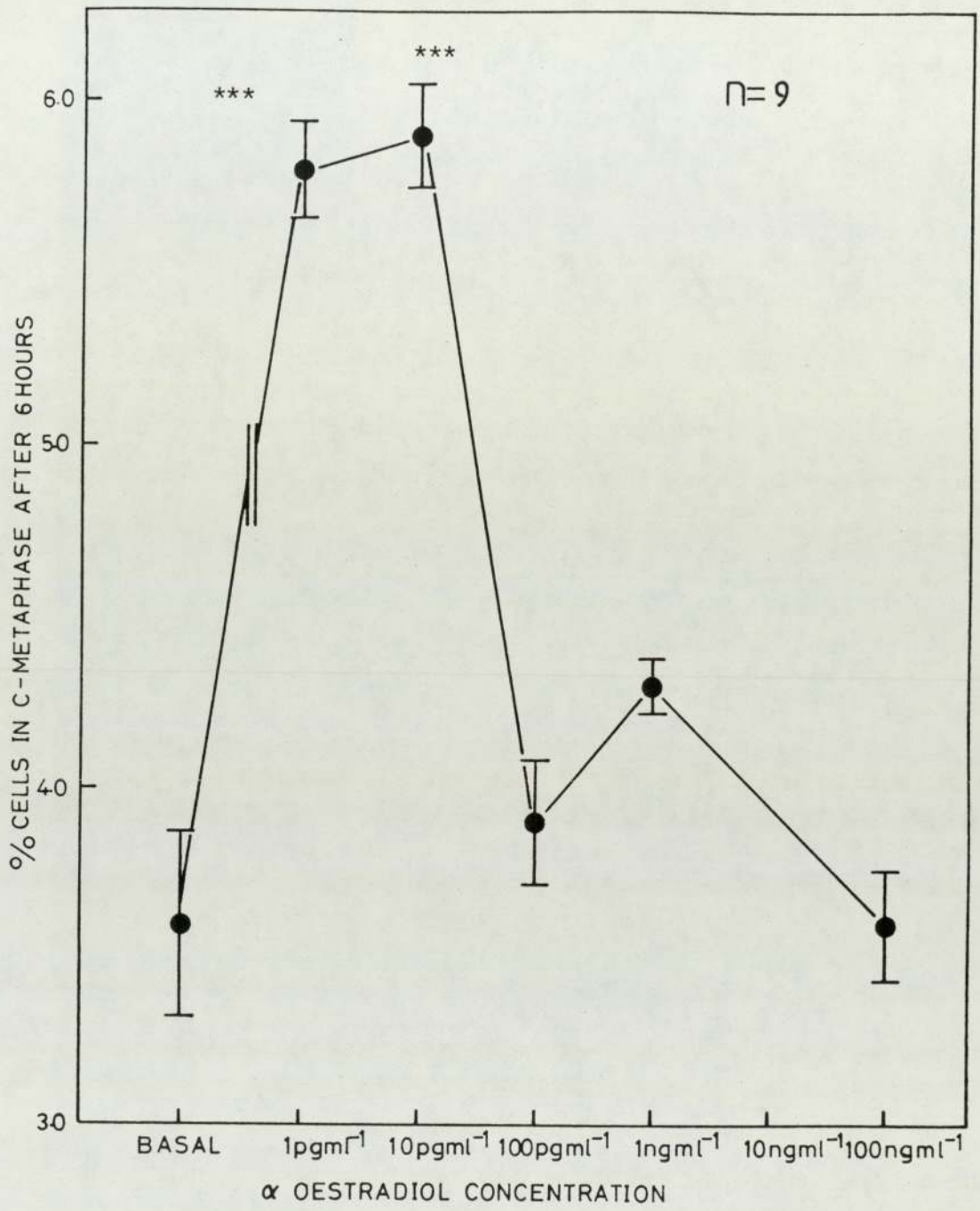


Figure 12 Effect of Alpha-Oestradiol Concentration on Mitotic Activity of Thymocytes Incubated in Basal Media.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}; values significantly different from those of basal mitotic activity in the absence of any alpha-oestradiol are indicated by asterisk(s).

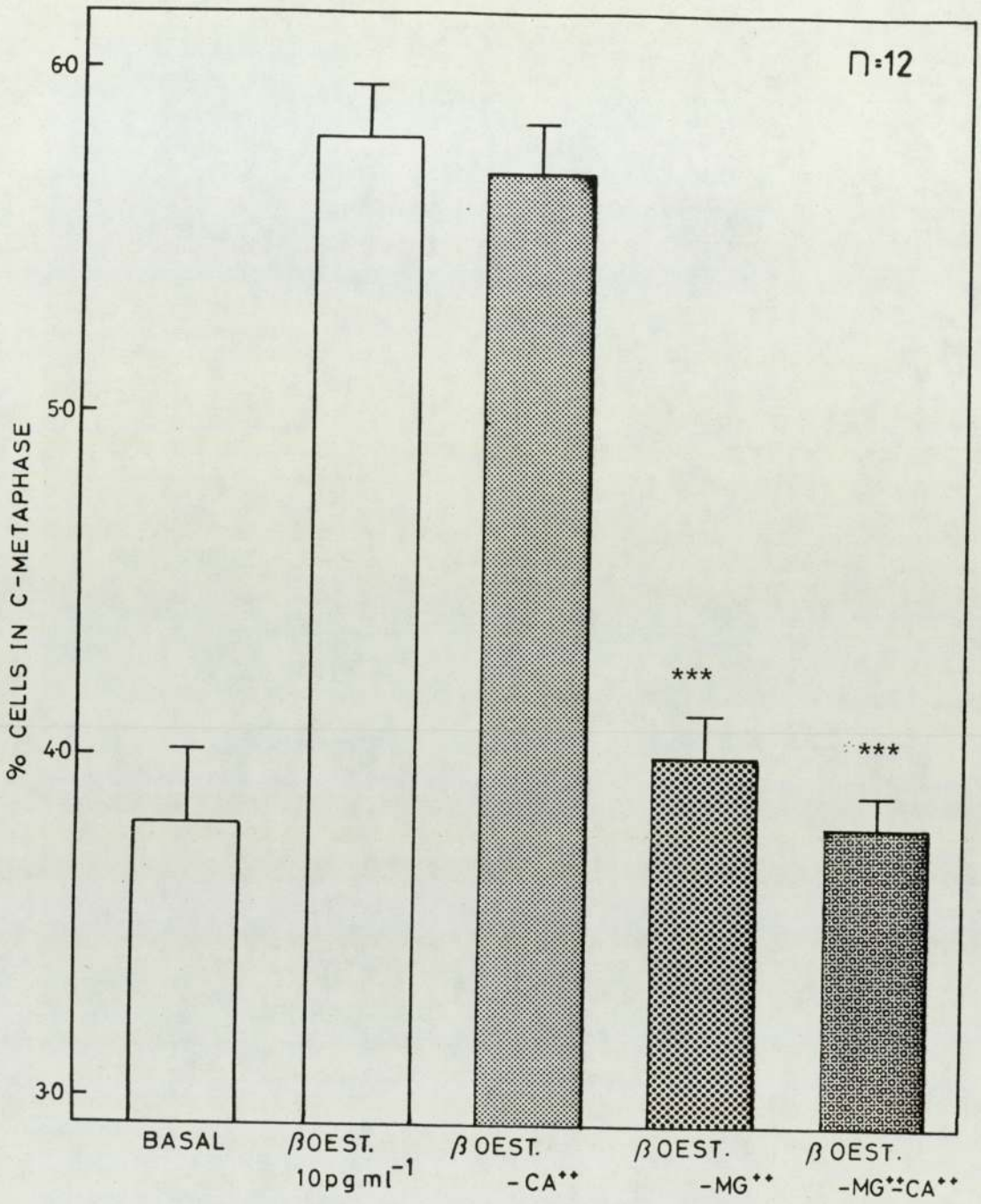


Figure 13 Ionic Dependency of Beta Oestradiol-Induced Mitosis

Oestradiol (10 $pgml^{-1}$) was included in 0.6 mM Ca^{++}, 1.0 mM Mg^{++} or in media where calcium ($-Ca^{++}$), magnesium ($-Mg^{++}$) or both divalent cations ($-Ca^{++}$ $-Mg^{++}$) had been omitted. Values significantly different from those of basal cultures incubated in the presence of oestradiol (10 $pgml^{-1}$) are indicated by asterisk(s).

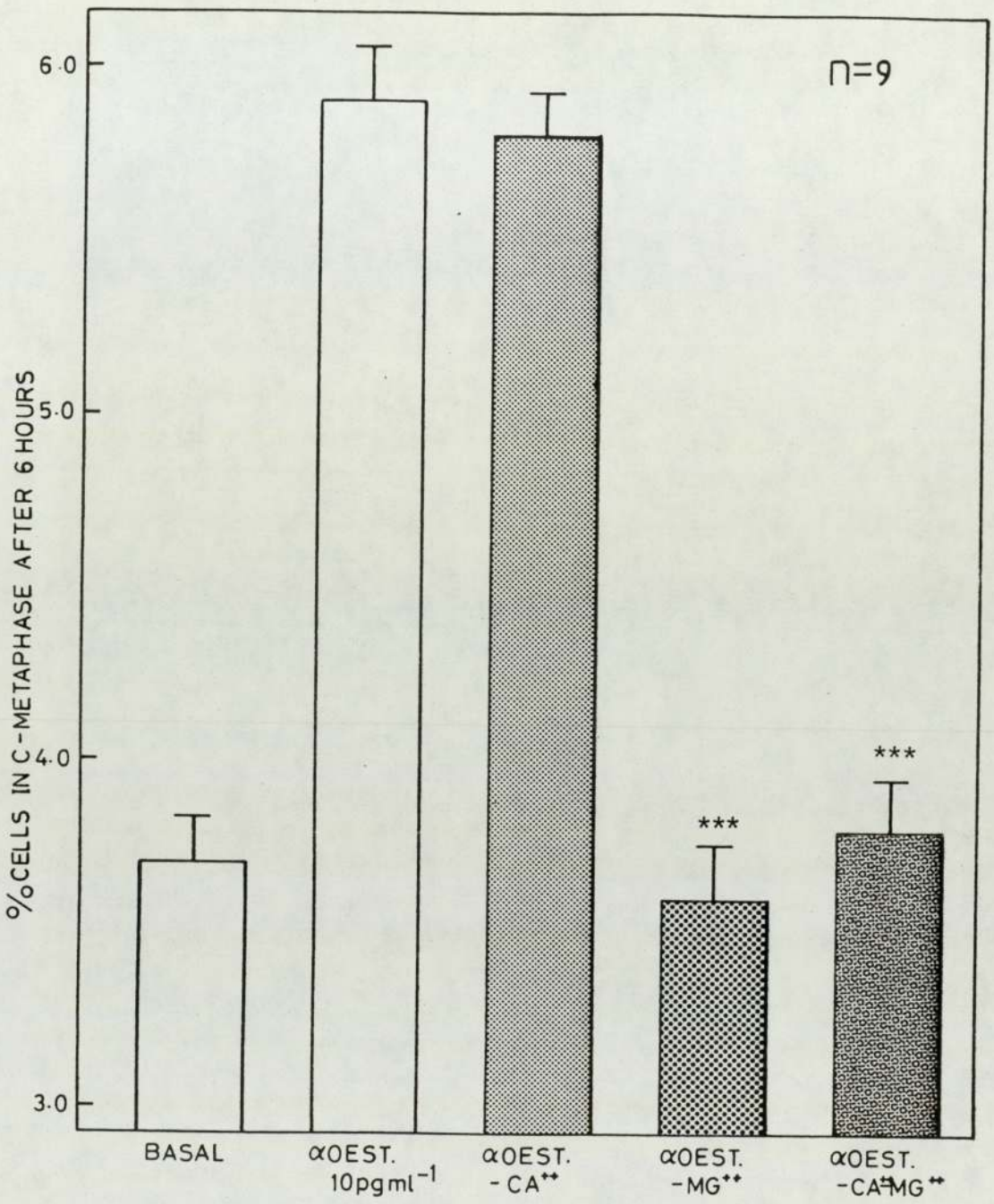


Figure 14 Ionic Dependency of Alpha-Oestradiol - Induced Mitosis.

Oestradiol (10 $pgml^{-1}$) was included in 0.6 mM Ca^{++}, 1.0 mM Mg^{++}, or in media where calcium ($-Ca^{++}$), magnesium ($-Mg^{++}$) or both divalent cations ($-Ca^{++}$ $-Mg^{++}$) had been omitted. Values significantly different from those of basal cultures incubated in the presence of oestradiol are indicated by asterisk(s).

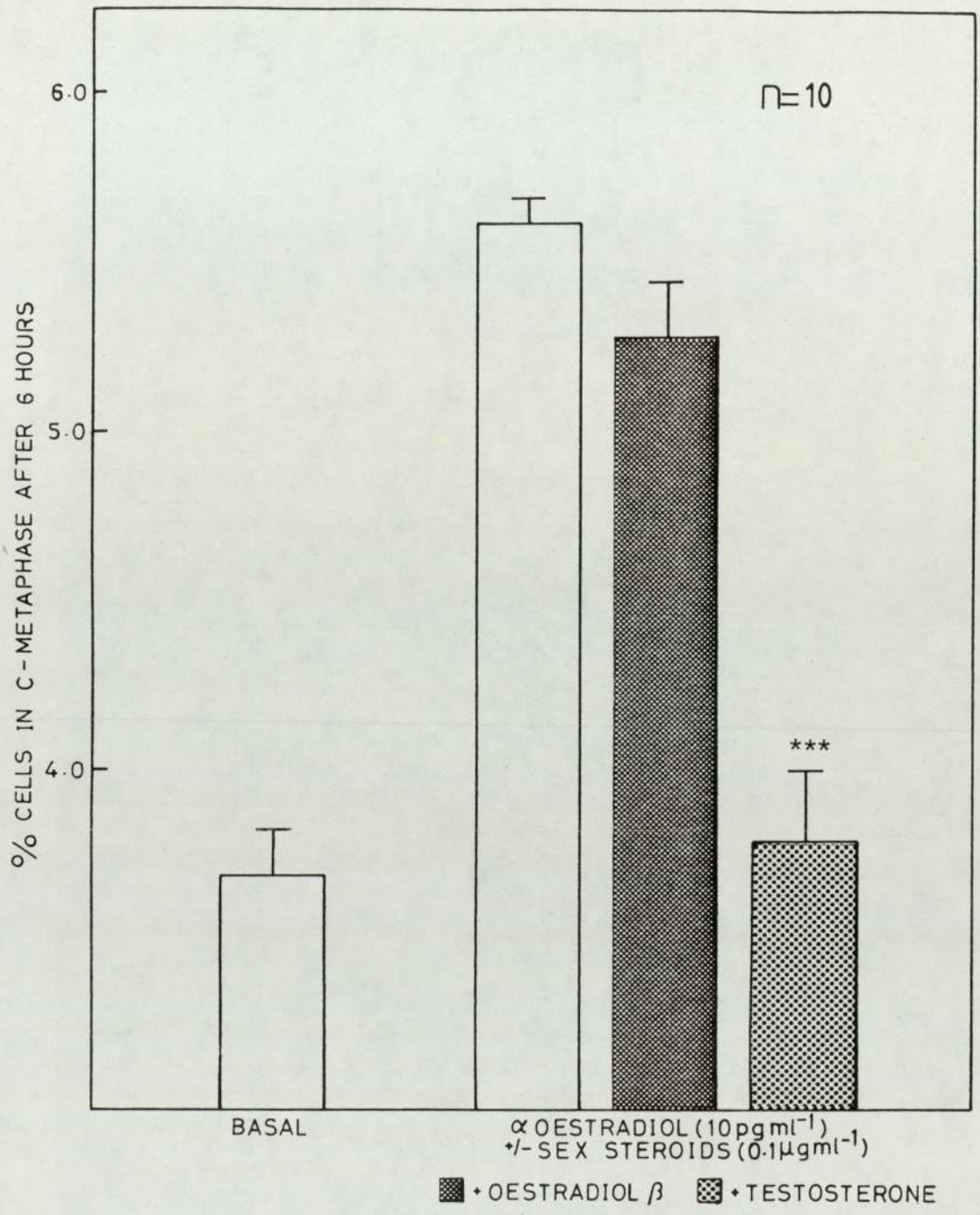


Figure 15 Steroid Blockade of Alpha-Oestradiol-Induced Mitosis.

The sex steroids beta-oestradiol and testosterone were added to the cultures at final concentrations of 0.1 $\mu g ml^{-1}$. Values significantly different from those of alpha-oestradiol at 10 $pg ml^{-1}$ incubated free of inhibitory doses of sex steroid are indicated by asterisk(s).

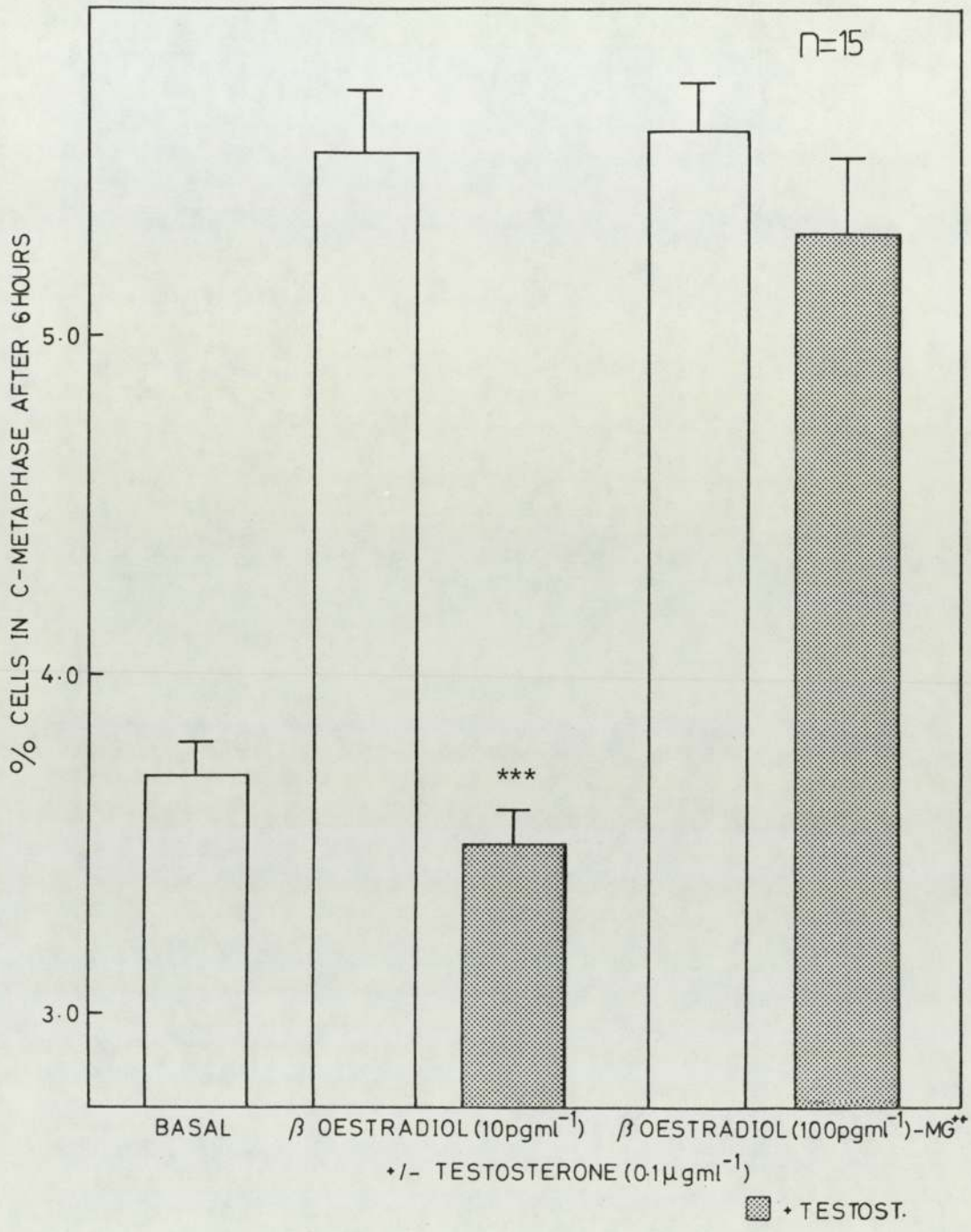


Figure 16 Effect of Testosterone on Beta-Oestradiol Induced Mitosis.

Mitogenic concentrations of beta-oestradiol employed were 10 $pgml^{-1}$ in basal media and 100 $pgml^{-1}$ in magnesium free media. Values significantly different from those of corresponding cultures incubated free of testosterone are indicated by asterisk(s).

low concentrations (10 pgm $^{-1}$) of alpha-oestradiol (figure 15). When the effect of a wide range of oestradiol concentrations on Ca^{++} influx was examined in the presence and absence of Mg^{++} it became clear that Mg^{++} omission reduced (to levels not significantly different from basal levels) the enhanced calcium influx normally observed in response to mitogenic oestradiol concentrations i.e. 1 $pgml^{-1}$ and 10 pgm^{-1} (figure 17). Removal of external magnesium ions almost totally abolished any increase in cytosolic calcium concentration induced by oestradiol (table 2) suggesting that the process of Ca^{++} mobilization promoted by oestradiol may be sensitive to Mg^{++} influx. Surprisingly, only when oestradiol concentrations were raised to 100 pgm^{-1} and 1 ngm^{-1} was enhanced calcium influx detected in the absence of magnesium (figure 17). This heightened influx of Ca^{++} in Mg^{++} free medium served to elevate intracellular calcium concentrations (table 3). Thus, if increased cytosolic calcium content is the crucial trigger for the mitogenic response then the higher oestradiol concentrations in Mg^{++} free media should also promote mitosis. Indeed, a parallel increase in mitosis accompanied the calcium uptake induced by oestradiol at both concentrations (100 pgm^{-1} and 1 ngm^{-1}) in the absence of Mg^{++} (figure 18). Not surprisingly mitosis induced by oestradiol at 100 $pgml^{-1}$ in the absence of extracellular magnesium was not abolished by testosterone application (figure 16). Preliminary experiments showed that this mitogenic concentration of oestradiol is dependent on extracellular calcium (results not shown) suggesting that oestradiol at this concentration does not release sufficient calcium from intracellular sources to provide a mitogenic signal. Thus, it is possible that oestradiol promotes the movement of calcium down its concen-

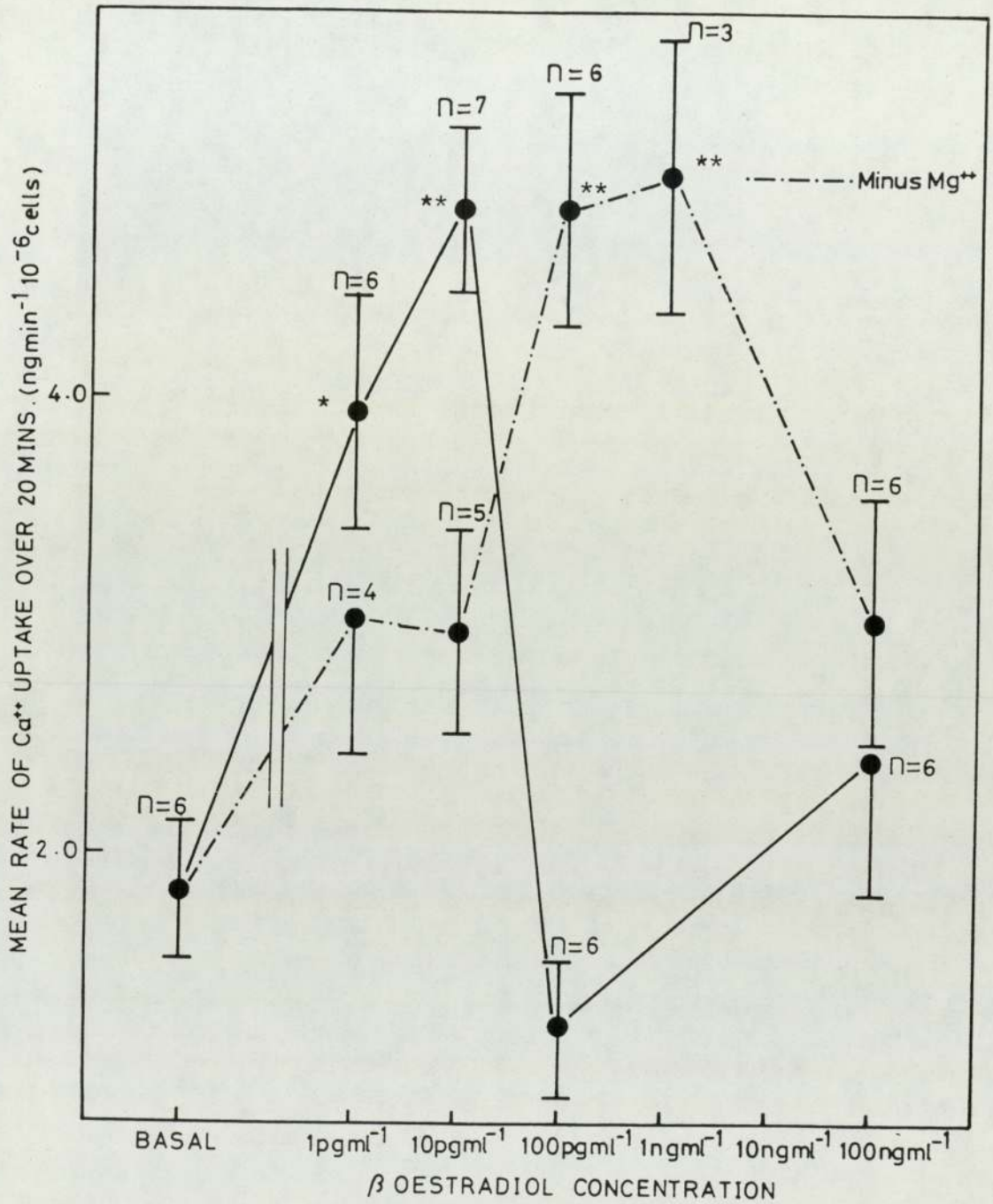


Figure 17 Effect of Beta-Oestradiol Concentration on $^{45}Ca^{++}$ Uptake Into Thymic Lymphocytes Incubated in Basal or Magnesium-Free Media.

Cultures included in media from which magnesium had been omitted are represented by the broken lines. Values significantly different from those of basal uptake rates are indicated by asterisk(s).

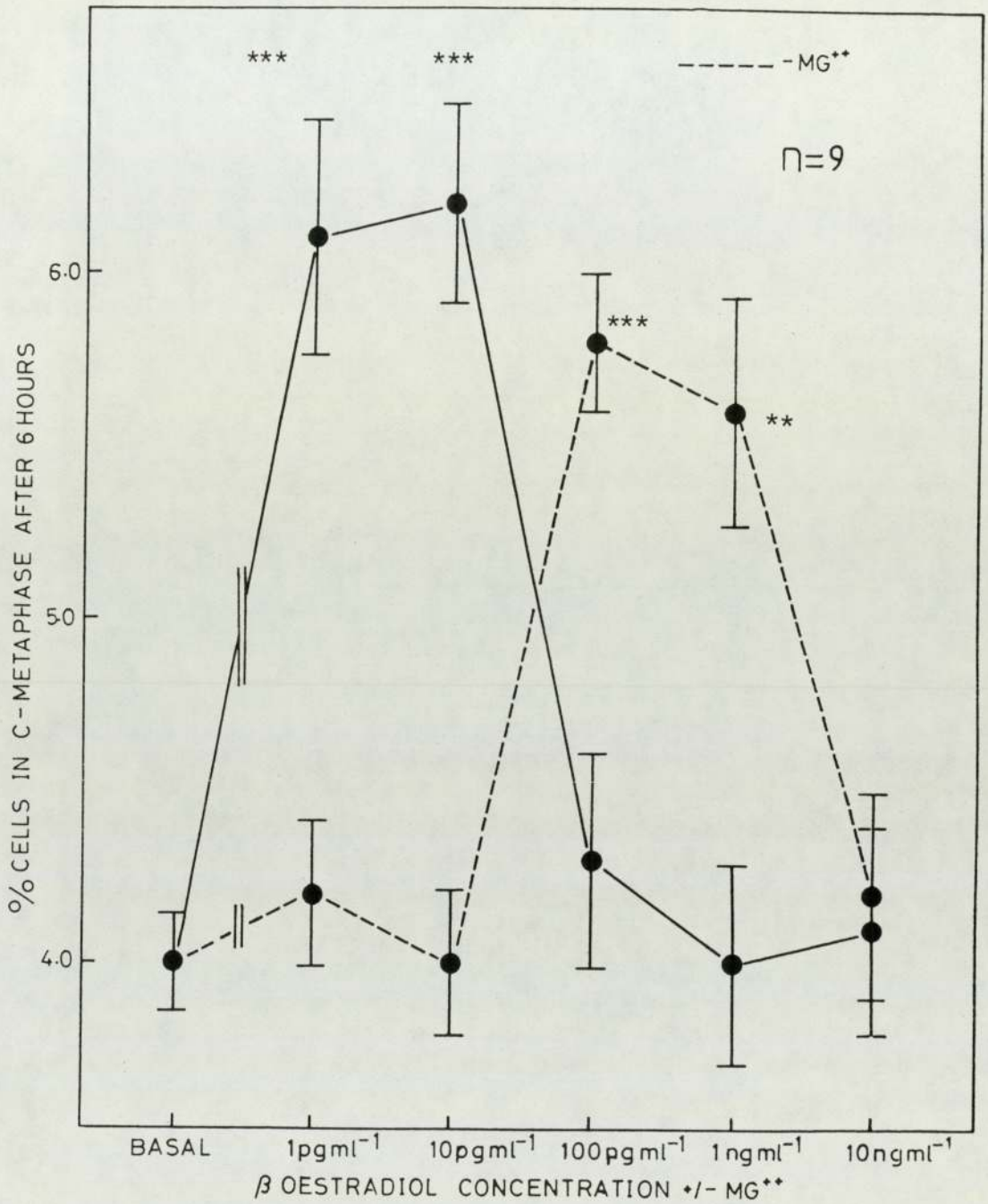


Figure 18 Effect of Beta-Oestradiol Concentration on Mitotic Activity of Thymic Lymphocytes Incubated in Basal or Magnesium-Free Media.

Cultures incubated in media from which magnesium had been omitted are represented by the broken lines. Values significantly different from those of basal mitotic activity are indicated by asterisk(s).

tration gradient across the plasma membrane or across the membranes of subcellular organelles by a process(es) which is facilitated by or possibly induced by magnesium ions. Whatever the source, the resultant increase in cytosolic calcium in some way triggers the initiation of DNA synthesis and ultimately mitosis. As anticipated the Ca^{++} antagonist verapamil significantly impaired oestradiol (10 $pgml^{-1}$)-induced Ca^{++} influx (figure 19). Likewise the agent inhibited the mitosis stimulated by alpha oestradiol (10 $pgml^{-1}$) and beta-oestradiol at 10$pgml^{-1}$ in normal media and 100 $pgml^{-1}$ in Mg^{++} free media (figures 20 and 21). Verapamil also inhibited the mitogenic response to oestradiol at 10 $pgml^{-1}$ in the absence of external calcium ions (table 4) which suggests that verapamil may also influence calcium movement across the membranes of subcellular organelles.

Table 4 Effect of Verapamil (5×10^{-6} gml^{-1}) on Oestradiol-Induced Mitosis in the Absence of Extracellular Calcium Ions

Beta-Oestradiol ($pgml^{-1}$)	Treatment Verapamil Concentration (gml^{-1})	Medium Cation Concentration (mM) Calcium	Magnesium	% Cells in c-metaphase after six hours ± S.E.M.
0	0	0.6	1.0	*** 3.7 ± 0.10(n=10)
10	0	0.6	1.0	5.8 ± 0.2 (n=10)
10	0	0	1.0	5.6 ± 0.1 (n= 8)
10	5×10^{-6}	0	1.0	* 4.3 ± 0.3 (n=13)

Significantly different from cultures incubated with oestradiol in Ca^{++} free media. * $P < 0.05$ *** $P < 0.001$

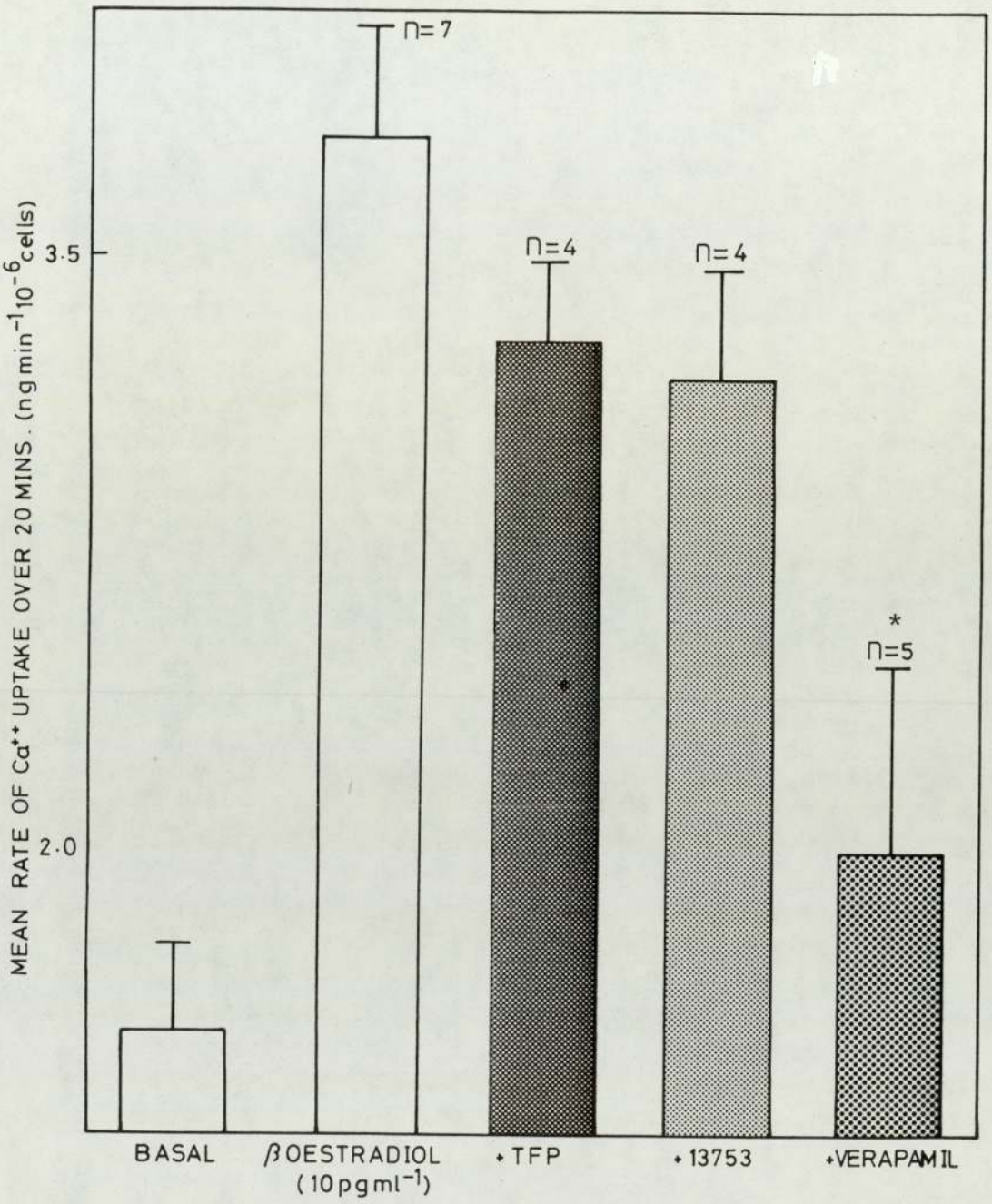


Figure 19 Effect of Calmodulin Inhibition and Calcium Channel Blockade on Beta-Oestradiol-Induced $^{45}Ca^{++}$ Uptake Into Thymocytes Incubated in Basal Medium.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}. Concentrations of both alpha & beta-oestradiol, TFP, M & B 13753 and Verapamil employed were 10 $pgml^{-1}$ 10^{-6}M, 10^{-5}M and 5 x 10^{-6} gml^{-1} respectively. Values significantly different from those of beta-oestradiol incubated free of treatment in basal media are indicated by asterisk(s).

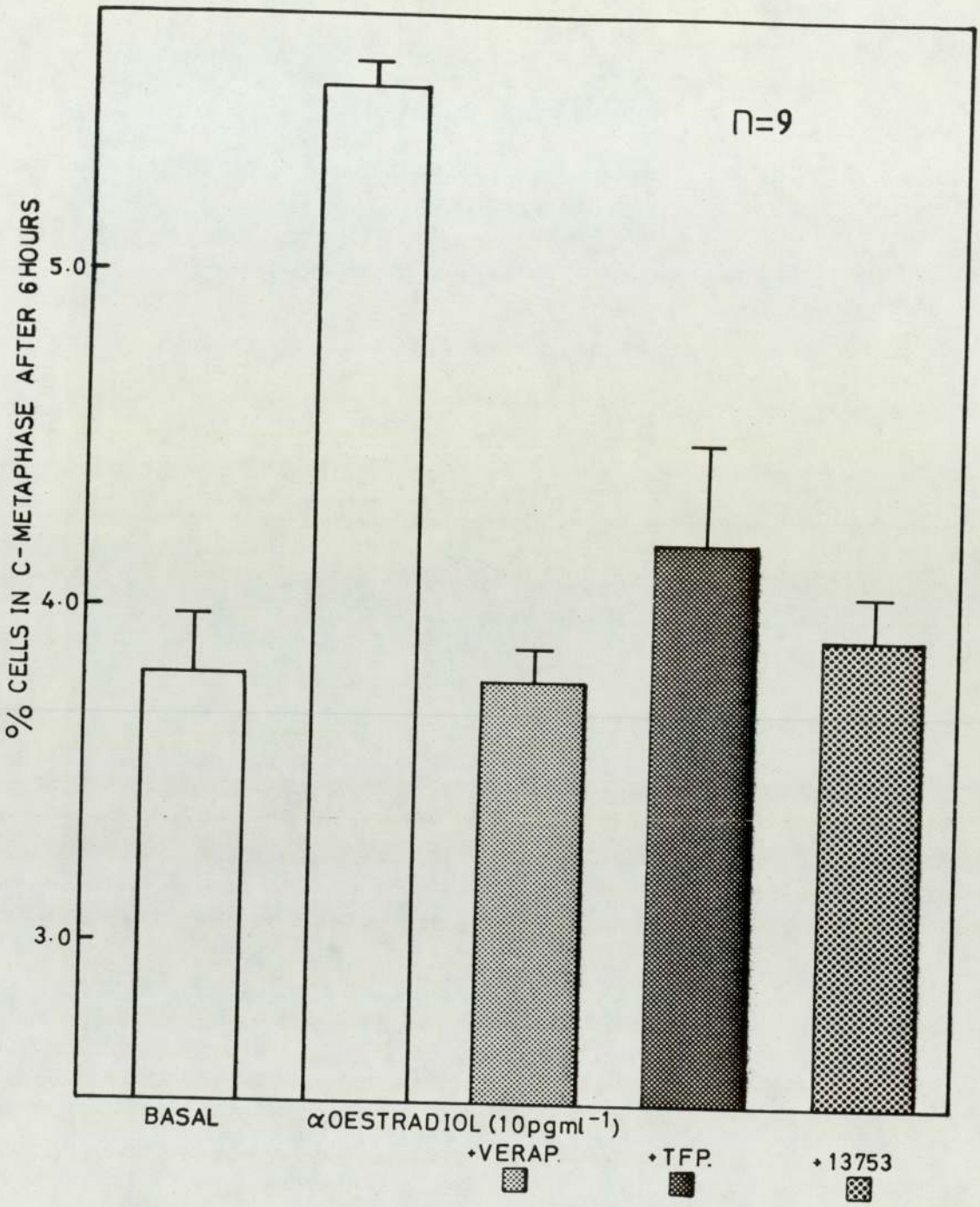


Figure 20 Effect of Calmodulin Inhibition and Calcium Channel Blockade on Alpha-Oestradiol-Induced Mitosis.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}. Concentrations of alpha-oestradiol, verapamil, TFP and M & B 13753 employed were 10 $pgml^{-1}$, 5 x 10^{-6} gml^{-1}, 10^{-6} M and 10^{-5} M respectively. Values significantly different from those of alpha-oestradiol incubated free of treatment in basal medium are indicated by asterisk(s).

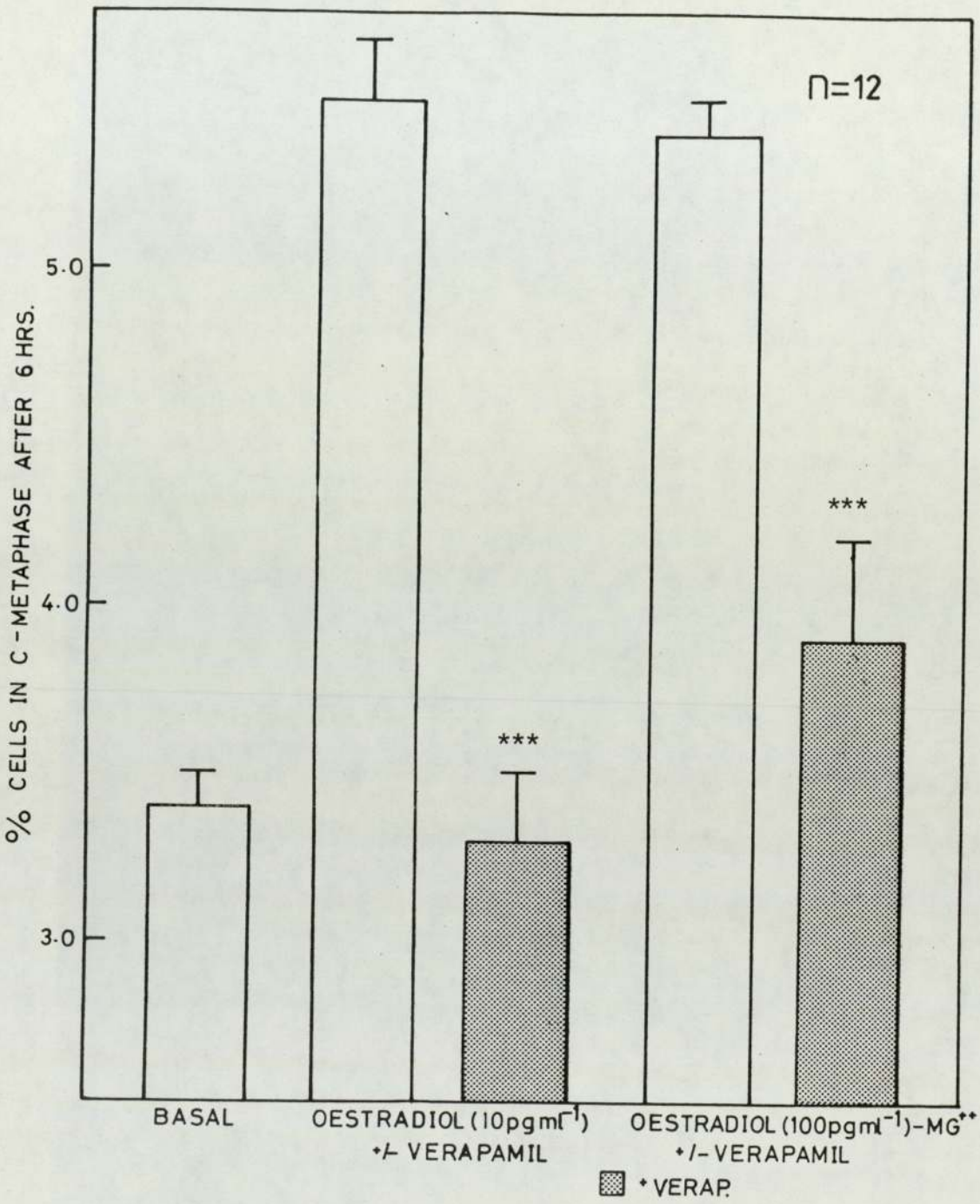


Figure 21 Effect of Calcium Channel Blockade on Beta-Oestradiol-Induced Mitosis in Thymic Lymphocytes Incubated in Basal or Magnesium-Free Media.

Basal medium contains 0.6 mM, 1.0 mM; magnesium free media has Mg^{++} omitted. Verapamil was employed at 5×10^{-6} gml^{-1} in each case. Values significantly different from those of corresponding cultures incubated free of verapamil are indicated by asterisk(s).

It is not unreasonable to assume that the rise in intracellular calcium ions probably activates the calcium regulatory protein, calmodulin (see section 1.2.1b). Trifluoperazine (a phenothiazone antipsychotic agent) selectively binds and inactivates the Ca^{++}-calmodulin complex probably by hydrophobically induced conformational changes of the Ca^{++} binding sites (Roufogalis, 1982; Weiss, Prozialeck & Wallace, 1982). If calmodulin is indeed activated in this proliferative system mitogen-induced mitosis should be compromised by the presence of such a calmodulin antagonist. Certainly the mitogenic response normally achieved by 1.8mM was inhibited by TFP in a dose dependent manner (figure 22). Concentrations 10^{-6} - 10^{-4}M were consistently effective as inhibitors of calcium induced mitosis. Higher levels (above 10^{-4}M) however proved highly cytotoxic to thymic lymphocytes. Significantly, TFP at 10^{-6}M did not prevent magnesium-stimulated proliferation (figure 23) suggesting that magnesium does not promote mitosis in a Ca^{++}-CaM dependent manner. Similarly an additional calmodulin inhibitor, a phenoxypropanolamine compound M & B 13753 (Brown (May & Baker Ltd), personal communication) at 10^{-5}M (figure 24) and 10^{-6}M (results not shown) abolished high calcium-induced mitosis but not that incurred by high magnesium (2.5 mM). Both TFP (10^{-6}M) and M & B 13753 (10^{-5} M) prevented beta-oestradiol-induced mitosis (10 $pgml^{-1}$ in normal media and 100 $pgml^{-1}$ in Mg^{++} free media) as shown in figures 25 and 26. Likewise the alpha-isomer of the steroid was ineffective as a mitogen in the presence of either calmodulin inhibitor (figure 20). These blockers did not impair the increase in calcium influx caused by high Ca^{++} (figure 27 and table 5) or a mitogenic concentration of oestradiol (figure 19).

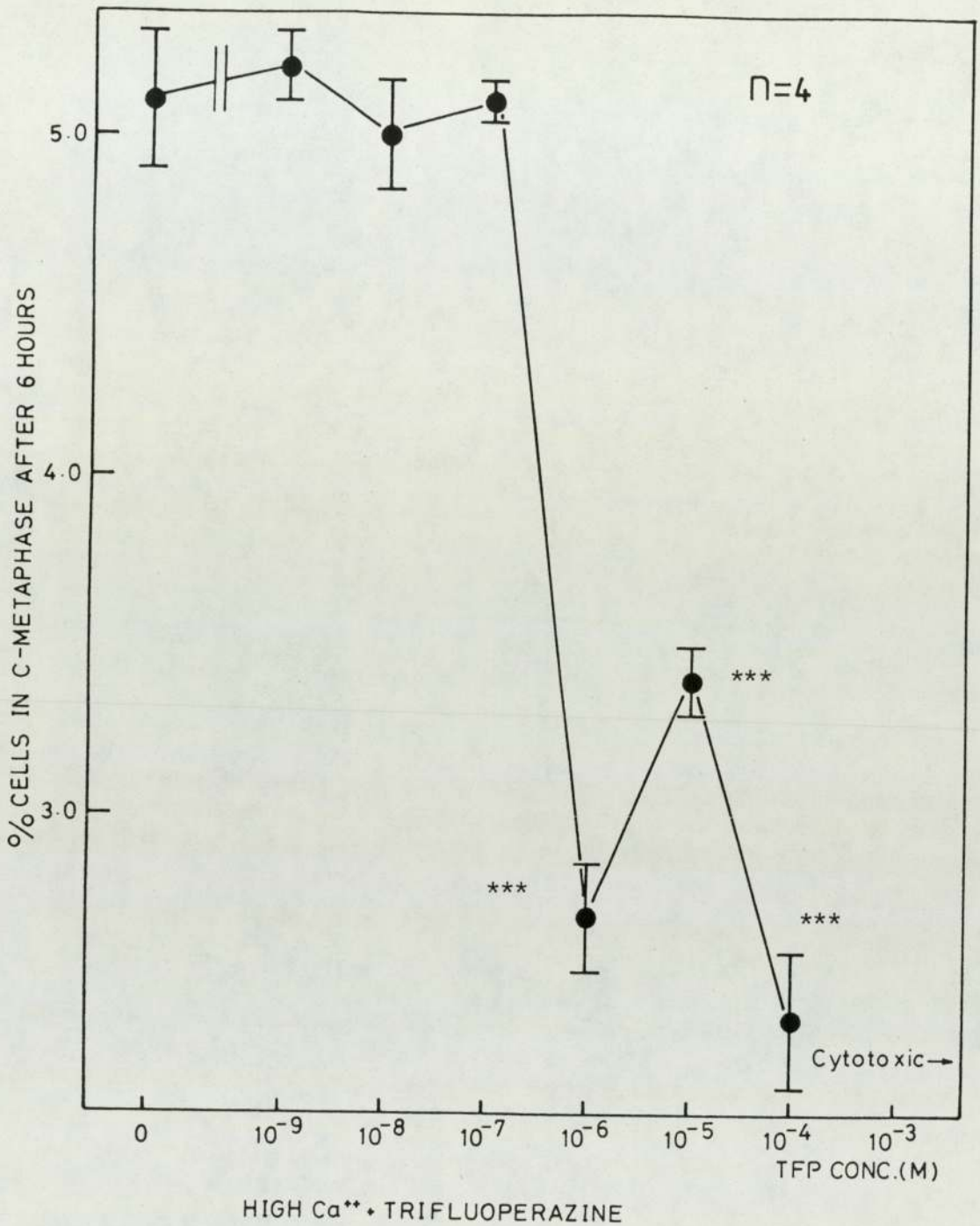


Figure 22 Effect of Calmodulin Inhibition on High Calcium-Induced Mitogenesis.

High Ca^{++} represents calcium concentration in culture medium of 1.8 mM. Values significantly different from those of cultures incubated free of trifluoperazine are indicated by asterisk(s).

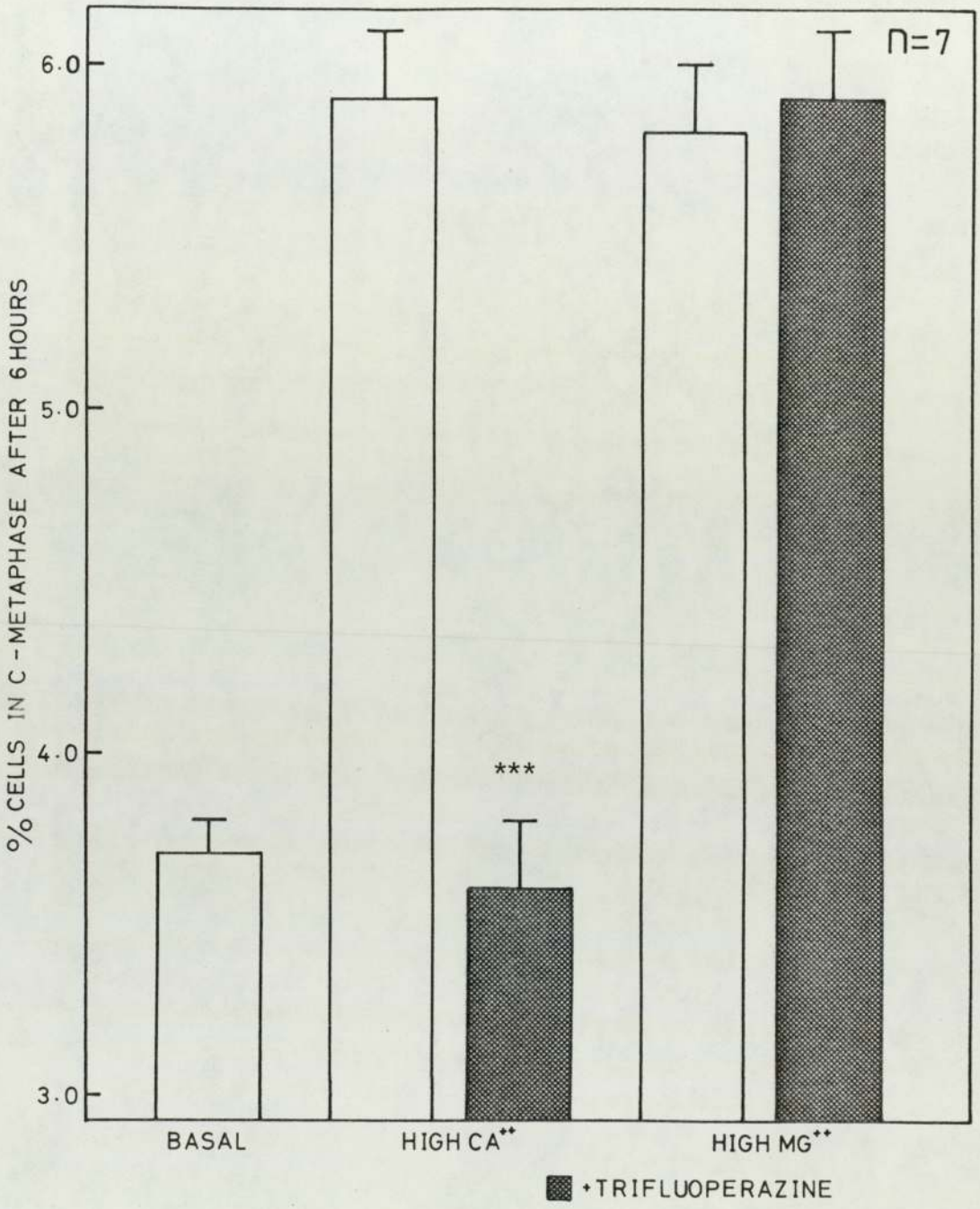


Figure 23 Effect of Trifluoperazine (10^{-6}M) on High Ca^{++}- and High Mg^{++}- Induced Mitogenesis.

High Ca^{++} and high Mg^{++} represent concentrations in culture media of 1.8 mM and 2.5 mM respectively. Values significantly different from those of corresponding cultures incubated free of trifluoperazine (10^{-6}M) are indicated by asterisk(s).

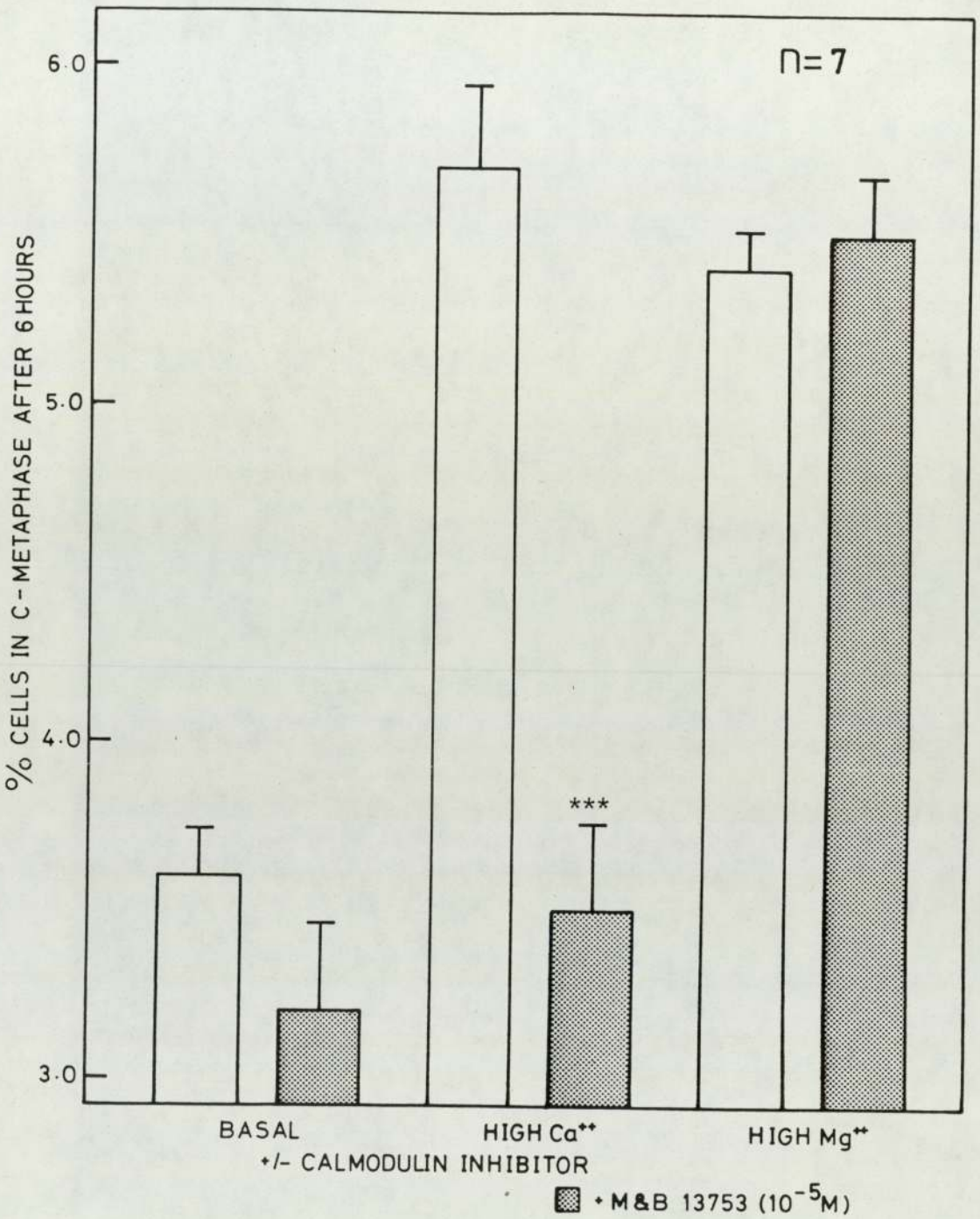


Figure 24 Effect of M & B 13753 (10^{-5}M) on Calcium-and Magnesium-Induced Mitogenesis.

High Ca^{++} and High Mg^{++} represent concentrations in culture medium of 1.8 mM and 2.5 mM respectively. Values significantly different from those of corresponding cultures incubated free of M & B (10^{-5}M) are indicated by asterisk(s).

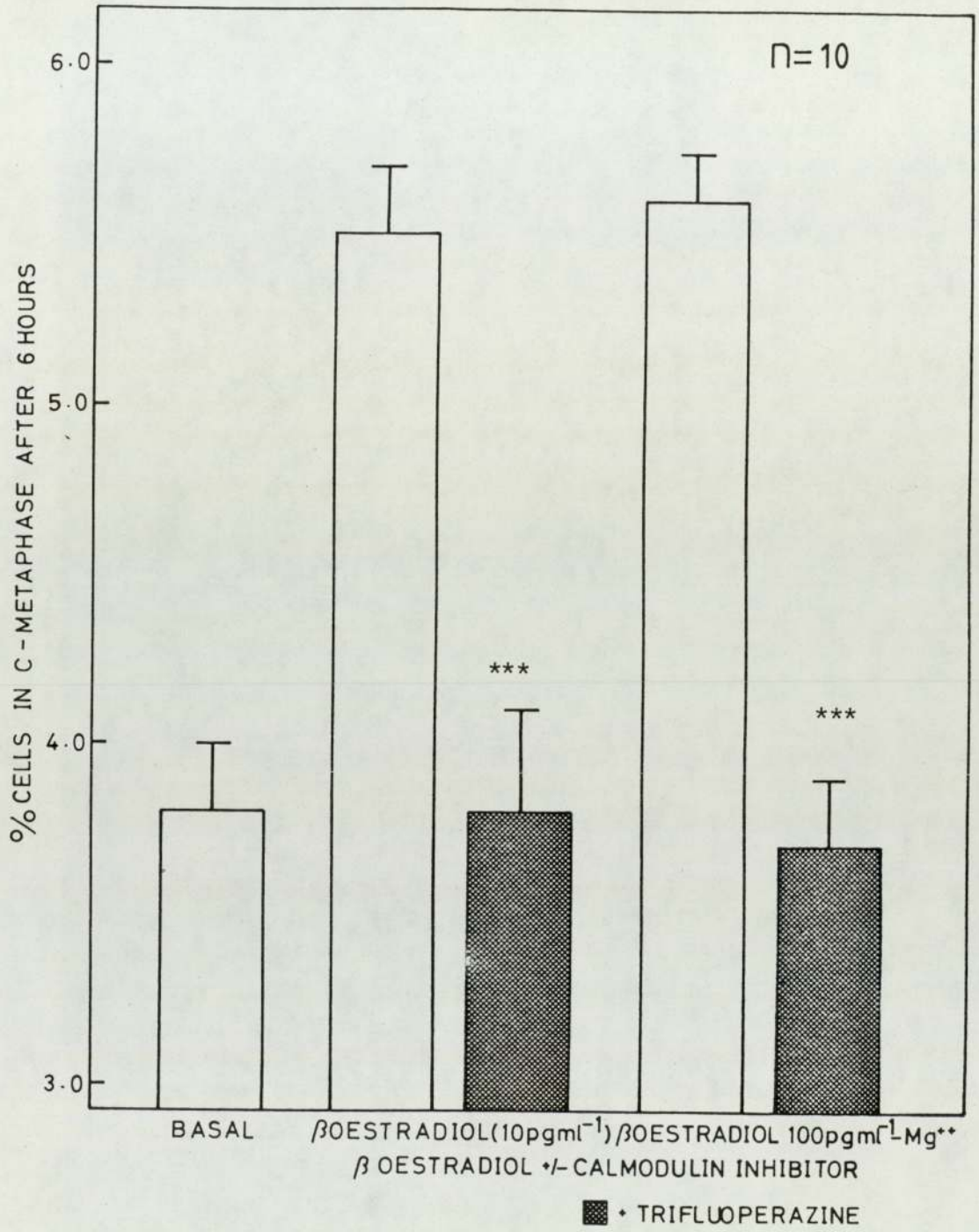


Figure 25 Effect of Trifluoperazine (10^{-6}M) on Beta-Oestradiol-Induced Mitosis in Thymocytes Incubated in Basal and Magnesium-Free Media.

Beta-oestradiol 10 $pgml^{-1}$ was included in basal conditions (0.6 mM Ca^{++}, 1.0 mM Mg^{++}), 100 $pgml^{-1}$ was incubated in magnesium free media (0.6 mM Ca^{++}, 0 mM Mg^{++}). Values significantly different to those of corresponding cultures incubated free of the calmodulin inhibitor are indicated by asterisk(s).

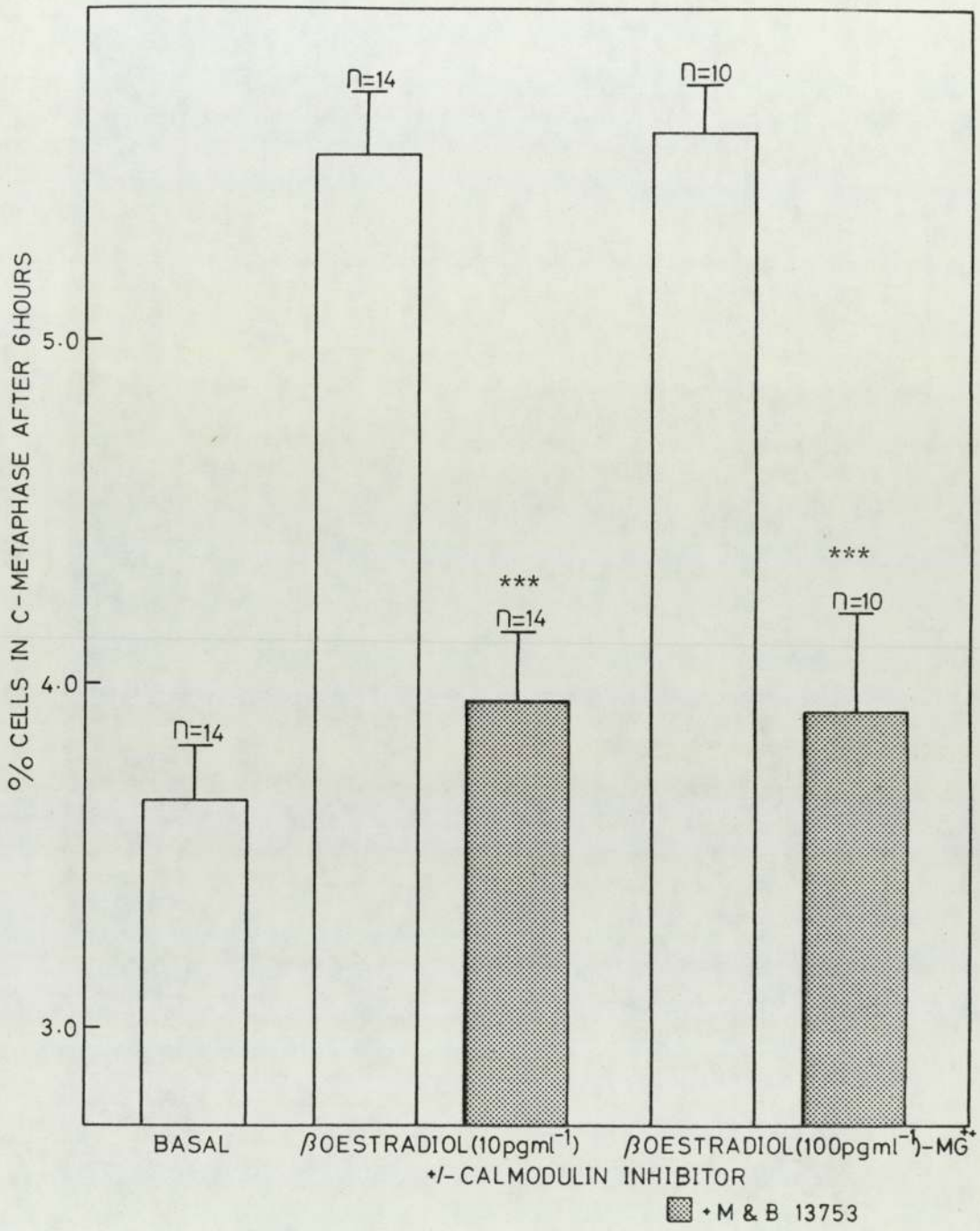


Figure 26 Effect of M & B 13753 (10^{-5}M) on Beta-Oestradiol-Induced Mitogenesis in Thymocytes Incubated in Basal and Magnesium-Free Media.

Beta oestradiol 10 $pgml^{-1}$ was included in 0.6 mM Ca^{++}, 1.0 mM Mg^{++}; 100 $pgml^{-1}$ was incubated in 0.6 mM Ca^{++}, 0 mM Mg^{++}. Values significantly different from those of corresponding cultures incubated free of the calmodulin inhibitor are indicated by asterisk(s).

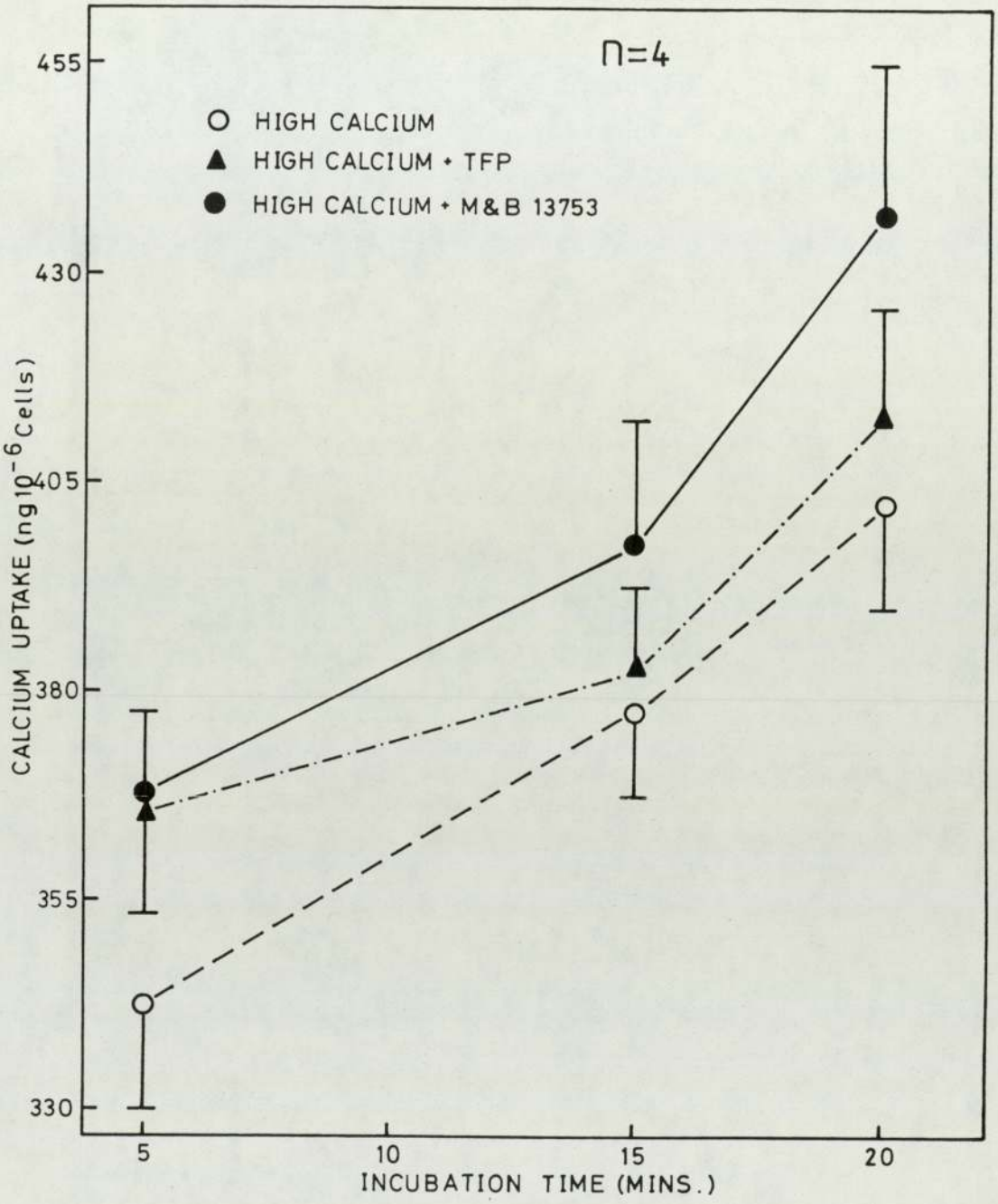


Figure 27 Effect of Calmodulin Inhibition on Calcium Uptake By Thymic Lymphocytes Incubated in High Ca^{++} Media.

High Ca^{++} indicates where calcium concentrations were raised to 1.8 mM. Trifluoperazine and M & B 13753 concentrations were 10^{-6}M and 10^{-5}M respectively. Values did not differ significantly from those of high Ca^{++} incubated free of calmodulin inhibitors.

Table 5 Effect of Calmodulin Inhibitors on High Ca^{++}-Induced Ca^{++} Influx over 20 Minutes (from figure 27).

Treatment Calmodulin Inhibitor	Medium Cation Concentration (mM) Calcium	Magnesium	Mean Rate of Ca^{++} Uptake Over 20 Minutes ± S.E.M. (ng min^{-1} 10^6 cells)
–	1.8	1.0	6.3 ± 0.4 (n=5)
Trifluoperazine (10^{-6}M)	1.8	1.0	5.8 ± 0.4 (n=4)
M & B 13753 (10^{-5}M)	1.8	1.0	5.6 ± 0.6 (n=4)

Therefore despite its magnesium-dependence, oestradiol (in a similar manner to calcium itself) raises intracellular calcium levels and via some calmodulin activated event(s) exerts its mitogenic action. For such an action however, it is not necessary to postulate the classical sequence of events for steroid hormone action i.e. combination with specific cytosolic and nuclear receptors followed by gene derepression (Jensen & DeSombre, 1973).

It was of particular interest therefore to examine the effects on thymic lymphocytes of the triphenyl ethylene compound tamoxifen which has been widely employed as an antioestrogen and anti-tumour agent in breast cancer therapy (Wakeling & Slater, 1980). Many of the actions of tamoxifen can be explained via a competitive inhibition with oestrogen for cytosolic receptors (Patterson, Furr, Wakeling & Battersby, 1982). In the mouse however tamoxifen is predominantly oestrogenic and in the rat it appears to be a partial oestrogen agonist (Jordan, Allen & Dix, 1980).

In rat thymic lymphocyte cultures high concentrations of tamoxifen (0.1 and 0.01 $\mu g ml^{-1}$), like high oestradiol concentrations (figure 1), blocked the mitogenic effect of raised extracellular calcium concentrations (figures 28 and 29). However in contrast to the inhibitory oestradiol, tamoxifen also blocked magnesium-induced mitosis (figures 28 and 29). The lower concentration (0.01 $\mu g ml^{-1}$) being slightly the more effective. Thus, in a sense it is acting like both oestradiol and testosterone. The action of tamoxifen on $^{45}Ca^{++}$ uptake followed a similar dose-dependent pattern to oestradiol in that the lower concentrations (1 $pg ml^{-1}$ and 10 $pg ml^{-1}$) significantly increased the rate of influx (figure 30). Measurement of the intracellular calcium concentration using Quin 2, supported this observation, although tamoxifen at 1 $pg ml^{-1}$ seemed to raise cytosolic levels above those achieved by the higher concentration (10 $pg ml^{-1}$), suggesting that tamoxifen may also exert a direct positive effect on calcium extrusion or sequestration processes (table 6).

Table 6 Effect of Tamoxifen on Intracellular Calcium Concentrations of Thymic Lymphocytes using Quin 2 as an Indicator.

Tamoxifen Concentration ($pg ml^{-1}$)	Medium Cation Concentration (mM)		Change in Intracellular Ca^{++} above basal as indicated by quin 2 fluorescence after twenty minutes. ($\triangle I$)
	Calcium	Magnesium	
1.0	0.6	1.0	0.27
10	0.6	1.0	0.13
1.0	0	1.0	0 (0.1 after 30 minutes)
10	0.6	0	0.046

(For traces see Appendix 1)

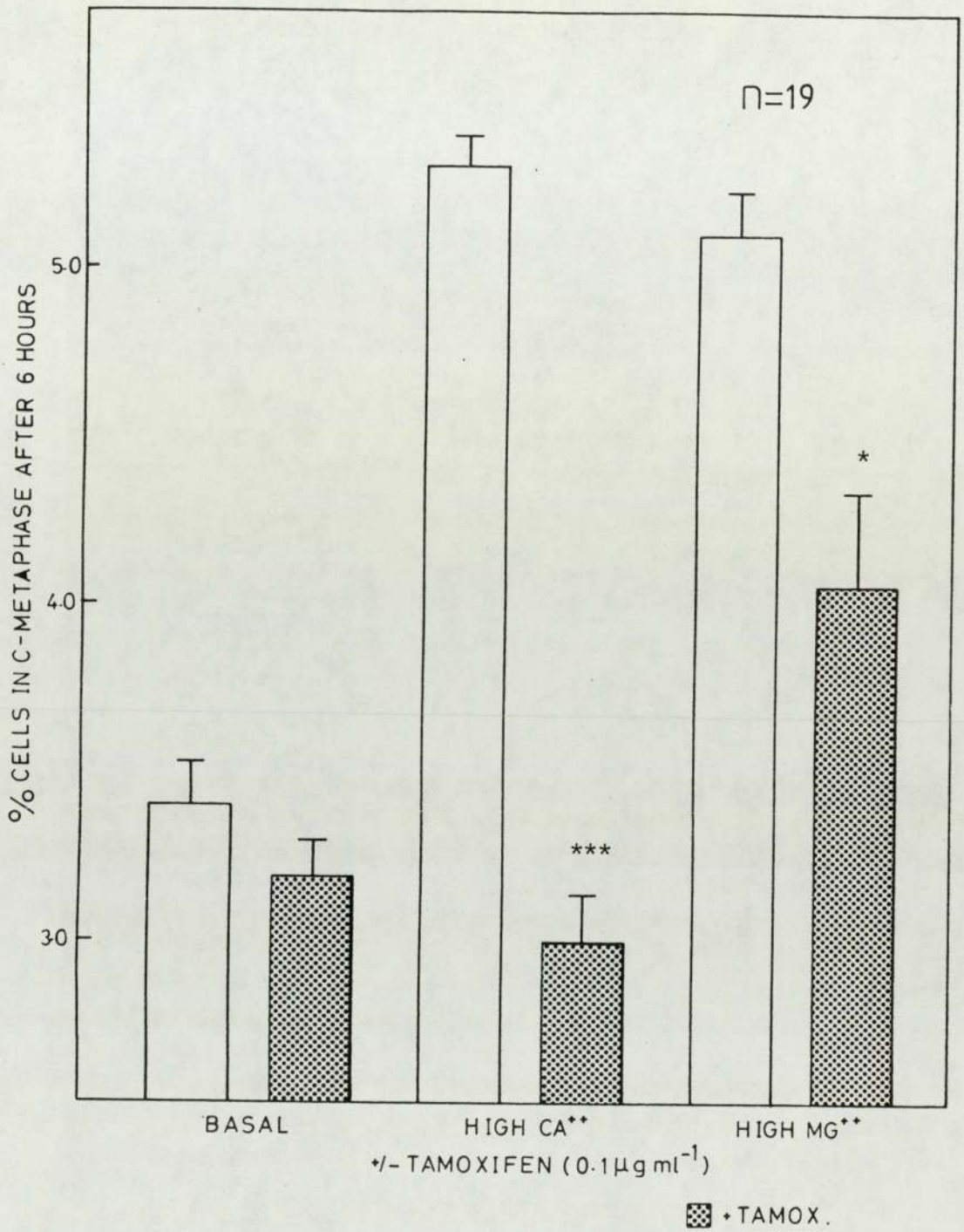


Figure 28 Effect of Tamoxifen ($0.1\,\mu g ml^{-1}$) on the Mitotic Stimulation Induced by High Concentrations of Calcium and Magnesium.

High Ca^{++} and high Mg^{++} indicates where basal (0.6 mM Ca^{++}, 1.0 mM Mg^{++}) concentrations were raised to 1.8 mM and 2.5 mM, respectively. Values significantly different from those of corresponding cultures incubated free of the antioestrogen are indicated by asterisk(s).

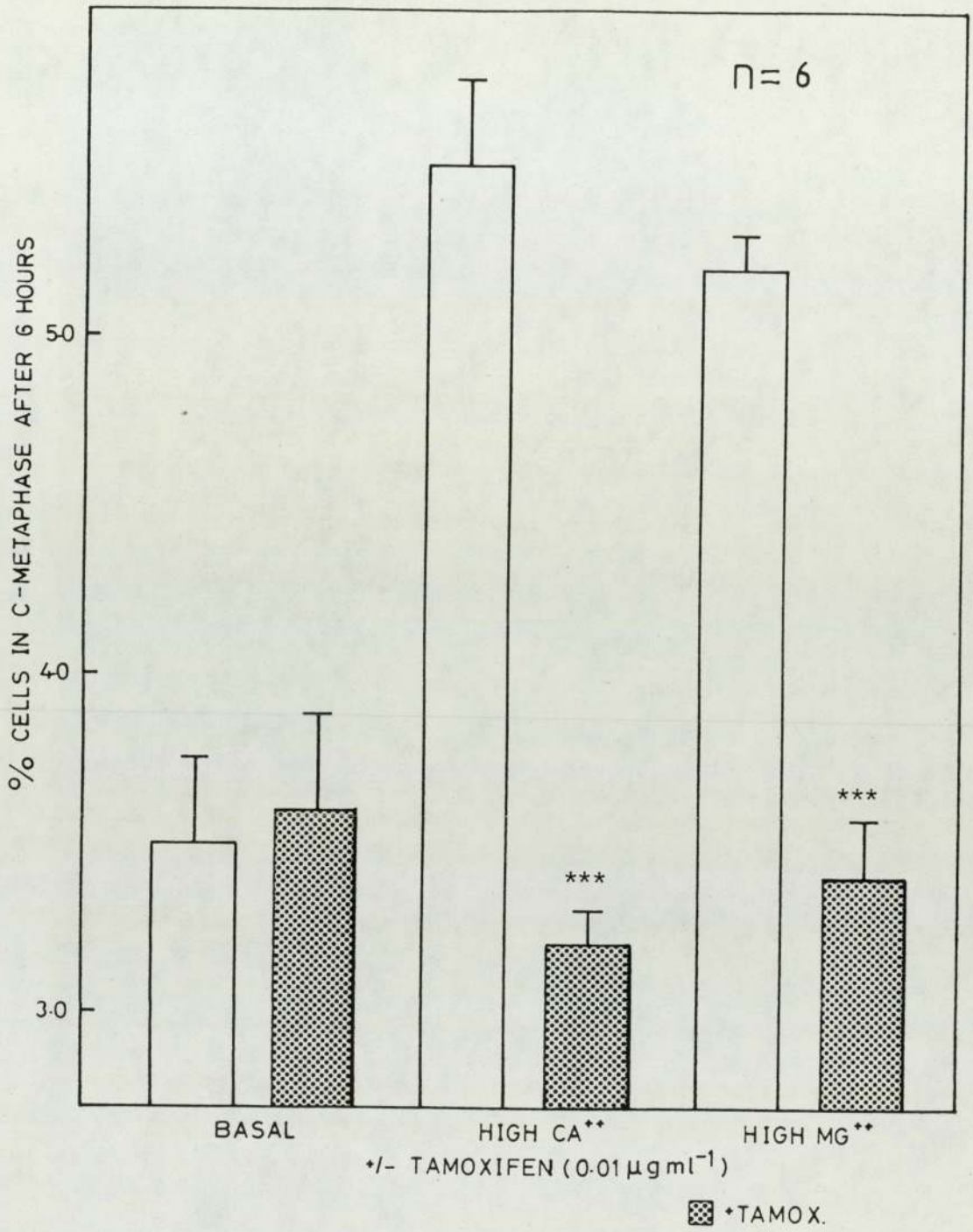


Figure 29 Effect of Tamoxifen ($0.01\mu g ml^{-1}$) on the Mitotic Stimulation of Thymocytes Induced by High Concentrations of Calcium and Magnesium.

The basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}. High Ca^{++} and high Mg^{++} indicate where these concentrations were raised to 1.8 mM and 2.5 mM respectively. Values significantly different from those of corresponding cultures incubated free of the antioestrogen are indicated by asterisk(s).

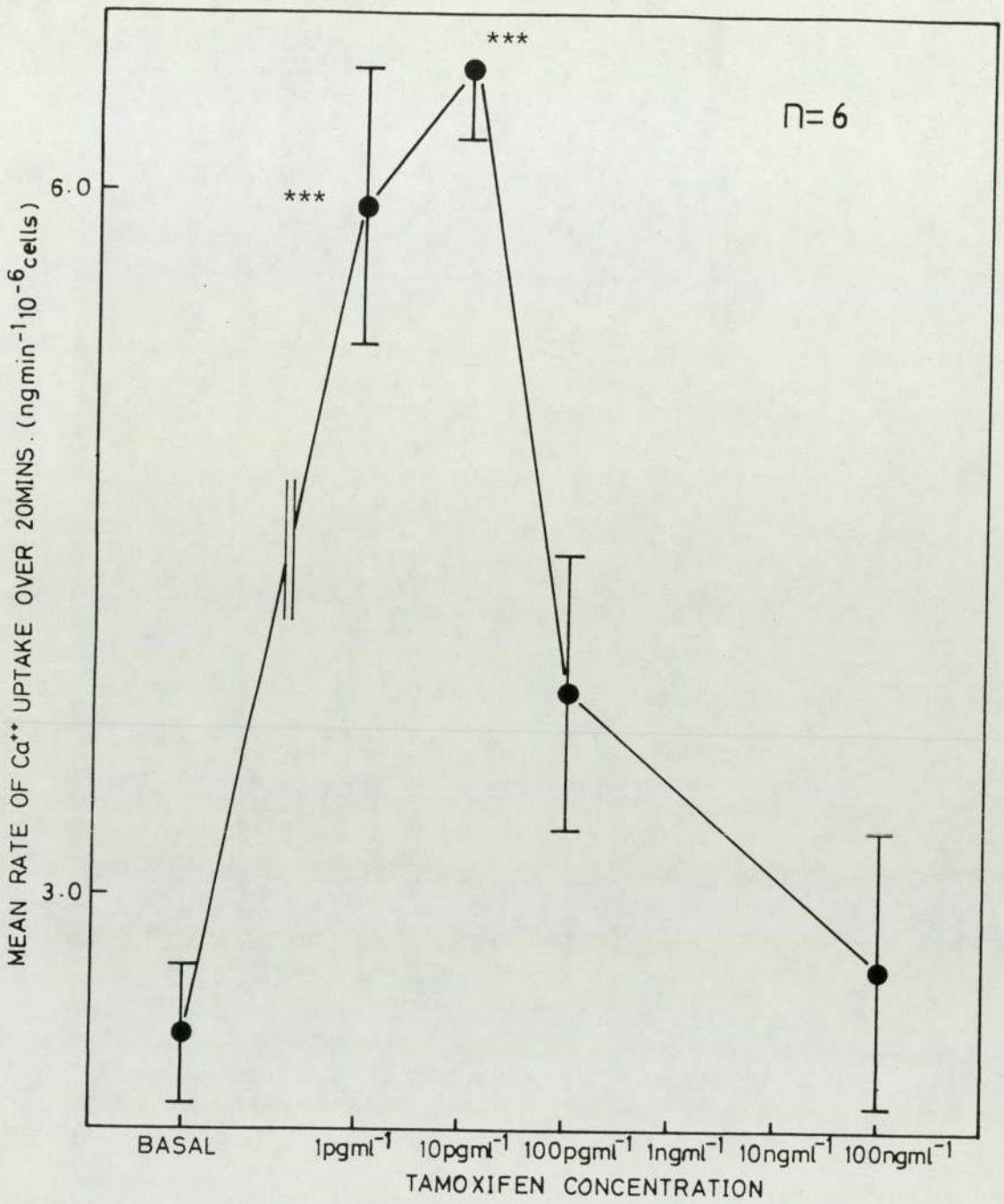


Figure 30 Effect of Tamoxifen Concentration on Calcium Influx Into Thymic Lymphocytes Incubated in Basal Media.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}. Values significantly different from those of cultures incubated free of any anti-oestrogen (basal) concentration are indicated by asterisk(s).

Despite the differences in the extent of elevation of intracellular Ca^{++} levels observed with Quin 2, both tamoxifen concentrations (1 and 10 $pgml^{-1}$) proved mitogenic whilst higher concentrations did not influence basal mitotic activity (figure 31). More detailed examination of the two mitogenic concentrations of tamoxifen revealed that the response to 1 $pgml^{-1}$ was calcium dependent and was inhibited both by oestradiol (but not testosterone) and the calcium channel blocker verapamil (figures 32 and 34). In contrast the mitogenic propensity of 10 $pgml^{-1}$ was abolished if magnesium ions were removed from the extracellular environment and its effect was inhibited by testosterone but not by oestradiol or verapamil (figures 33 to 35). The elevation of intracellular Ca^{++} content promoted by tamoxifen at 1 $pgml^{-1}$ was completely abolished (at least during the first 20 minutes of its addition) if Ca^{++} was removed from the incubation medium (table 6). There was however an apparent delayed moderate increase in $[Ca^{++}]i$ (reaching $\triangle I = 0.1$ at 30 minutes) presumably caused by mobilization from intracellular stores. Because of the time scale though it is most unlikely that this calcium would be involved in recruitment of cells from "GO" into the cycle. The replacement of calcium to the external medium at this time initiated an immediate influx of Ca^{++} (see trace in Appendix 1) raising $[Ca^{++}]i$ to levels comparable to those induced by tamoxifen (1 $pgml^{-1}$) in basal conditions (0.6 mM Ca^{++}), $\triangle I = 0.32$ after only five minutes. Thus, extracellularly located Ca^{++} is obviously the primary source of the 'trigger' calcium pertinent for the mitotic response to 1 $pgml^{-1}$ tamoxifen. Removal of magnesium ions from the medium, significantly reduced the tamoxifen (10 $pgml^{-1}$)-induced elevation of cytosolic calcium content (table 6), suggesting that despite the magnesium dependence of this concentration calcium probably

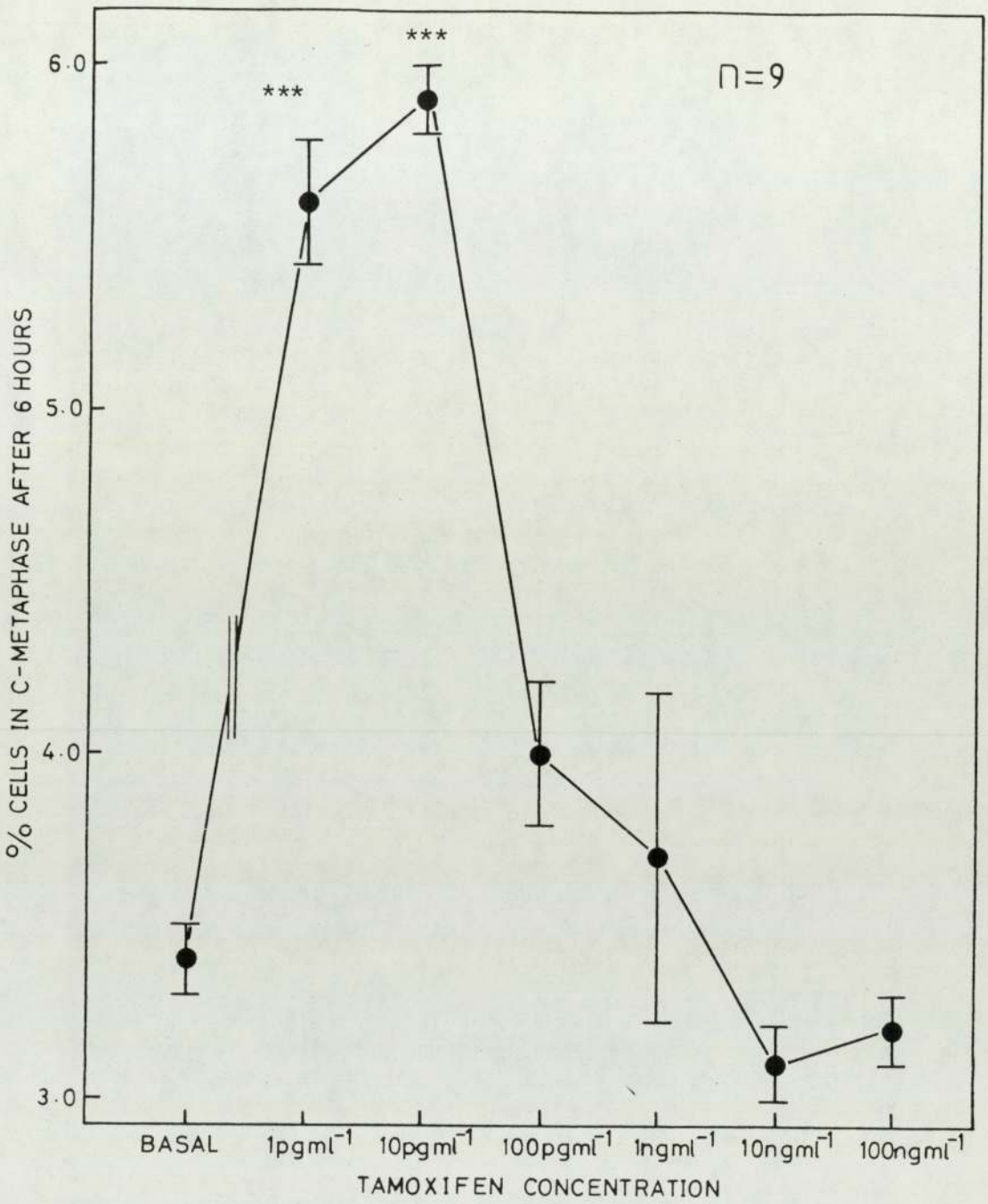


Figure 31 Effect of Tamoxifen Concentration on Mitotic Activity of Thymocytes Incubated in Basal Media.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}. Values significantly different from those of cultures incubated free of any antioestrogen (basal) are indicated by asterisk(s).

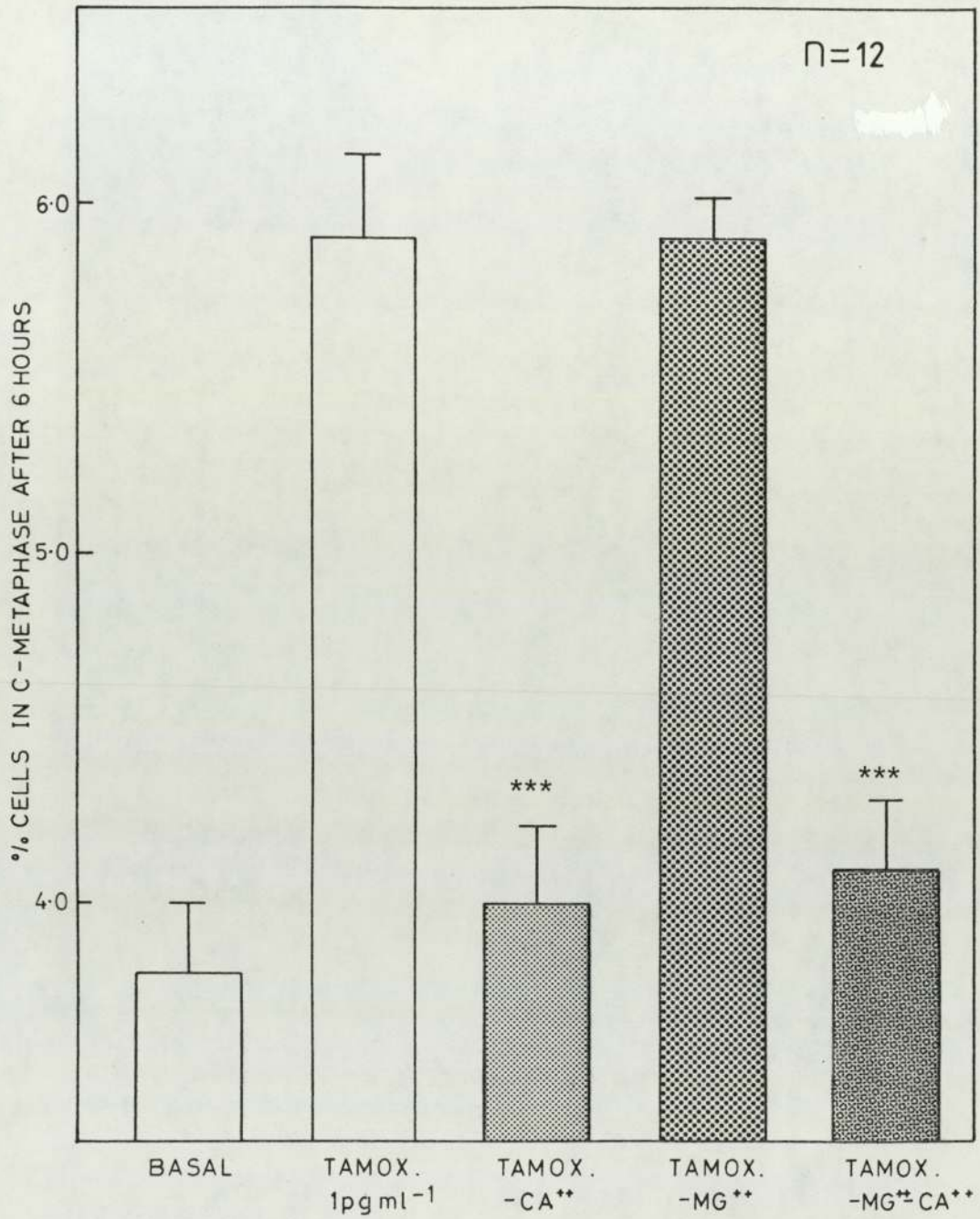


Figure 32 Ionic Dependency of Tamoxifen (1 $pgml^{-1}$)- Induced Mitogenesis.

Tamoxifen was included in 0.6 mM Ca^{++}, 1.0 mM Mg^{++}, or in media where calcium ($-Ca^{++}$), magnesium ($-Mg^{++}$), or both divalent cations ($-Ca^{++}$ $-Mg^{++}$) had been omitted. Values significantly different from those of Tamoxifen incubated in basal media are indicated by asterisk(s).

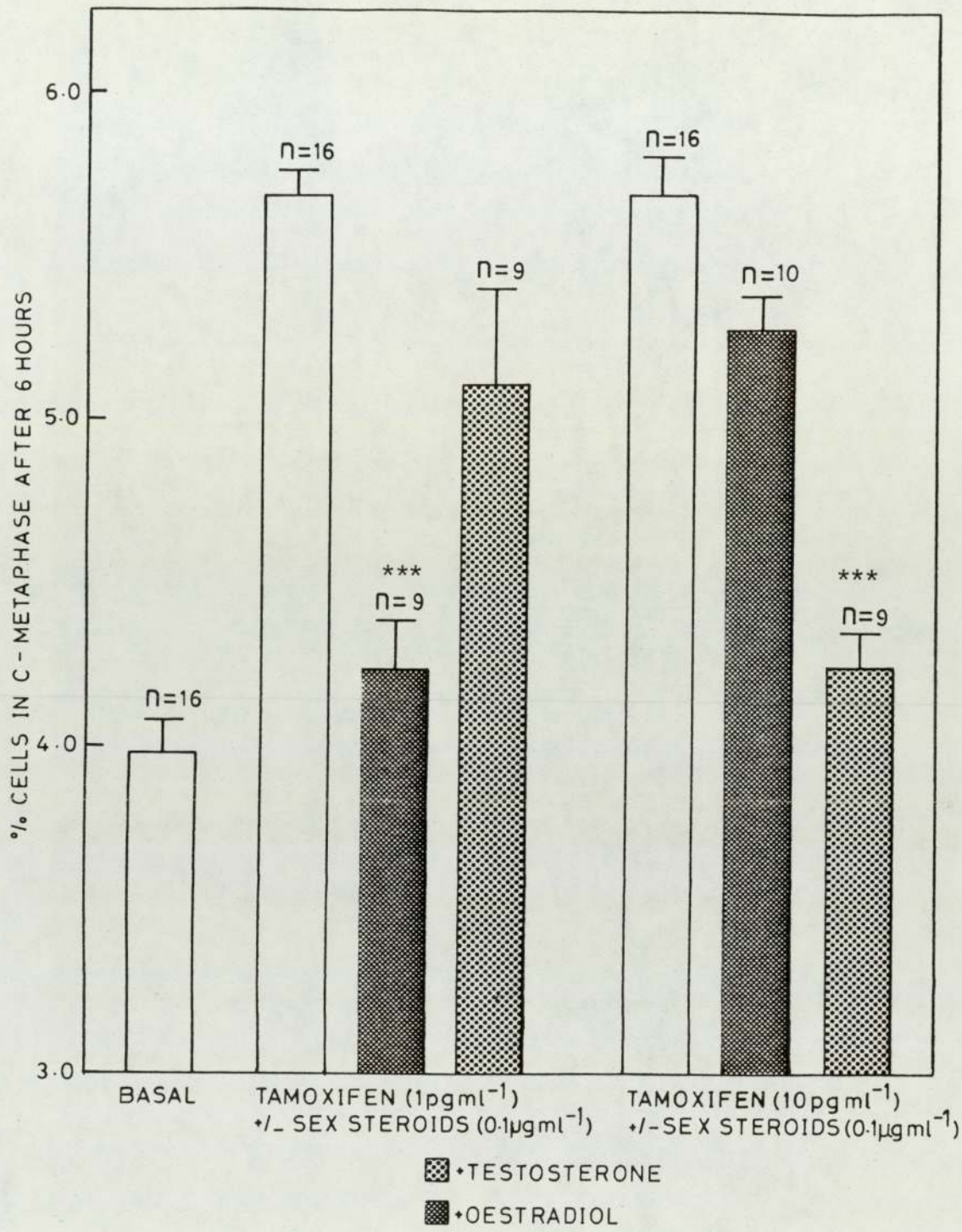


Figure 33 Steroid Blockade of Tamoxifen-Induced Mitogenesis.

Testosterone and beta-oestradiol were added to cultures at a final concentration of 0.1 $\mu g ml^{-1}$. Values significantly different from those of corresponding cultures incubated free of sex steroids are indicated by asterisk(s).

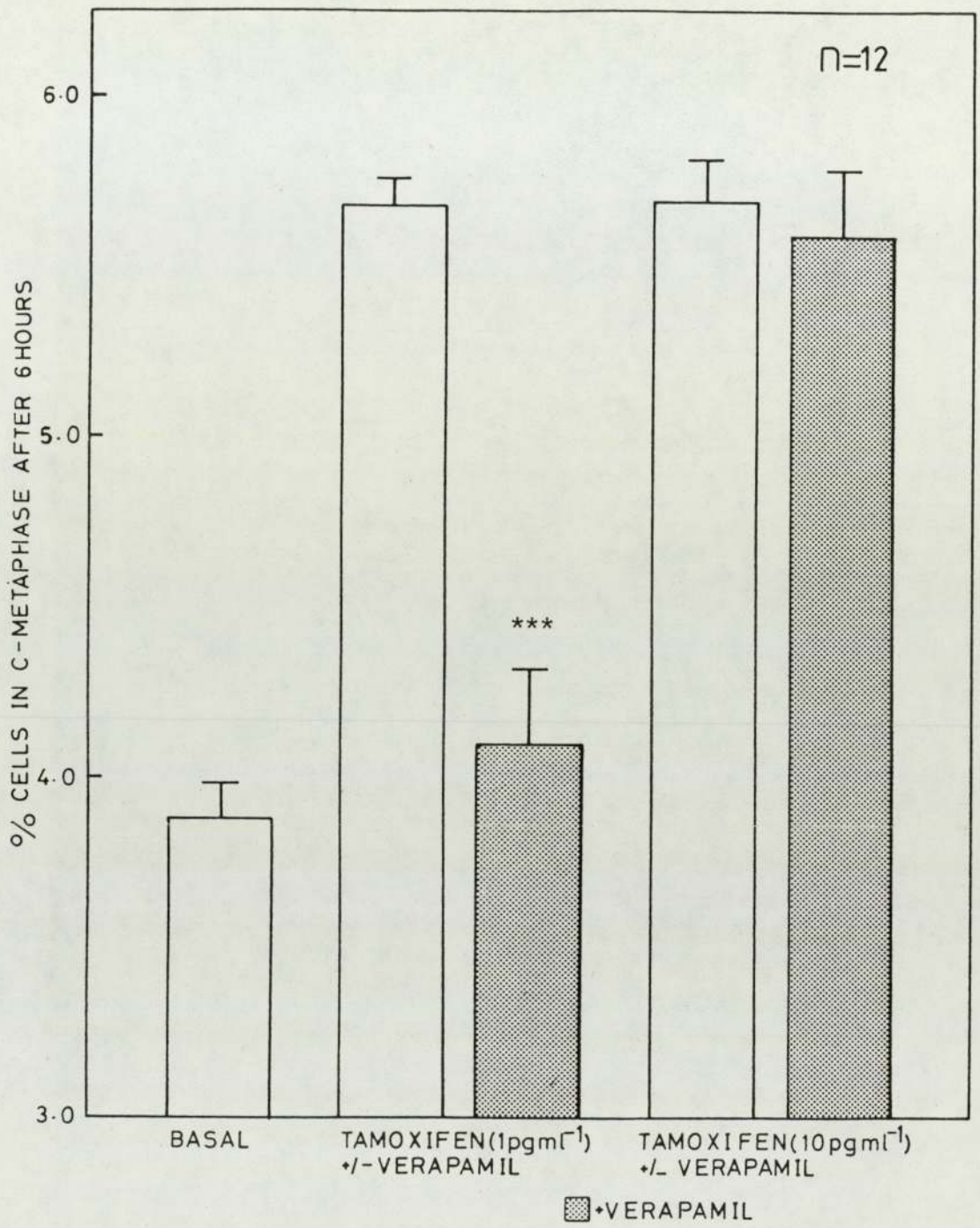


Figure 34 Effect of Calcium Channel Blockade on Tamoxifen-Induced Mitogenesis.

Tamoxifen 1 $pgml^{-1}$ and 10 $pgml^{-1}$ was included, in the presence or absence of the calcium channel blocker verapamil (5×10^{-6} gml^{-1}). Values significantly different from those of corresponding cultures incubated free of verapamil are indicated by asterisk(s).

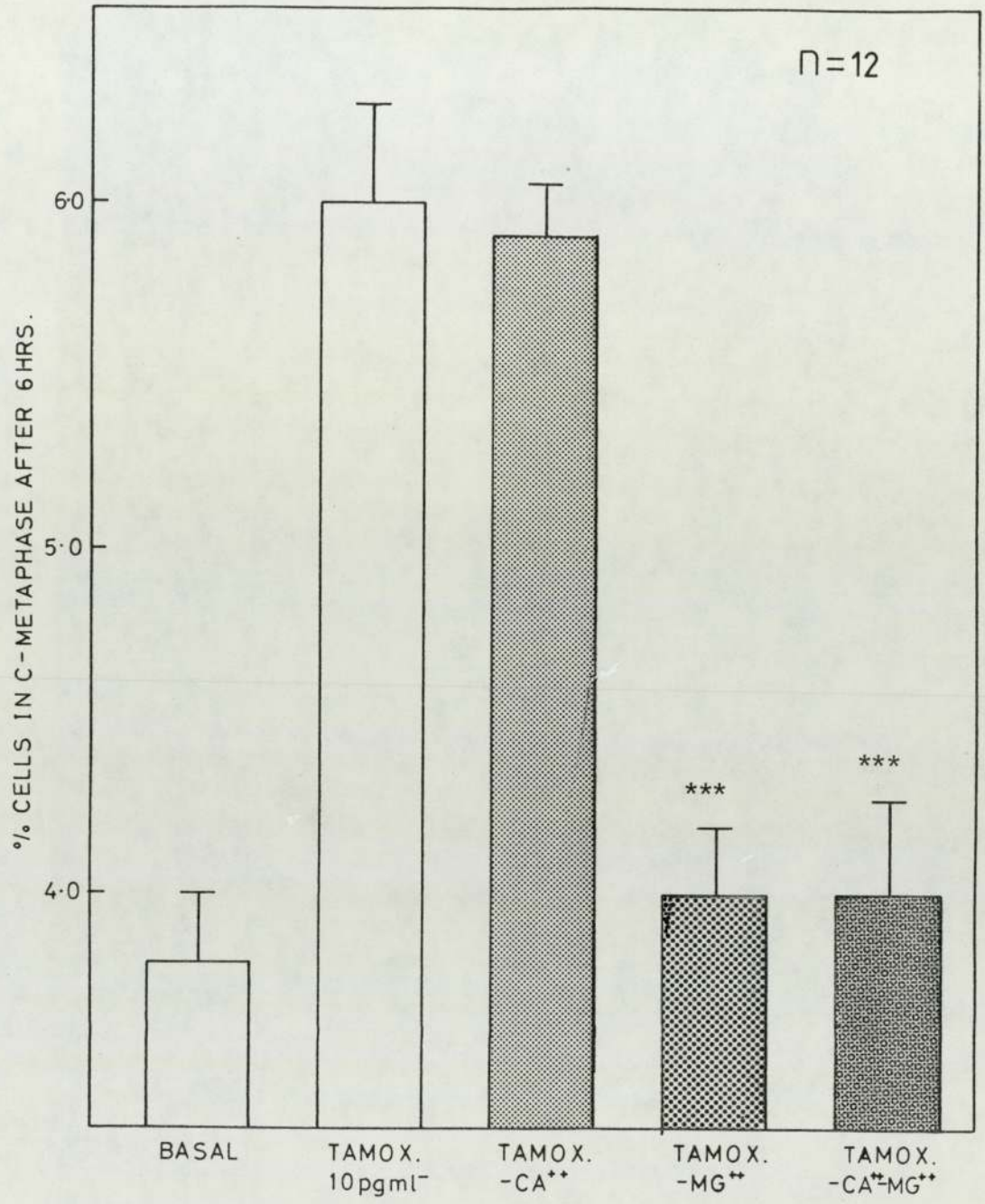


Figure 35 Ionic Dependency of Tamoxifen (10 $pgml^{-1}$)-Induced Mitogenesis.

Tamoxifen was included in 0.6 mM Ca^{++} and 1.0 mM Mg^{++} or in media where calcium ($-Ca^{++}$), magnesium ($-Mg^{++}$) or both divalent cations ($-Ca^{++}$ - Mg^{++}) had been omitted. Values significantly different from those of cultures incubated in the presence of tamoxifen in basal conditions are indicated by asterisk(s).

has an important role in the activation process. Certainly, the presence of calmodulin inhibitors abolished the response to both mitogenic concentrations of tamoxifen (figure 36) implicating calcium-calmodulin interactions in tamoxifen-induced mitogenesis. The process however, appears more complex than in the case of oestradiol action. Because the mitotic response to tamoxifen at 10 $pgml^{-1}$ is not inhib-ibited by the blockade of calcium influx by verapamil (figure 34) the observed enhanced uptake of Ca^{++} (figure 30) becomes superfi-cially redundant. However, if tamoxifen (10 $pgml^{-1}$) can rapidly stimulate a plasma membrane Ca^{++} extrusion pump this may counteract (to some extent) the $^{45}Ca^{++}$ inflow, (unfortunately, the effects of tamoxifen on Ca^{++} efflux rates have not been investigated) and would help to explain why oestradiol (0.1 μgml^{-1}) which has been shown to potentiate enhanced Ca^{++} influx (at least in high Ca^{++} conditions (figure 6)) did not inhibit the mitogenic response to this antioestrogen (figure 34). Therefore, it seems that tamoxifen increases cytosolic calcium either via enhanced influx across the plasma membrane or via a magnesium-dependent mobilization of sequestered calcium from intracellular organelles. Whether any of the antitumour properties of tamoxifen can be ascribed to its ability to influence divalent cation metabolism remains to be seen.

To find that the steroid oestradiol could modulate calcium ion movement was unanticipated although another steroid, vitamin D_3 and its more active metabolites are known to contribute to calcium homeostasis in the whole animal by enhancing calcium translocation across the intestinal mucosa (Omdahl, Holick, Suda, Tanaka and DeLuca, 1971). Its mechanism of action involves cytosolic and nuclear receptor binding of the steroid followed by gene derepression, messenger RNA

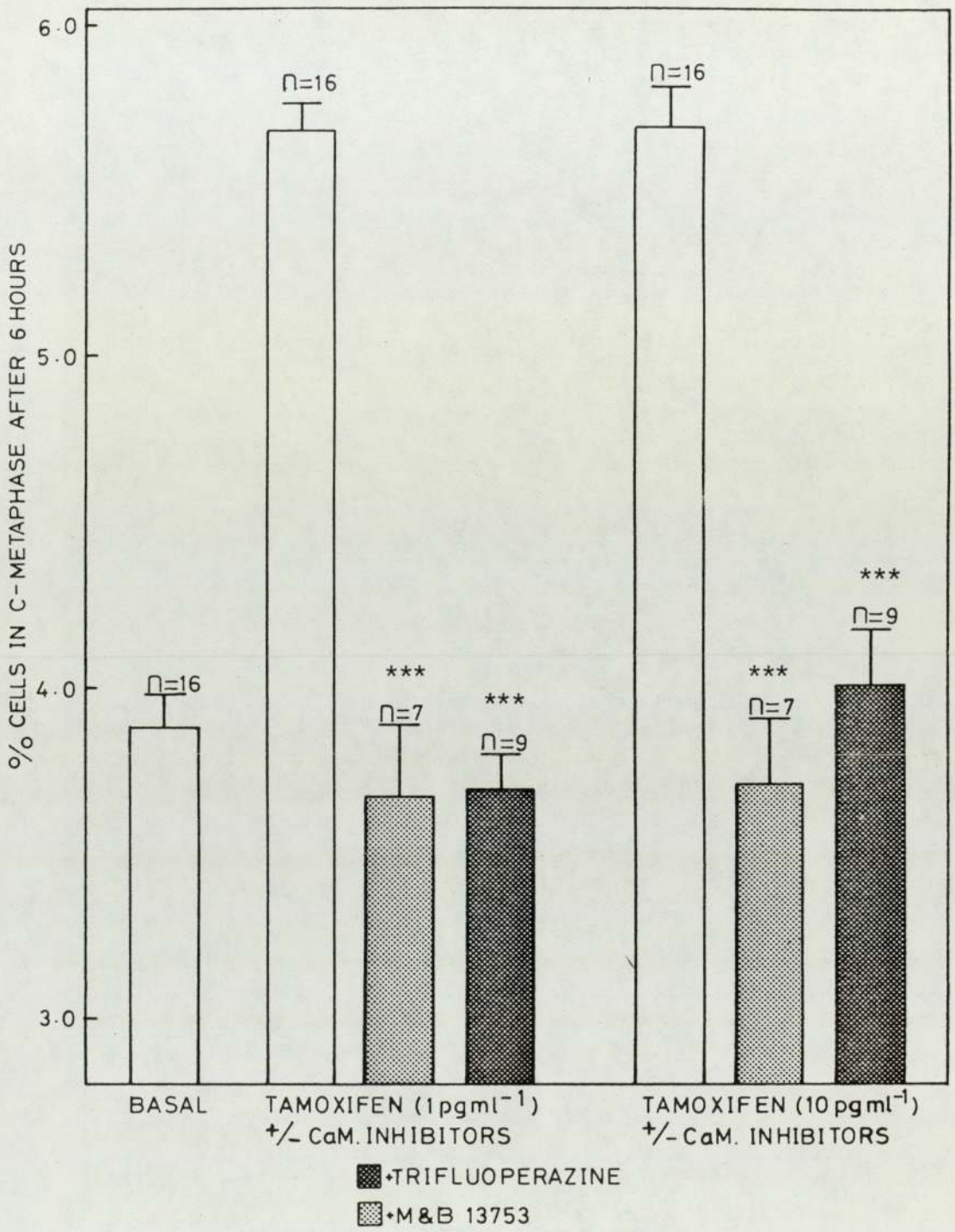


Figure 36 Effect of Calmodulin Inhibition on Tamoxifen-Induced Mitogenesis.

M & B 13753 and trifluoperazine were at final concentrations of 10^{-5} M and 10^{-6} M respectively. Values significantly different from those of corresponding cultures incubated free of calmodulin inhibitors are indicated by asterisk(s).

synthesis and ultimately the production of a specific calcium binding protein which facilitates calcium transport into and across the intestinal epithelia (Norman, Roth & Orci, 1982). The sequence of events involved in this process occurs over a protracted period of time. In contrast, vitamin D_3 and its hydroxylated derivatives enhanced the rate of calcium uptake within 20 minutes of their addition to cultured thymic lymphocytes (figure 37). This appears to correlate to increased cytosolic calcium content seen with quin 2 $\triangle I = 0.20$ (see table 8). The stimulated calcium influx was not significantly reduced by the presence of either the transcription inhibitor actinomycin D or the translation inhibitor cycloheximide (figure 10). There was exact parallelism between calcium uptake and the mitotic potential of vitamin D_3 and its mono and di-hydroxylated metabolites (figure 38). The effective molar concentrations of the steroid and its derivatives, for promoting calcium uptake and mitosis were in keeping with their relative physiological potencies in other tissues (Stern, 1981). Not surprisingly, their mitogenic propensities showed an absolute requirement for calcium but not magnesium ions in the extracellular environment (figures 39 and 40). Accordingly, oestradiol but not testosterone (at 0.1 $\mu g ml^{-1}$) abolished this calcium-dependent process (figures 41 and 42). Likewise, calcium channel blockade (which reduced vitamin D_3-stimulated Ca^{++} uptake (table 7)) and calmodulin inhibition (which did not influence the Ca^{++} uptake (table 7)), both prevented the vitamin D_3-induced mitogenesis (figure 43 to 45). This suggests that the vitamin, via calcium from an extracellular source increases the intracellular Ca^{++} concentration and subsequently initiates the mitogenic process via calmodulin activation.

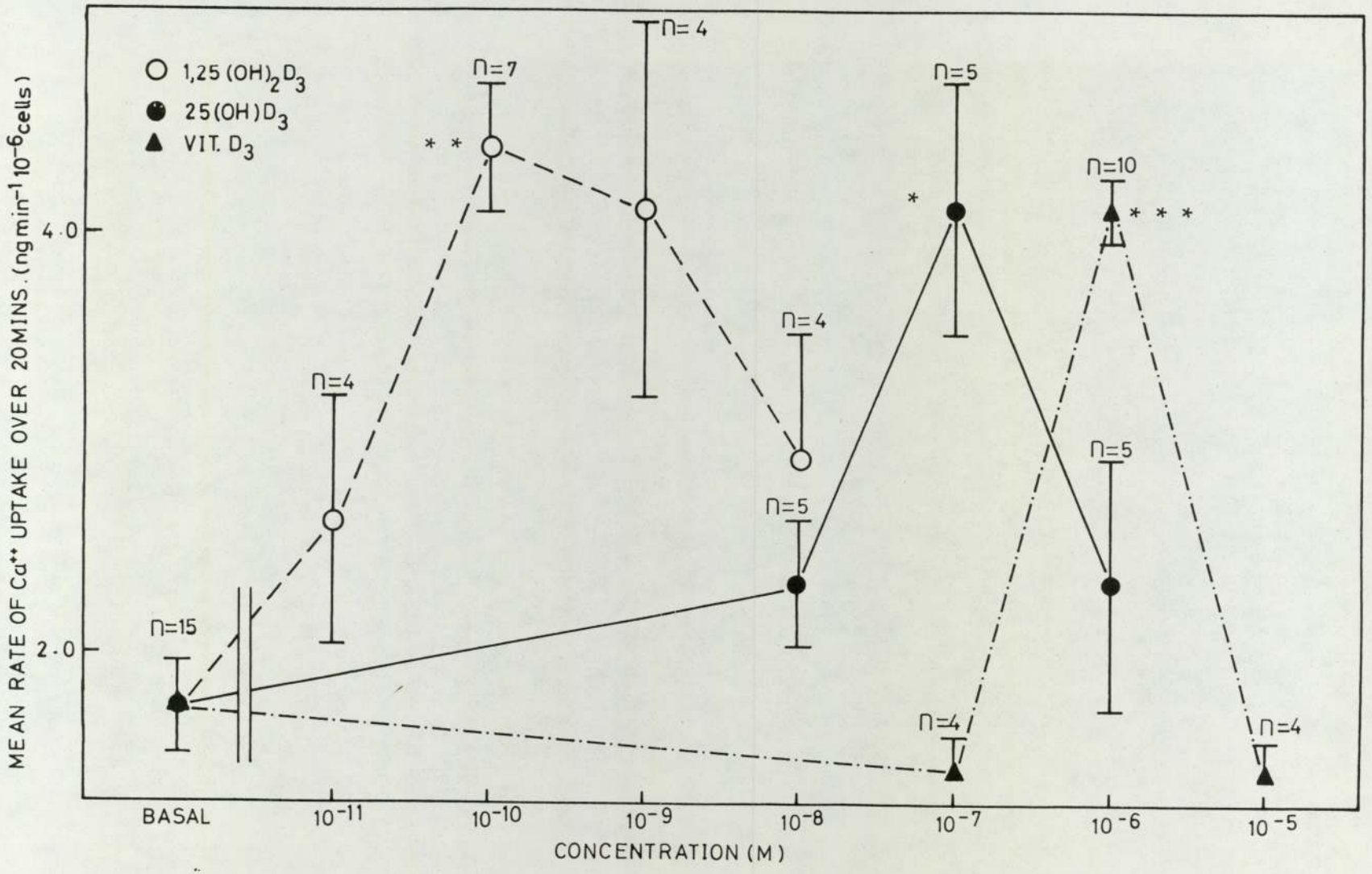


Figure 37 Effects of Vitamin D_3 and its Hydroxylated Metabolites on Calcium Uptake Into Thymic Lymphocytes. Values significantly different from those of basal calcium uptake are indicated by asterisk(s).

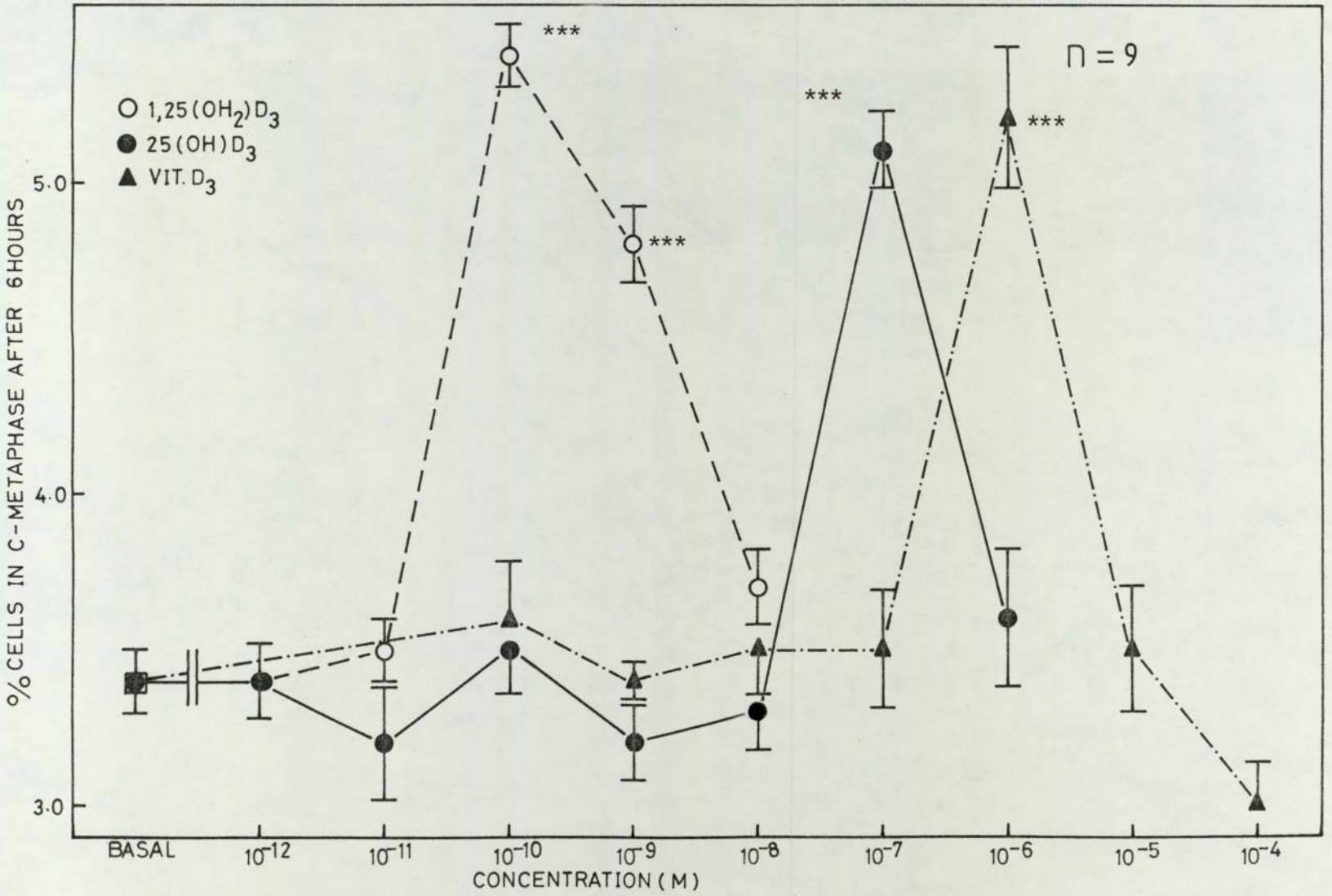


Figure 38 Effect of Vitamin D_3 and its Hydroxylated Metabolites on Mitotic Activity of Thymic Lymphocytes Incubated in Basal Media.

Values significantly different from basal mitotic activity are indicated by asterisk(s).

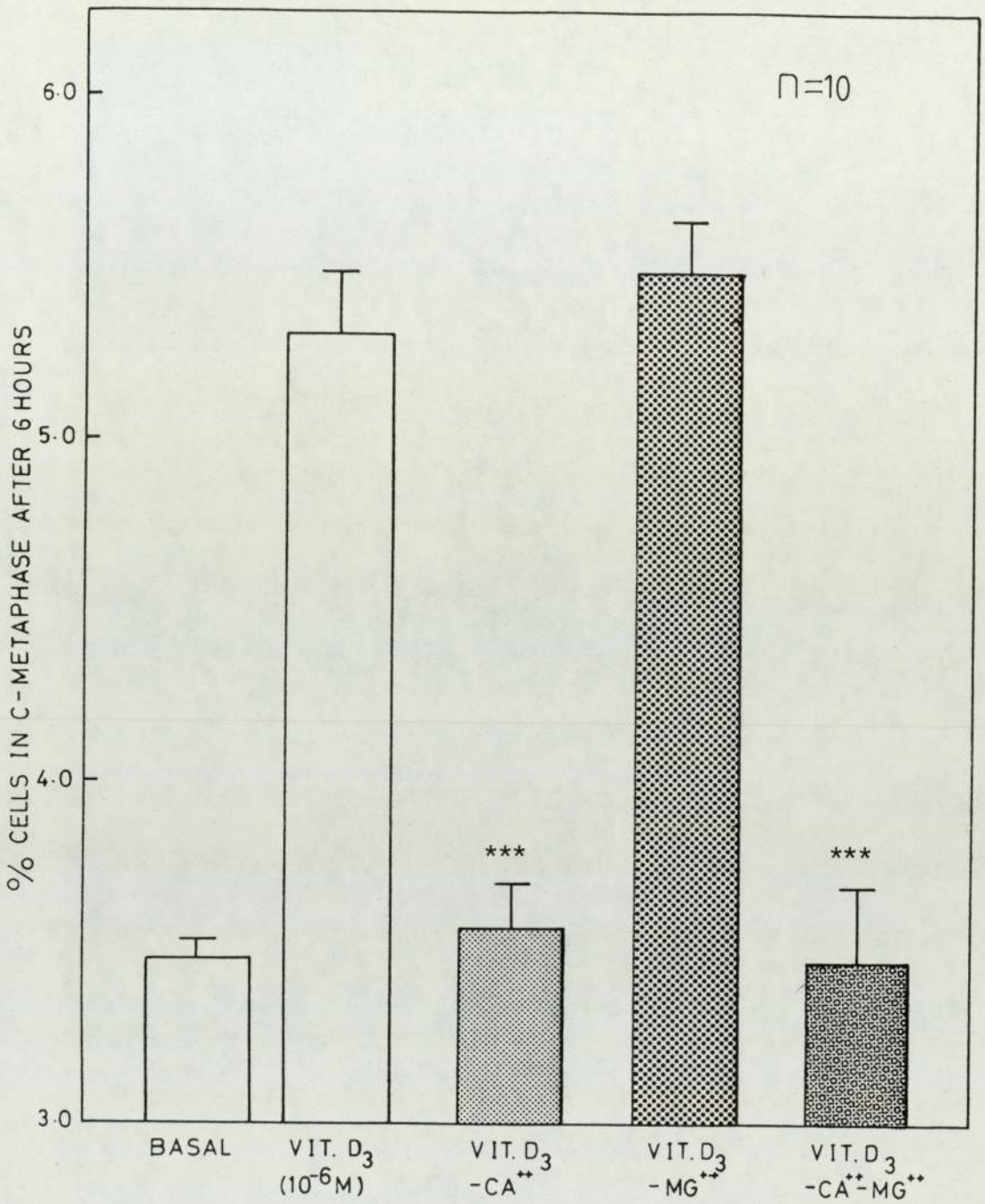


Figure 39 Ionic Dependency of Vitamin D_3-Induced Mitogenesis.

Vitamin D_3 (10^{-6}M) was included in basal (0.6 mM Ca^{++}, 1.0 mM Mg^{++}) calcium free ($-Ca^{++}$) and magnesium free ($-Mg^{++}$) media or in the absence of both divalent cations. Values significantly different from those of Vitamin D_3 cultures incubated in basal media are indicate by asterisk(s).

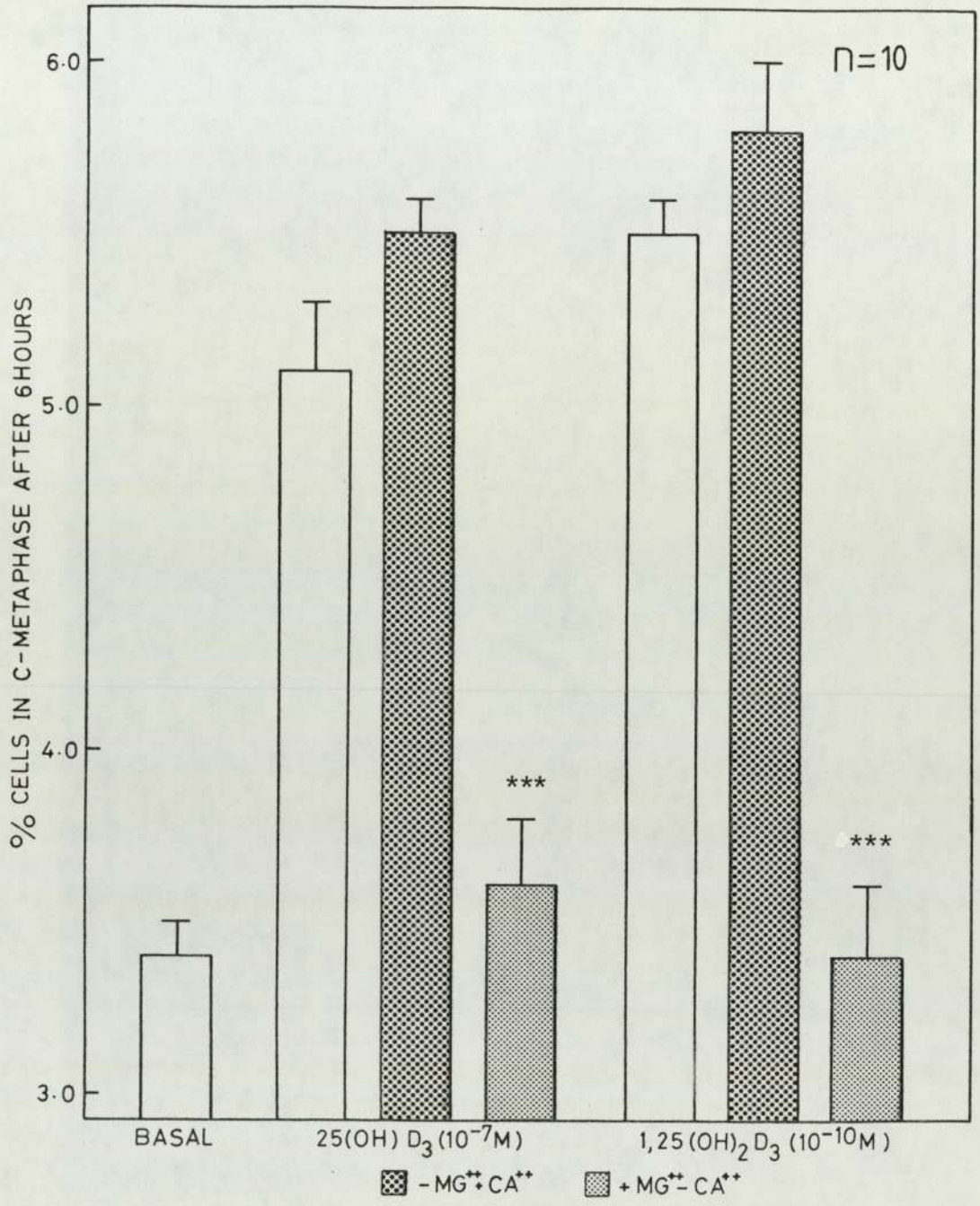


Figure 40 Ionic Dependency of 25-Hydroxycholecalciferol- and 1,25-Dihydroxycholecalciferol.

Vitamin D_3 metabolites were included in basal, calcium free ($-Ca^{++}$) and magnesium free ($-Mg^{++}$) media. Values significantly different from those of corresponding cultures incubated in basal conditions are indicated by asterisk(s).

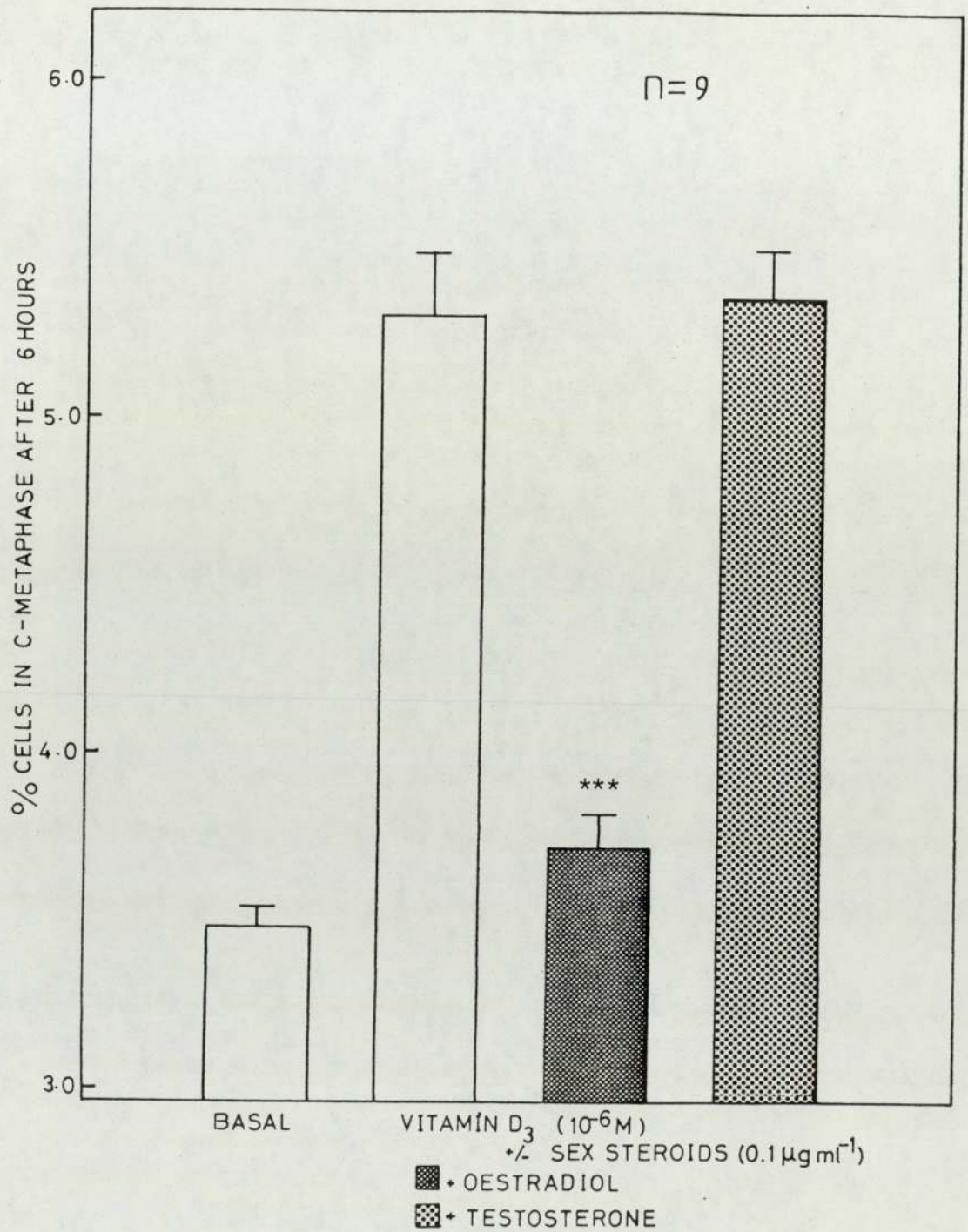


Figure 41 Steroid Blockade of Vitamin D_3-Induced Mitogenesis

Testosterone and oestradiol were added to Vitamin D_3 cultures at a final concentration of 0.1 $\mu g ml^{-1}$. Values significantly different from those of Vitamin D_3 incubated free of sex steroids are indicated by asterisk(s).

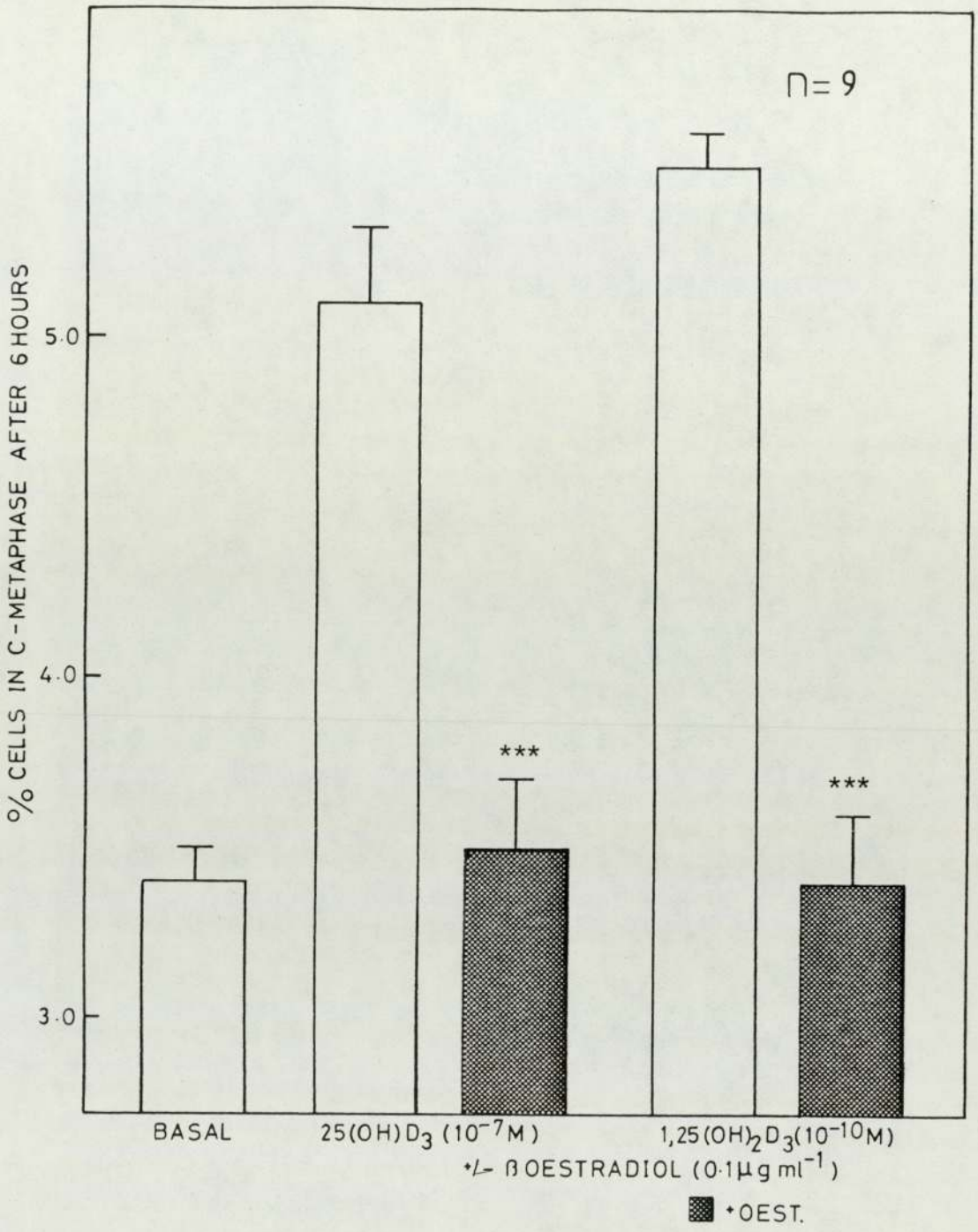


Figure 42 Steroid Blockade of 25-Hydroxycholecalciferol- and 1,25-Dihydroxycholecalciferol-Induced Mitosis.

Oestradiol was added to the cultures at a final concentration of $0.1\ \mu g ml^{-1}$. Values significantly different from those of corresponding cultures incubated free of the sex steroid are indicated by asterisk(s).

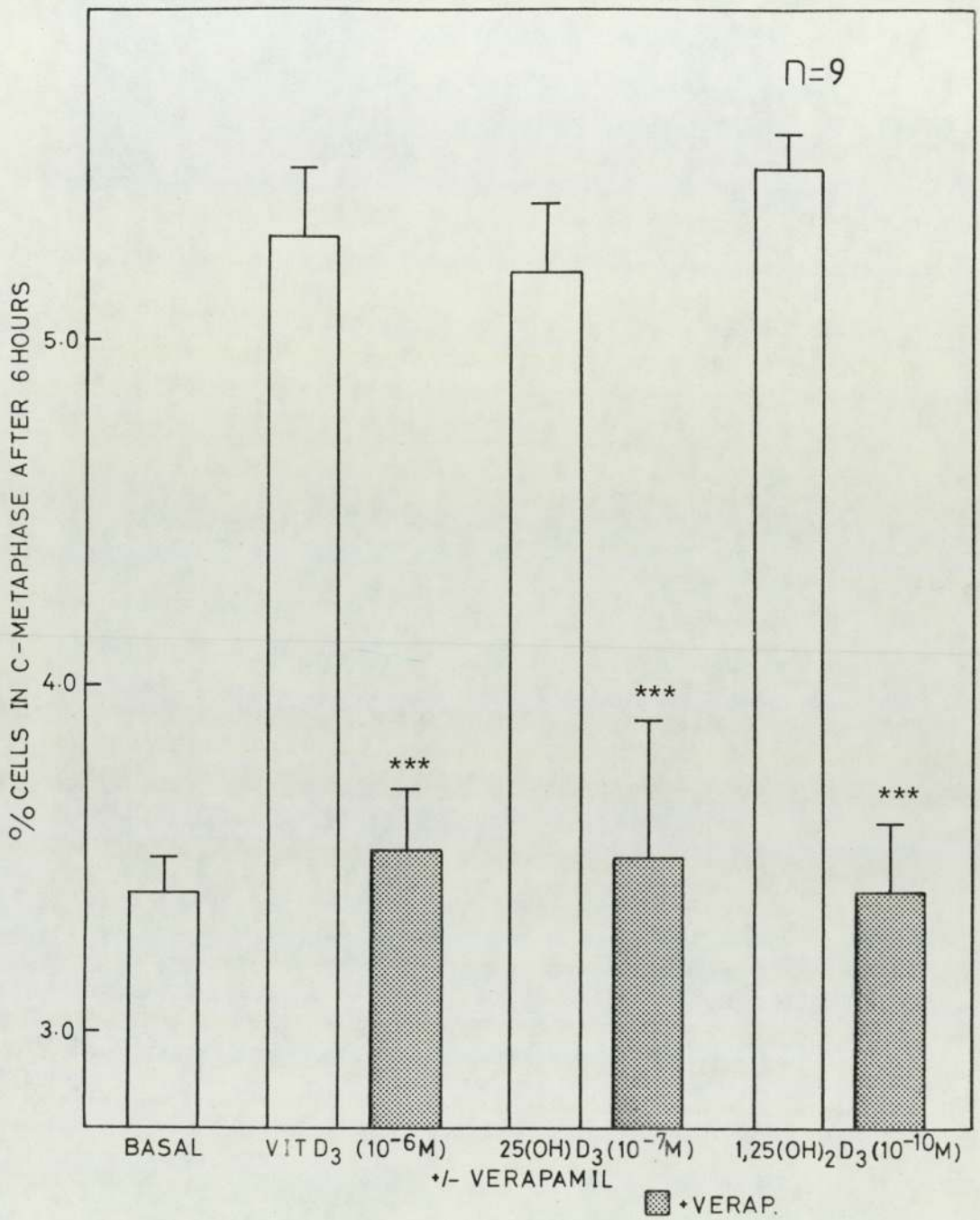


Figure 43 Effect of Calcium Channel Blockade on Mitosis Induced by Vitamin D_3 and its Hydroxylated Metabolites in Basal Media.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}. Verapamil was employed at a final concentration of 5×10^{-6} gml^{-1} in each case. Values significantly different from those of corresponding cultures incubated free of the calcium antagonist are indicated by asterisk(s).

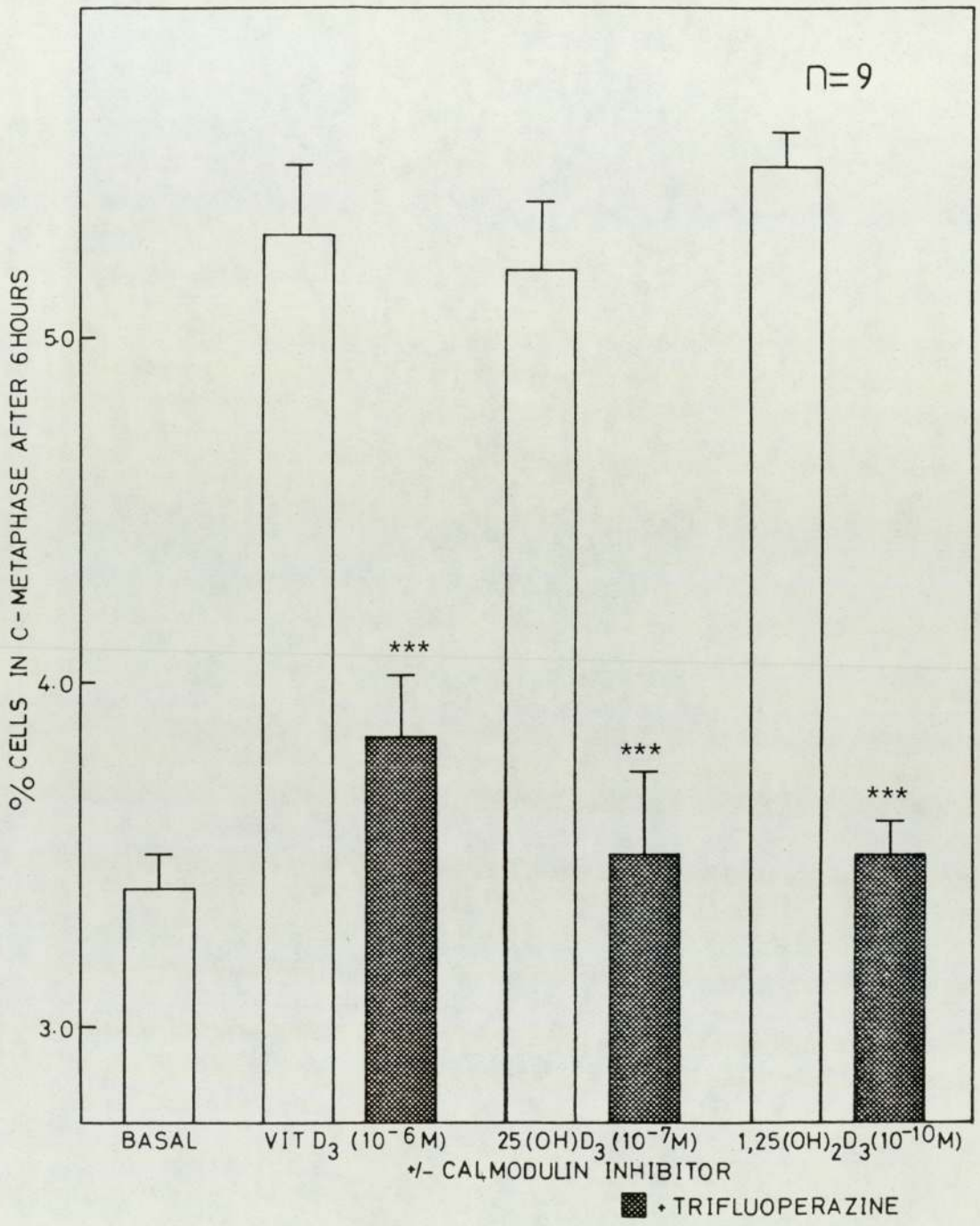


Figure 44 Effect of Trifluoperazine on Mitosis Induced by Vitamin D_3 and its Hydroxylated Metabolites.

The calmodulin inhibitor TFP was employed at a final concentration of 10^{-6}M in each case. Values significantly different from those of corresponding cultures incubated in the absence of the calmodulin inhibitor are indicated by asterisk(s).

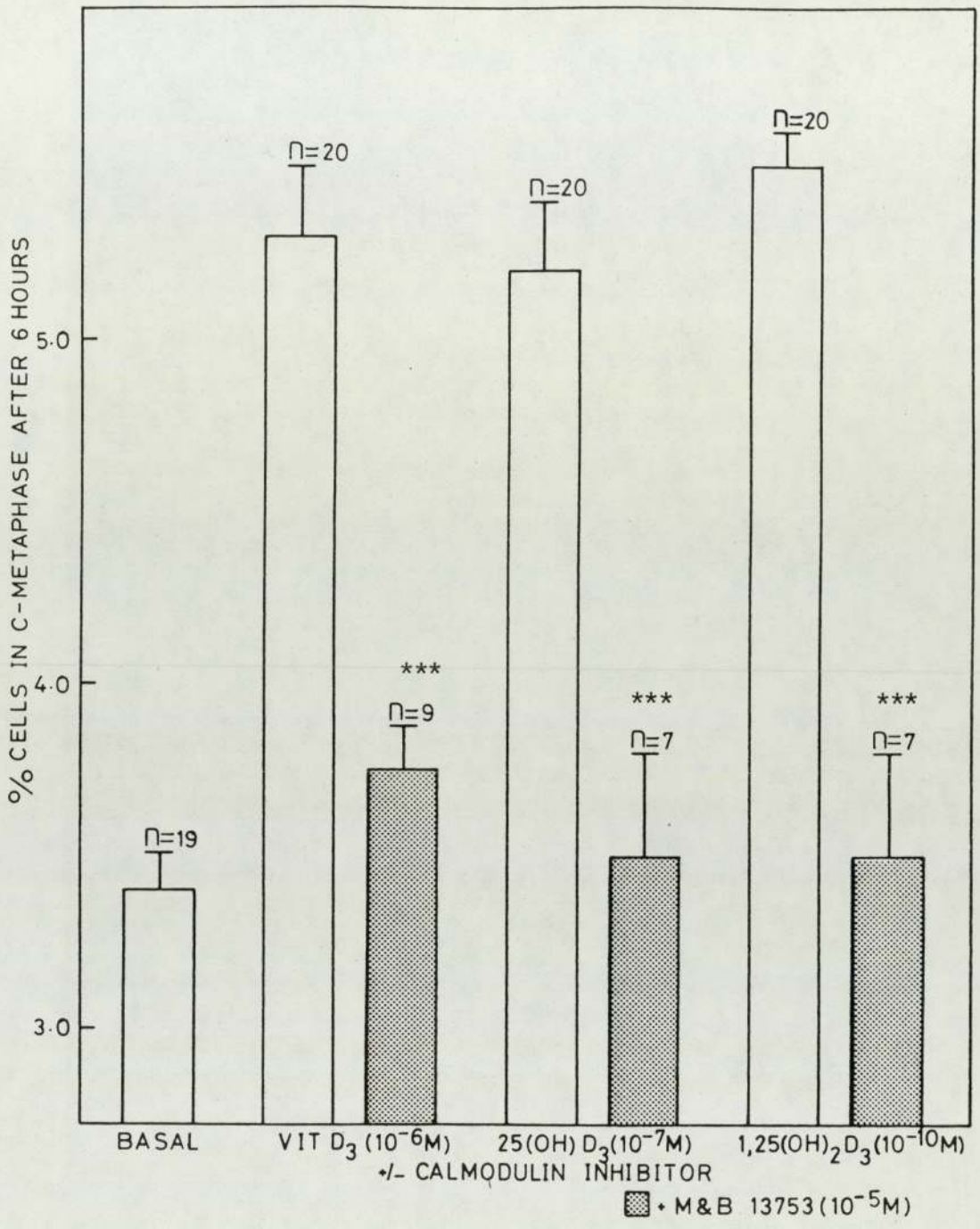


Figure 45 Effect of Calmodulin Inhibitor M & B 13753 on Mitosis Induced by Vitamin D_3 and its Hydroxylated Metabolites.

M & B 13753 was employed at a final concentration of 10^{-5}M in each case. Values significantly different from those of corresponding cultures incubated in the absence of the calmodulin inhibitor are indicated by asterisk(s).

Table 7 Effect of a Calcium Channel Blocker (Verapamil) and Calmodulin Inhibitors (TFP and M & B 13753) on Vitamin D_3-Induced Calcium Influx into Thymic Lymphocytes.

Vitamin D_3 Conc. (M)	Treatment (Concentration)	Medium Cation Concentration (mM) Calcium	Magnesium	Mean Rates of Calcium Uptake over 20 minutes +/- S.E.M. (ng min^{-1} 10^{-6} cells)
10^{-6}	0	0.6	1.0	4.4 ± 0.3 (n=5)
10^{-6}	Trifluoperazine (10^{-6}M)	0.6	1.0	3.8 ± 0.4 (n=5)
10^{-6}	M & B 13753 (10^{-5}M)	0.6	1.0	3.8 ± 0.2 (n=4)
10^{-6}	Verapamil ($5X10^{-6}$ gml^{--})	0.6	1.0	* 1.9 + 0.2 (n=4)
0	0	0.6	1.0	* 1.6 ± 0.1 (n=5)

Significantly different from untreated vitamin D_3 cultures * $P < 0.05$

A further physiological agent which is known to be related to mammalian calcium homeostasis, serving to raise plasma calcium levels in response to hypocalcaemia is the peptide parathyroid hormone (PTH). In the present study a biologically active PTH fragment containing the first 34 amino acids (PTH 1-34) has been employed. This NH_2-terminal fragment clearly promoted calcium uptake into thymic lymphocytes in a dose related manner (figure 46) which served to raise the cytosolic calcium concentration (table 8).

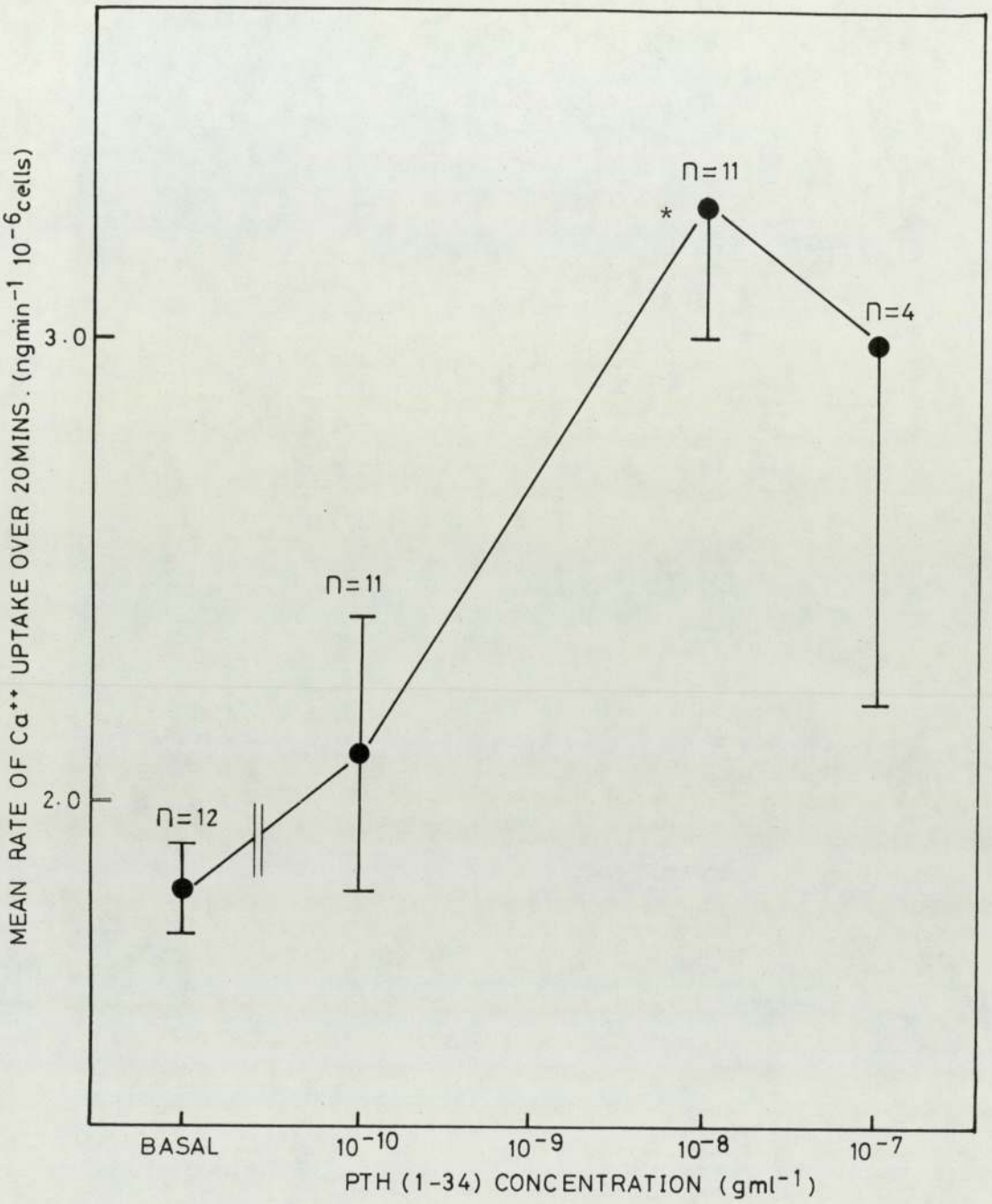


Figure 46 Effect of PTH (1-34) Concentration on Calcium Uptake from Basal Media Into Thymic Lymphocytes.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}. Values significantly different from those of basal cultures incubated in the absence of any PTH(1-34) concentration are indicated by asterisk(s).

Table 8 The Effect of External Calcium Levels and Calcium Homeostatic Hormones (PTH and Vitamin D_3) on Intra-Cellular Ca^{++} Concentration of Thymic Lymphocytes Employing Quin 2 as an Indicator.

Treatment Concentration	Medium Cation Concentration (mM) Calcium	Magnesium	Change in Intracellular Ca^{++} above basal after 20 mins. ($\triangle I$)
PTH(1-34) (10^{-8} gml^{-1})	0.6	1.0	0.30
Vitamin D_3 (10^{-6} M)	0.6	1.0	0.20
None	1.8	1.0	0.32
None	1.8	1.0	0.31
None	1.8	1.0	0.28

(for traces see figure 8 and Appendix 1)

Such increased $[Ca^{++}]i$ presumably somehow initiates the mitogenic process as PTH (1-34) at 10^{-7} and 10^{-8} gml^{-1} stimulated mitosis (figure 47). This is a Ca^{++} dependent phenomenon since removal of calcium but not magnesium ions prevented the mitotic response (table 9). Diminuition of PTH induced mitosis was also achieved, following application of the calcium channel blocker verapamil or the sex steroid oestradiol at concentrations of 5×10^{-6} gml^{-1} and 0.1 μgml^{-1} respectively (table 10). Testosterone at 0.1 μgml^{-1} had no effect.

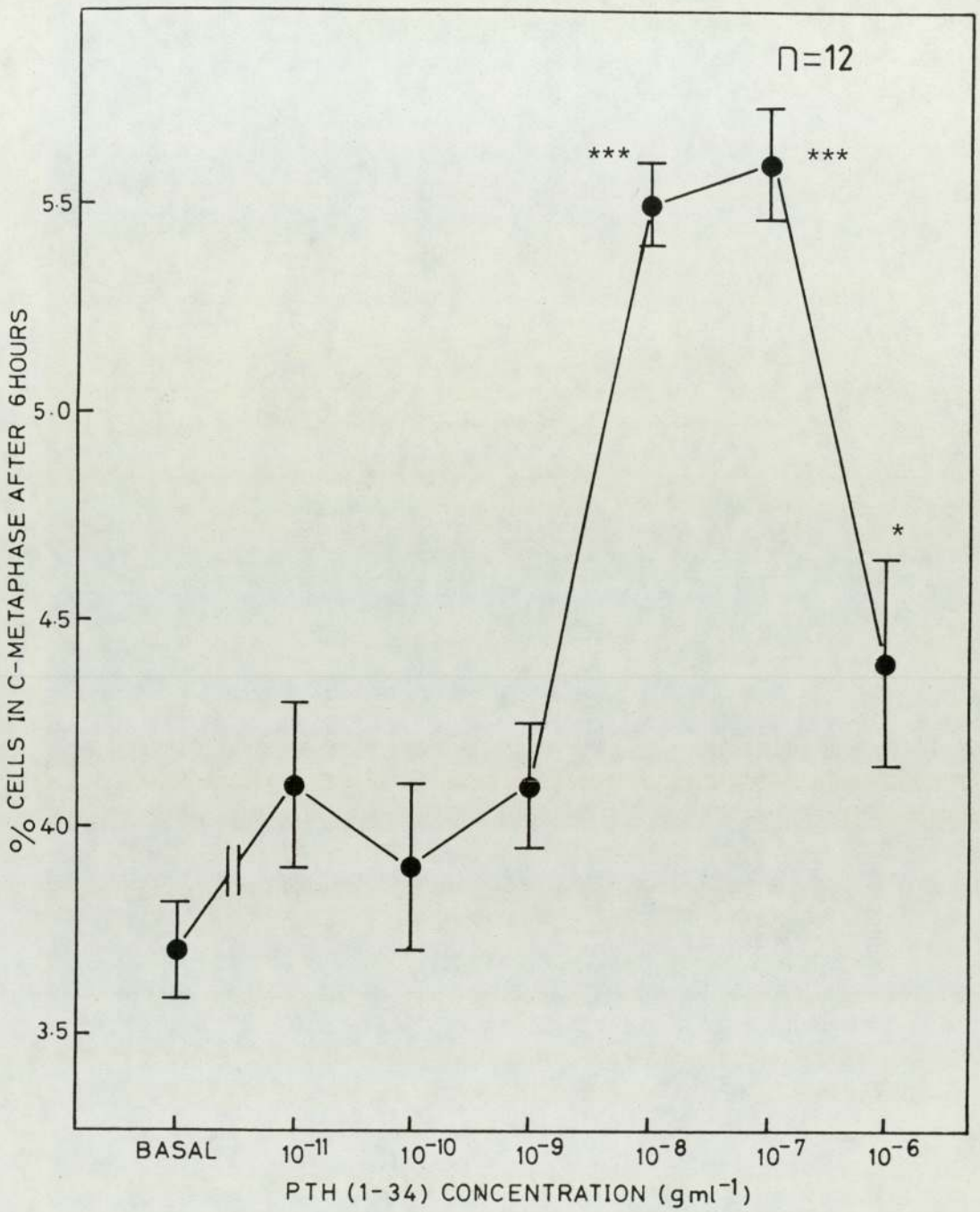


Figure 47 Effect of PTH(1-34) Concentration on Mitotic Activity of Thymic Lymphocytes Incubated in Basal Media.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}. Values significantly different from those of basal cultures incubated free of any PTH(1-34) concentration are indicated by asterisk(s).

Table 9 Influence of the Extracellular Divalent Cation Concentration Upon PTH (1-34)-Induced Mitosis in the Rat Thymic Lymphocyte

PTH (1-34) Concentration (gml^{-1})	Medium Cation Concentration (mM) Calcium	Magnesium	% Cells c-metaphase after 6 hours. ± S.E.M.
10^{-7}	0.6	1.0	*** 5.6 ± 0.2 (n =10)
10^{-8}	0.6	1.0	*** 5.5 ± 0.1 (n =12)
10^{-7}	0	1.0	3.4 ± 0.3 (n = 7)
10^{-8}	0	1.0	3.3 ± 0.2 (n = 9)
10^{-7}	0.6	0	*** 5.7 ± 0.4 (n = 7)
10^{-8}	0.6	0	*** 5.6 ± 0.1 (n = 9)
10^{-7}	0	0	3.5 ± 0.3 (n = 5)
10^{-8}	0	0	3.4 ± 0.2 (n = 5)
0	0.6	1.0	3.8 ± 0.2 (n =12)

Significantly different from basal mitotic activity *** $P < 0.001$

Table 10 The Influence of Verapamil and the Sex Steroids on the PTH-Induced Mitotic Response

PTH (1-34) Concentration (gml^{-1})	Treatment Concentration (μgml^{-1})	Medium Cation Concentration (mM) Calcium	Magnesium	% Cells in c-metaphase after 6 hrs. ± S.E.M.
10^{-8}	0	0.6	1.0	5.5 ± 0.2 (n=11)
10^{-8}	Oestradiol (0.1)	0.6	1.0	***3.6 ± 0.3 (n=9)
10^{-8}	Testosterone (0.1)	0.6	1.0	5.6 ± 0.2 (n=9)
10^{-8}	Verapamil (5)	0.6	1.0	***3.8 ± 0.1 (n=9)
0	0	0.6	1.0	***3.8 ± 0.1 (n=10)

Significantly different from untreated PTH cultures *** $P < 0.001$

The calcium ion thus also appears to serve as an important mediator of PTH mitogenicity and in a similar manner to other cases studied, exerts its effect via calmodulin activation since both trifluoperazine and M & B 13753 inhibited the mitotic response to PTH (1-34) application (figure 48).

The evidence thus far favours a dominant role for calcium in the sequence of events which triggers recruitment of normally quiescent cells into the cycle and their progression towards mitosis. Magnesium seems to play a supporting role, facilitating the action of certain mitogens which are able to enhance calcium influx from extracellular sources or mobilize calcium from cytosolic sequestration organelles.

When considering the methods of elevating the intracellular Ca^{++} concentration one must also include the effect of modulating the activity of the plasma membrane Ca^{++} pump. An increase in cell calcium content could therefore be achieved by inhibiting Ca^{++} extrusion from the cell. As mentioned previously (see introduction), Ca^{++} export across the plasma membrane may be directly linked to the influx of sodium ions into the cell against their concentration gradient (Blaustein, 1974). An increase in the transmembrane sodium gradient thus provides a driving force for calcium ion extrusion whereas a decrease retards Ca^{++} exit and therefore favours its accumulation within the cell. In this study it was reasoned that, manipulation of the transmembrane monovalent cation equilibria by inhibition of the $(Na^{+} - K^{+})$-ATPase or by alterations in extracellular sodium concentration should reduce the sodium gradient, impair Ca^{++} extrusion and therefore prove mitogenic.

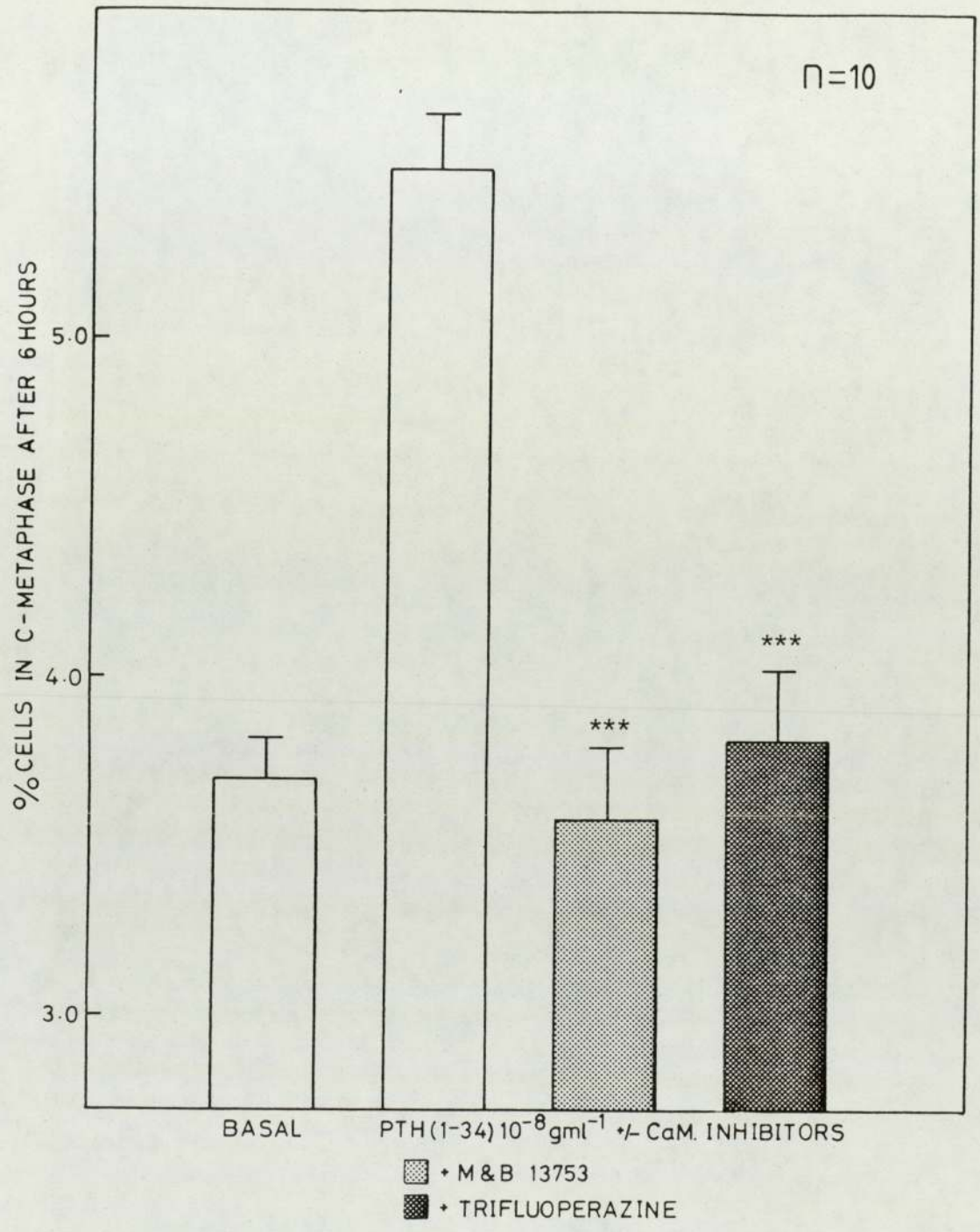


Figure 48 Effect of Calmodulin Inhibition on PTH-Induced Mitogenesis in Thymic Lymphocytes.

The calmodulin inhibitors M & B 13753 and TFP were employed at final concentrations of 10^{-5}M and 10^{-6}M respectively. Values significantly different from those of PTH(1-34) cultures incubated free of calmodulin inhibitors are indicated by asterisk(s).

In an attempt to raise intracellular sodium content and thus diminish the transmembrane gradient the sodium ion concentration in the culture medium was increased above the normal 145 mM in a progressive manner up to 185 mM. Mitotic stimulation was observed (figure 49), with maximum stimulation corresponding to increments of 10 mM and 20 mM, Na^+. The response could not be attributed to changes in osmolarity since equiosmolar sucrose increments were without mitogenic effect (table 11).

Table 11 Effect of Sucrose Upon Rat Thymic Lymphocyte Proliferation

Sucrose Concentration (mM)	Medium Cation Concentration (mM)		% Cells in c-metaphase after 6 hours ± S.E.M.
	Calcium	Magnesium	
0	0.6	1.0	3.8 ± 0.2 (n = 4)
20	0.6	1.0	3.7 ± 0.2 (n = 4)
40	0.6	1.0	3.9 ± 0.3 (n = 4)
0	1.8	1.0	*** 5.9 ± 0.2 (n = 4)

Significantly different from basal mitotic activity *** $P < 0.001$

The theory that the elevated mitotic activity was provoked by increased intracellular sodium content of the cell was further supported by the observations achieved using the cardiac steroid ouabain. Ouabain is a specific, competitive inhibitor of the (Na^+/K^+)-ATPase which was expected to raise intracellular sodium by preventing its active efflux. Indeed, over a thirty minute incubation period ouabain significantly reduced the Na^+ efflux rate out of cells as compared to basal levels, (table 12).

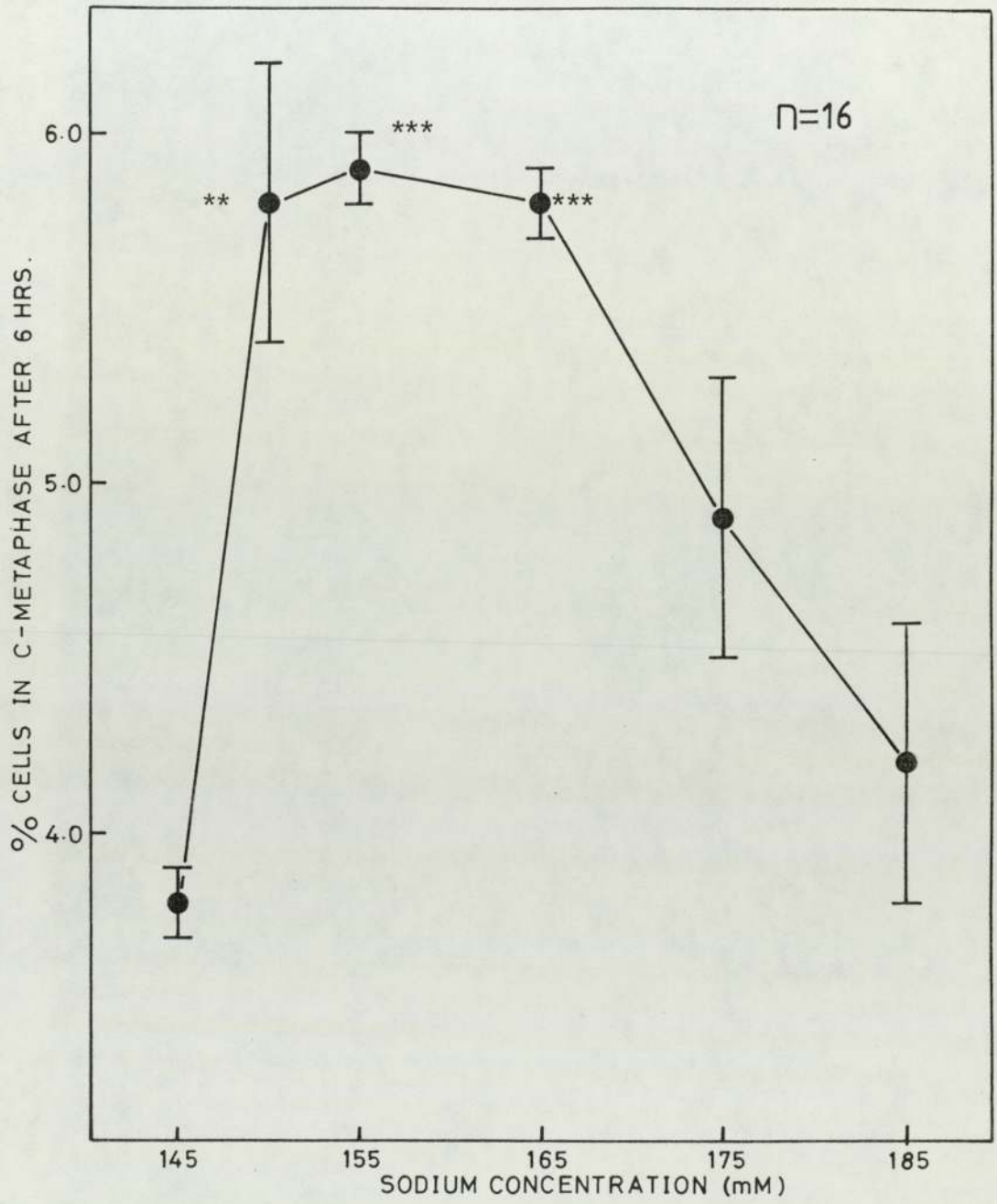


Figure 49 The Mitotic Response of Cultured Thymic Lymphocytes to Raised Extracellular Sodium Ion Concentrations.

Basal medium contains 145 mM Na^{+}, 0.6 mM Ca^{++}, 1.0 mM Mg^{++}. Values significantly different from basal mitotic activity (145 mM Na^{+}) are indicated by asterisk(s).

Table 12 Effect of the (Na^+/K^+)-ATPase Inhibitor, Ouabain on the Relative Na^+ Efflux Rate Compared to Controls

Treatment Ouabain Concentration (M)		% Reduction in rate of Na^+ efflux out of cells relative to basal levels of efflux. Mean $\pm$ S.E.M.	
0		0	(n = 4)
10^{-4}	**	15.6 $\pm$ 1.40	(n = 4)
10^{-7}	**	15.9 $\pm$ 2.10	(n = 4)
10^{-11}	**	16.8 $\pm$ 2.70	(n = 4)

Significantly different from controls ** $P < 0.01$

When ouabain was added to thymocyte cultures a biphasic mitogenic response was observed (figure 50), suggesting something more complex than a simple elevation of intracellular Na^+ levels was involved. Stimulation of mitosis was apparent at 10^{-7}M and 10^{-11} M and indeed the higher mitogenic concentration proved calcium-dependent whilst the lower concentration required extracellular magnesium to be effective (table 13). In accord with previous studies oestradiol (but not testosterone) blocked the action of the high, calcium-dependent mitogenic concentration of ouabain whereas testosterone (but not oestradiol) prevented the stimulation of mitotic activity by the low ouabain concentration (table 13).

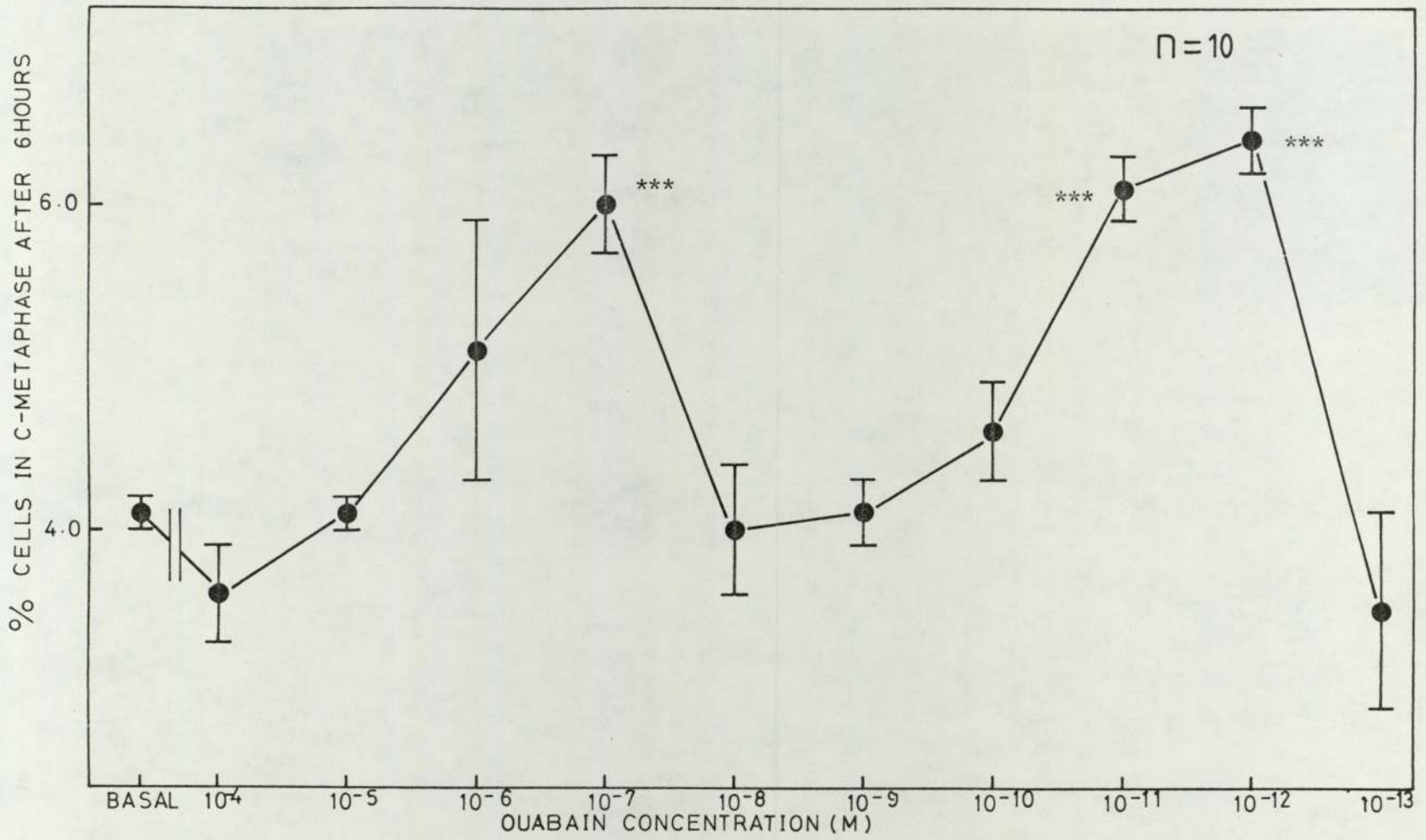


Figure 50 The Mitotic Response of Cultured Thymic Lymphocytes to Ouabain.

Cells were incubated in basal conditions, 0.6 mM Ca^{++}, 1.0 mM Mg^{++}, 145 mM Na^{+}. Values significantly different from basal mitotic activity are indicated by asterisk(s).

Table 13 Ionic Dependency and Sex Steroid Blockade of Ouabain Induced Mitogenesis

Ouabain Concentration (M)	Sex Steroid at 0.1 $\mu g ml^{-1}$	Medium Cation Concentration (mM) Calcium	Magnesium	% Cells in c-metaphase after 6 hrs. ± S.E.M.
10^{-7}	None	0.6	1.0	*** 6.0 ± 0.2 (n=23)
10^{-7}	None	0	1.0	4.1 ± 0.2 (n=5)
10^{-7}	None	0.6	0	*** 6.3 ± 0.1 (n=5)
10^{-7}	Oestradiol	0.6	1.0	4.0 ± 0.2 (n=5)
10^{-7}	Testosterone	0.6	1.0	*** 6.0 ± 0.2 (n=5)
10^{-11}	None	0.6	1.0	*** 6.0 ± 0.2 (n=23)
10^{-11}	None	0	1.0	*** 6.2 ± 0.2 (n=5)
10^{-11}	None	0.6	0	3.9 ± 0.2 (n=5)
10^{-11}	Oestradiol	0.6	1.0	*** 5.5 ± 0.2 (n=5)
10^{-11}	Testosterone	0.6	1.0	4.0 ± 0.2 (n=5)
0	None	0.6	1.0	4.1 ± 0.1 (n=40)

Significantly different from basal mitotic activity *** $P < 0.001$

Studies on other cell types have indicated that ouabain inhibits cell division (Quastel & Kaplan, 1968; Szamel et al., 1980; Tupper et al., 1977). However, the concentrations employed were invariably much higher than the concentration which enhanced mitotic activity here. High levels of ouabain (10^{-4}M) had no effect on the basal mitotic activity observed in thymocyte cultures (figure 50), although they were found to prevent high calcium - or high magnesium-induced mitogenesis (figure 51).

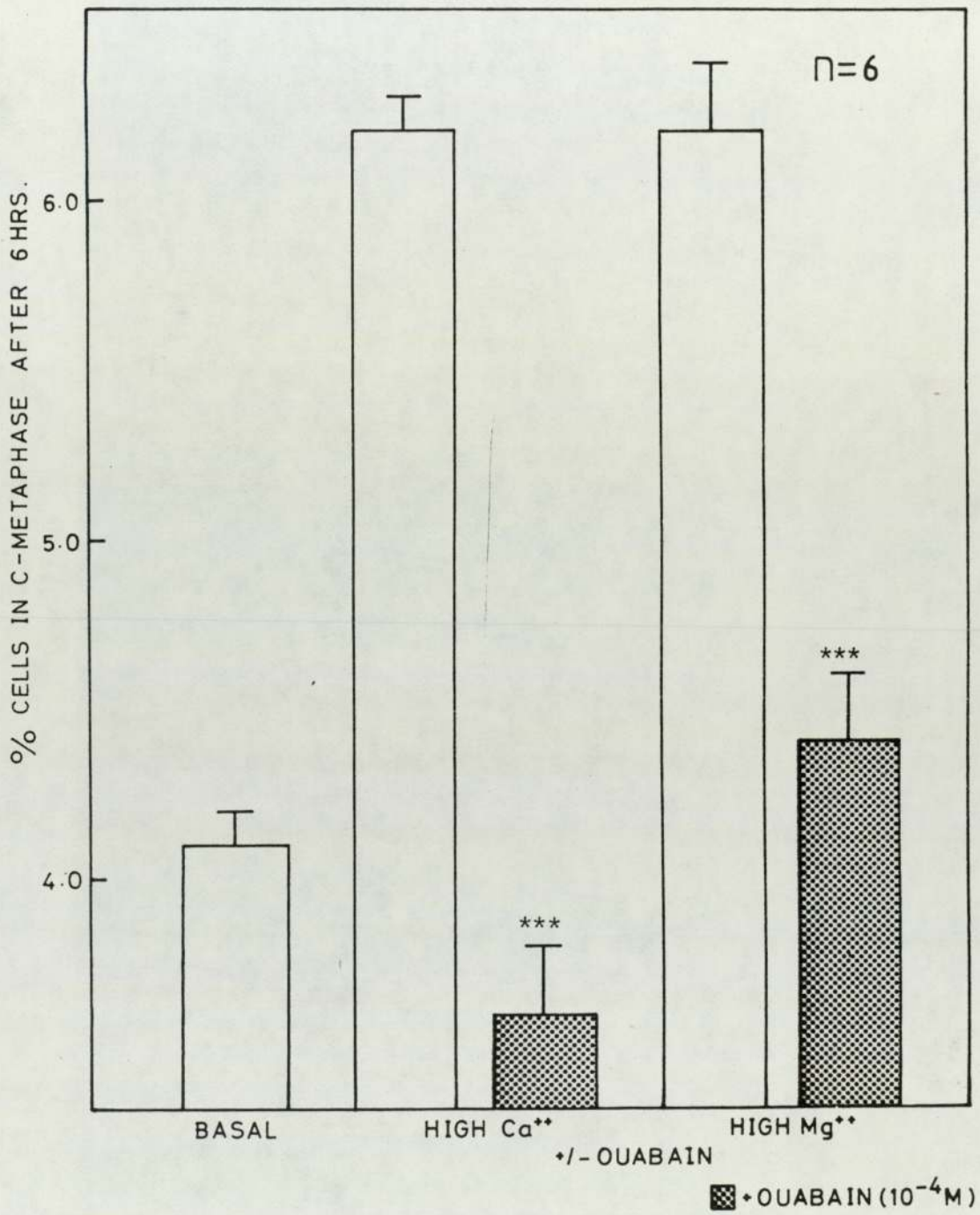


Figure 51 Effect of High Concentrations (10^{-4}M) of Ouabain on Calcium- and Magnesium-Induced Mitogenesis.

Elevated mitotic activity was induced by exposure of thymic lymphocytes to either 1.8 mM Ca^{++} (High Ca^{++}) or 2.5 mM Mg^{++} (High Mg^{++}). Values significantly different from those of corresponding cultures incubated free of ouabain are indicated by asterisk(s).

If the primary mitogenic action of ouabain was to inhibit the membrane pump and therefore increase intracellular sodium concentration it follows that the mitogenicity of sodium increments (10 mM and 20 mM) should exhibit similar divalent cationic dependency. Indeed the effect of the 10 mM increment proved to be calcium dependent whereas the 20 mM increment required external magnesium ions (table 14).

Table 14 The Effect of Calcium or Magnesium Deprivation on Sodium Induced Mitogenesis

Sodium Concentration Increment from Basal Levels of 145 mM (mM)	Medium Cation Concentration (mM)		% Cells in c-metaphase after 6 hours ± S.E.M.
	Calcium	Magnesium	
0	0.6	1.0	4.1 ± 0.1 (n=40)
10	0.6	1.0	*** 5.9 ± 0.1 (n=18)
10	0	1.0	4.1 ± 0.2 (n=7)
10	0.6	0	*** 5.7 ± 0.3 (n=7)
20	0.6	1.0	*** 5.8 ± 0.1 (n=18)
20	0	1.0	*** 6.0 ± 0.2 (n=7)
20	0.6	0	4.4 ± 0.2 (n=6)

Significantly different from basal mitotic activity *** $P < 0.001$

For ouabain thus, it was predicted that provided extracellular calcium was available the steroid should reduce the transmembrane sodium gradient, prevent calcium extrusion via Ca^{++}/Na^{+} exchange and in this way be mitogenic. This may be the case when ouabain was present at 10^{-7}M. However, at 10^{-11} M although this compound was mitogenic there was an absolute

requirement for magnesium and no dependency on extracellular calcium (table 13). It could be argued that a modest accumulation of sodium within the cell, promoted by this low concentration of ouabain (by partial inhibition of the pump), may mobilize calcium from mitochondria provided normal cytosolic magnesium levels obtain (Nicholls, 1978; Crompton, Kunzl & Carafoli, 1977). Such assumptions implicate calcium ions as the ultimate mitotic activator. Consistent with these predictions the intracellular calcium concentration was elevated by the addition of both ouabain concentrations to the thymocyte cultures (table 15).

Table 15 Effect of Ouabain on Intracellular Calcium Concentrations of Thymic Lymphocytes

Ouabain Concentration (M)	Medium Cation Concentration (mM)		Change in Intracellular Ca^{++} above Basal ($\triangle I$)		
	Calcium	Magnesium	20 mins	22 mins	30 mins
10^{-7}	0.6	1.0	0.17		
10^{-11}	0.6	1.0	0.12		0.21
10^{-7}	0	1.0	0.038	0^{+}	0.13^{+}
10^{-11}	0.6	0	-0.13		-

$^{+}$ denotes I after external Ca^{++} replacement which occurred at time 21 minutes

These elevations were not achieved via enhanced Ca^{++} uptake from the extracellular fluid (as expected) since ouabain concentrations failed to influence the basal rate of calcium influx (table 16).

Table 16 Influence of Ouabain on the rate of $^{45}Ca^{++}$-Uptake into Thymic Lymphocytes

Ouabain Concentration (M)	Medium Cation Concentration (M)		Mean Rate of Ca^{++} Uptake over 20 mins ± S.E.M. ng min^{-1} 10^{-6} cells
	Calcium	Magnesium	
0	0.6	1.0	1.8 ± 0.1 (n=5)
10^{-7}	0.6	1.0	2.0 ± 0.2 (n=5)
10^{-11}	0.6	1.0	1.7 ± 0.3 (n=5)

Removal of extracellular calcium seemed to abolish the elevation of cytosolic calcium content in response to 10^{-7} M ouabain. However, after readdition of calcium ions a somewhat delayed increase was detected (see trace in Appendix 1 and table 14). This supports the contention that ouabain does not activate transmembrane calcium channels. It is more likely that raised $[Ca^{++}]i$ arises because basal calcium diffusion into the cell can no longer be counteracted by the coupled Na^+/Ca^{++} exchange (due to ouabain induced-diminuition of the transmembrane Na^+ gradient). Omission of external magnesium clearly reduced intracellular calcium levels in the presence of ouabain at 10^{-11} M (table 15) suggesting that if as predicted Na^+ promotes calcium mobilization this process requires magnesium. Since it is unknown if the sodium increments employed are sufficient or insufficient to activate the (Na^+/K^+)-ATPase it is difficult to say if the Na^+ gradient actually diminishes. It is therefore unclear whether the mitogenic effect of extracellular Na^+ is the result of modulation of intracellular calcium concentration or some other

action. With respect to ouabain however, if as appears to be the case, calcium ions are serving as the primary mediator of the mitogenic response then, in view of our previous findings it is not unreasonable to assume that calmodulin activation is the next step in the sequence of events leading ultimately to mitosis. Certainly, calmodulin inhibition (by TFP and M & B 13753) prevented the mitotic response to the higher (10^{-7}M) mitogenic concentration of ouabain (figure 52). For ouabain at 10^{-11} M the situation appears more complex. Although M & B 13753 significantly ($P < 0.01$) blocked the action of the steroid, TFP did not prove consistently effective (figure 52). The reasons for such observations are unknown. On the face of it however, it appears that calmodulin may be involved in the mitogenic responses evoked by both ouabain concentrations.

Despite the observed predominant role of the calcium ion as the primary mediator of the mitogenic response to the mitogens studied thus far, it is possible that Mg^{++} may in certain cases serve to control the coordinated array of events associated with the initiation of DNA synthesis and cell division. The mitogenic effect of raising the extracellular magnesium concentration (figure 1) does not influence the rate of basal Ca^{++} uptake into thymic lymphocytes (figure 53) and although the magnesium does appear to elevate the $[Ca^{++}]i$ (presumably by mobilization from intracellular stores) I - 0.19 (see table 17), it is possible that this is an indirect effect, (which is somehow overridden by the Mg^{++} process). It may occur simply as a result of the greatly elevated cytosolic Mg^{++} levels competitively displacing Ca^{++} from internal binding sites. This assumption is supported somewhat by the observations that neither TFP nor M & B 13753 inhibited the high Mg^{++}-induced mitosis (figures 23 and 24), suggesting that whatever its mechanism of action the activated Ca^{++}-calmodulin complex is not involved.

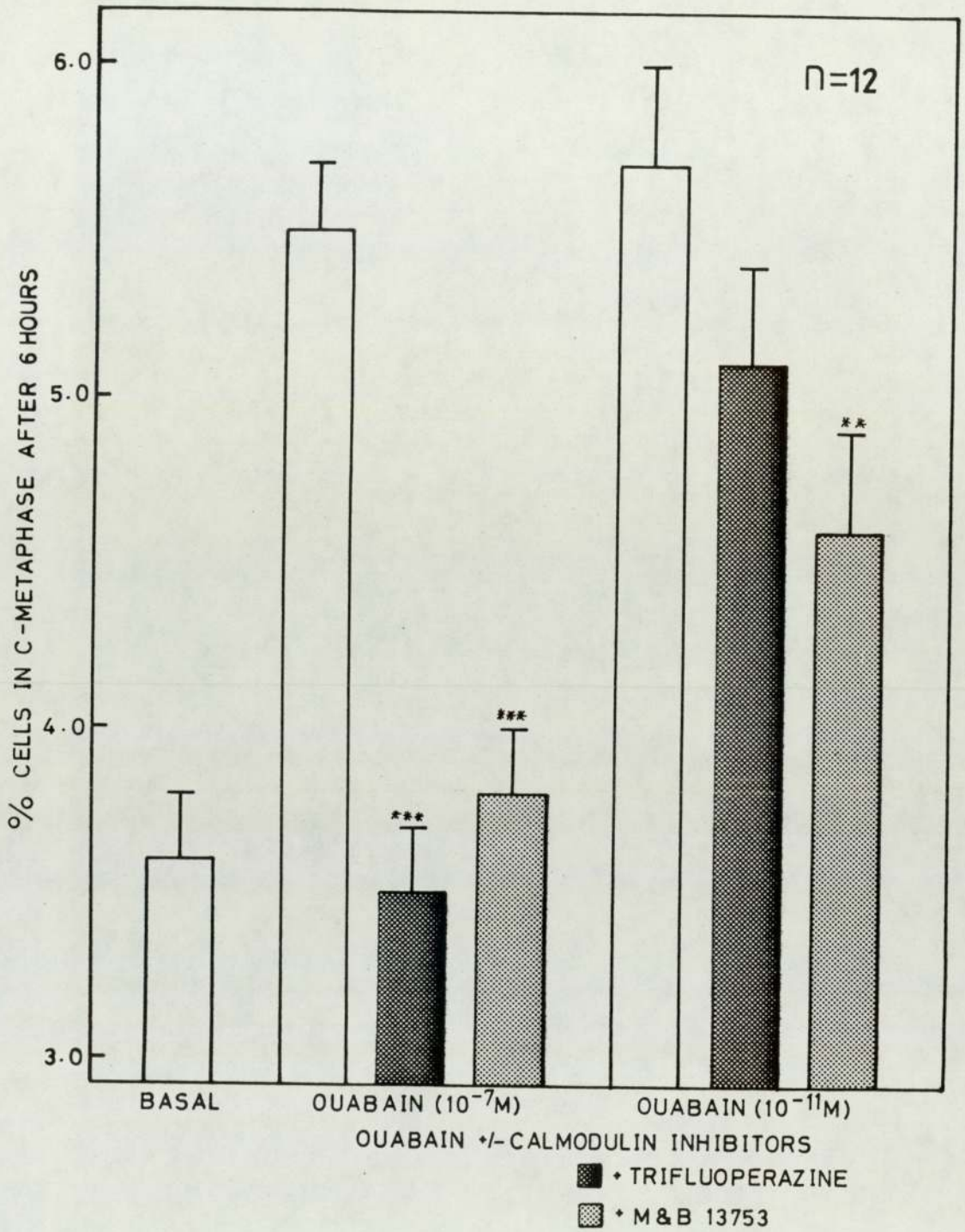


Figure 52 Effect of Calmodulin Inhibition on Ouabain-Induced Mitosis in Rat Thymic Lymphocytes.

Calmodulin inhibitors employed were TFP at 10^{-6}M and M & B 13753 at 10^{-5}M. Values significantly different from those of corresponding cultures incubated in the absence of the calmodulin inhibitors are indicated by asterisks(s).

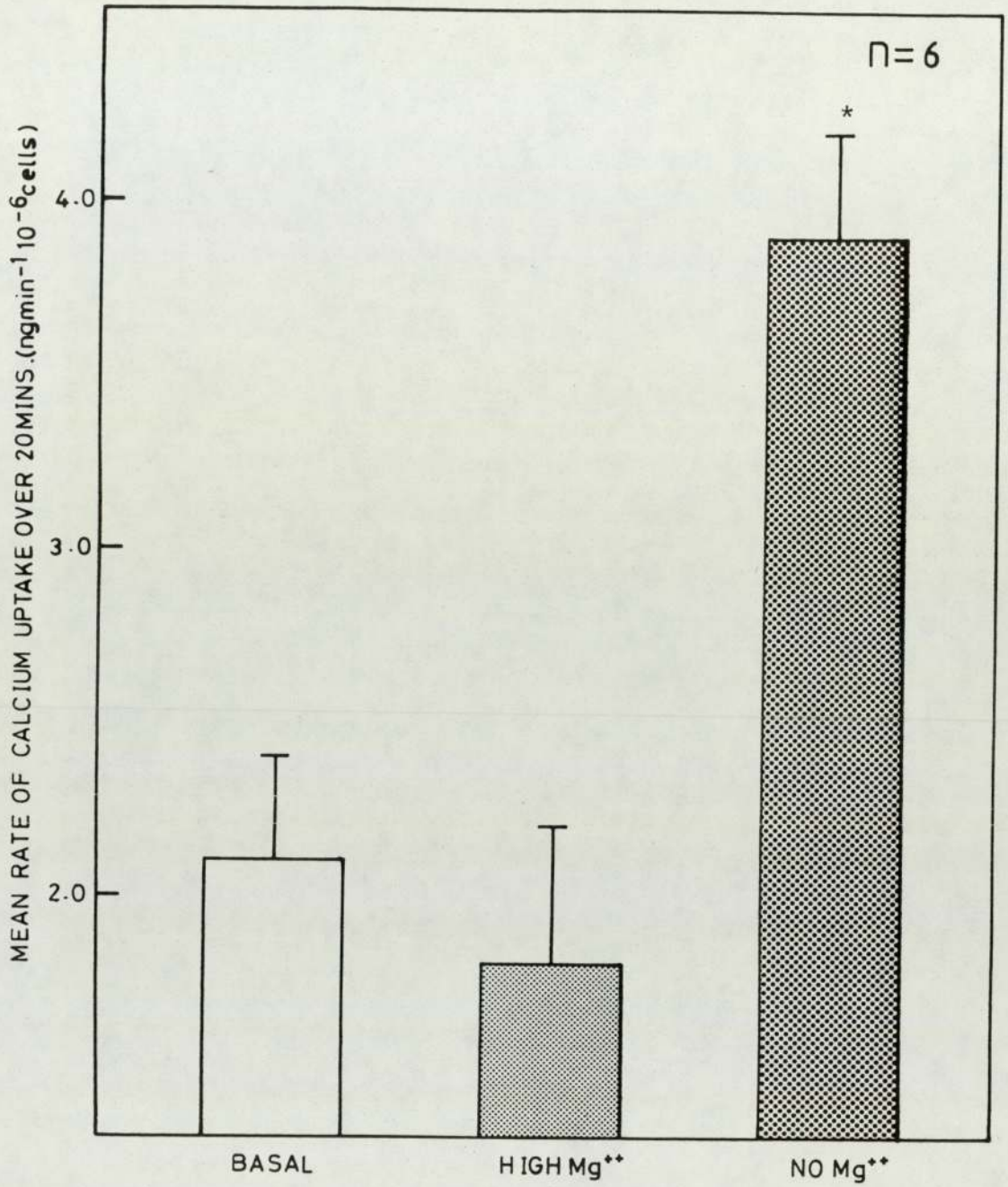


Figure 53 Effect of Modulation of Extracellular Magnesium Ion Concentration on the Calcium Uptake Process in Rat Thymocytes.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}. For high Mg^{++} levels, magnesium concentration was raised to 2.5 mM, for no Mg^{++}, magnesium was omitted from the culture media. Values significantly different from those of basal cultures are indicated by asterisk(s).

Table 17 The Effect of Magnesium or Intracellular Calcium Concentration of Thymic Lymphocytes

Medium Cation Concentration (mM)		Change in Intracellular Calcium Concentration above Basal ($\triangle$I)
Calcium	Magnesium	
0.6	2.5	0.19
0	2.5	0.31
1.8	1.0	0.27
1.8	0	0.40
0.6	0	0.01

Paradoxically perhaps, as shown in table 17, the removal of external calcium ions potentiated the high magnesium induced elevation in $[Ca^{++}]i$. This however, is probably a direct result of increased membrane permeability and subsequent Mg^{++}-mediated displacement of Ca^{++} from internal binding sites. It is unlikely therefore to have a specific role in stimulus-mitosis coupling. In a similar manner to above, the removal of external magnesium ions resulted in the potentiation of the response to high Ca^{++} (table 17). This further increase in cytosolic Ca^{++} levels may be due to diminished Ca^{++} efflux since Mg^{++} is required for maximum activity of the calcium plasma membrane pump (Schatzman & Burgin, 1978). In the light of these findings thus, it is not surprising that the high Ca^{++} concentration retains its mitogenicity in the absence of the other divalent cation (magnesium). In basal calcium (0.6 mM) the acute reduction in extracellular magnesium concentration was also associated with an enhanced rate of calcium uptake (figure 53). This however, did not lead to an elevated intracellular calcium concentration

(table 16) or enhanced mitotic activity (results not shown). The calcium homeostatic system certainly appears to be able to rapidly buffer the transient calcium increase observed in these conditions, consistent with its non-mitogenicity. It is clear therefore that Mg^{++} can influence the calcium regulatory mechanism but whether or not this plays a role in mediation of its mitogenic action is as yet unknown.

In a further attempt to focus more precisely on the role of magnesium ions in mitotic activation the magnesium dependent mitogen, isoprenaline was selected for further examination. This beta-adrenergic compound was mitogenic at 10^{-6} M (figure 54). At this concentration, and at non-mitogenic concentrations, isoprenaline had no effect whatsoever on calcium influx (figure 55) or intracellular mobilization, $\triangle I = 0$ (see Appendix 1 for trace). Its mitogenic potency was not compromised by calcium channel blockade (figure 56) and similarly calmodulin inhibition by trifluoperazine was without effect (figure 57). This suggests that whatever magnesium dependent events are set in motion after isoprenaline combines with its specific receptor on the plasma membrane, and which ultimately lead to mitosis, calmodulin activation is not amongst them. Such a conclusion is not necessarily contradicted by the observation that the calmodulin inhibitor M & B 13753 appeared to inhibit isoprenaline-induced mitosis ($P < 0.001$) as shown in figure 57. The compound is a phenoxypropanolamine and may well be a beta-antagonist and thus, like propanalol, is likely to inhibit the action of isoprenaline.

Because cyclic nucleotides have frequently been implicated as mediators of the mitogenic action of various growth factors in thymus and other tissues (section 2.3.3b and 2.4), their effect on various aspects of divalent cation metabolism were examined. In

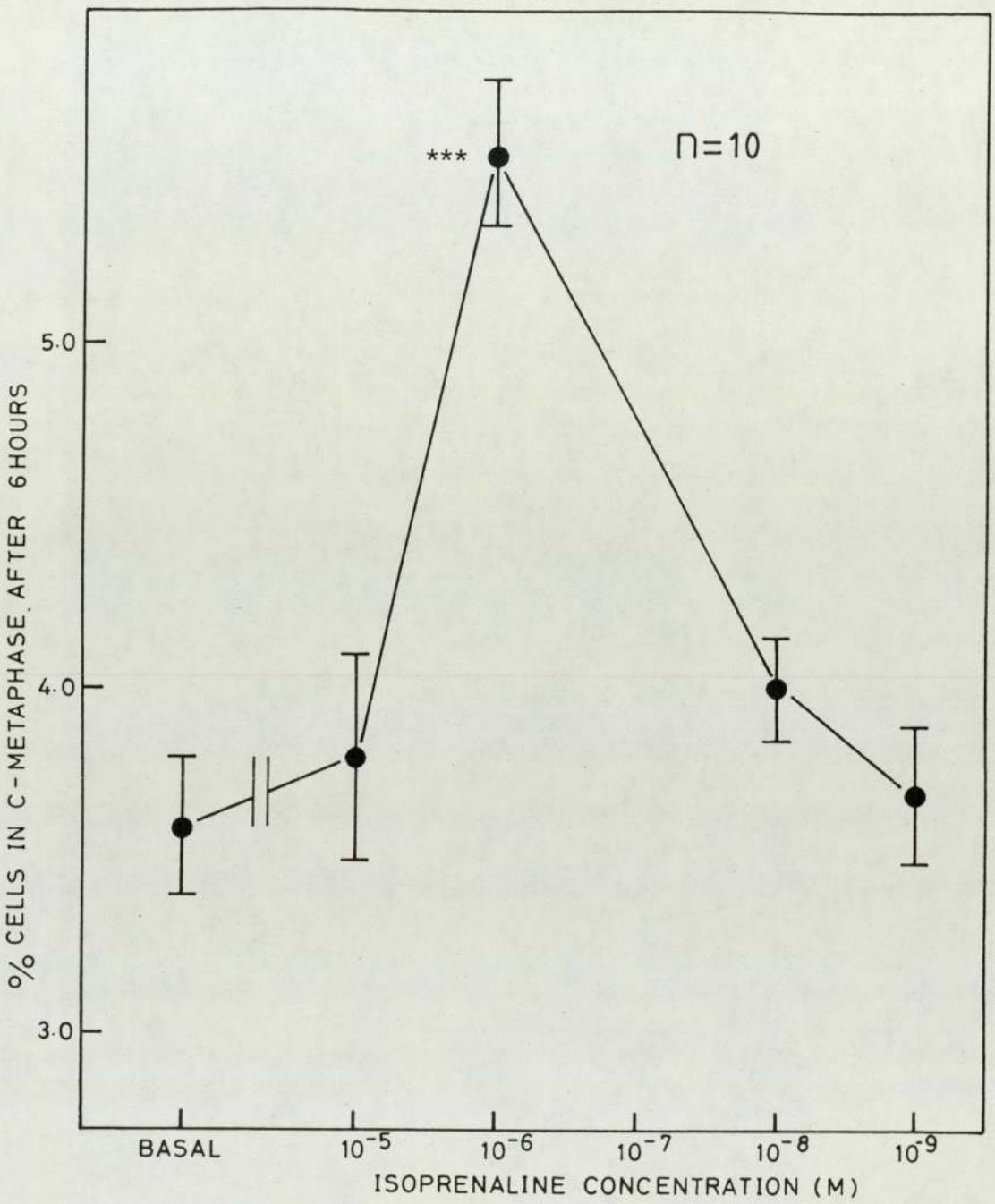


Figure 54 Effect of Isoprenaline Concentration on Mitotic Activity of Rat Thymic Lymphocytes in Basal Media.

Basal medium contains 0.6 mM Ca^{++}, 1.0 mM Mg^{++}. Values significantly different from those of cultures incubated in the absence of any isoprenaline concentration (basal controls) are indicated by asterisk(s).

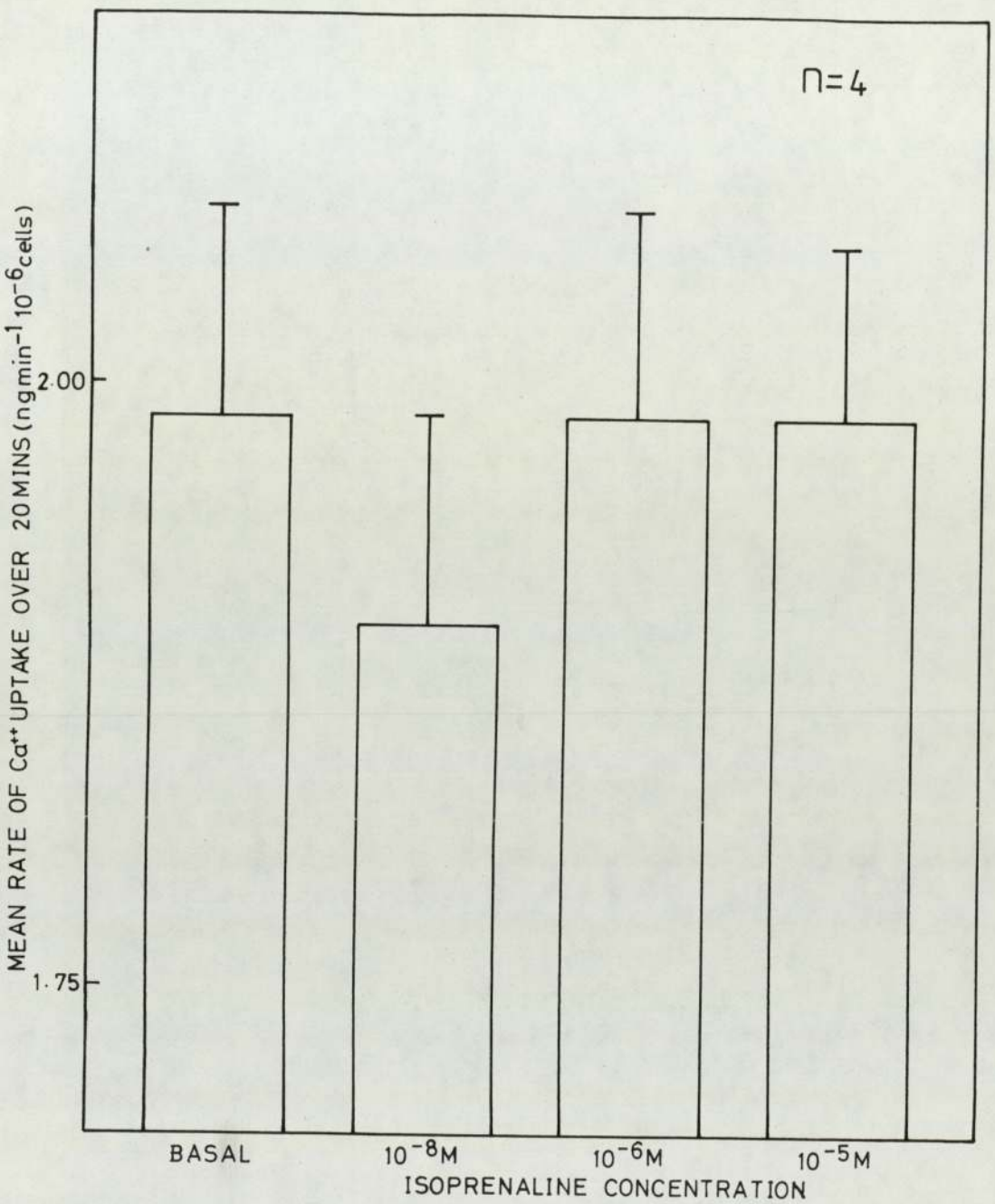


Figure 55 Effect of Isoprenaline Concentration on Calcium Uptake from Basal Media Into Thymic Lymphocytes.

Values do not differ significantly from those of basal calcium uptake.

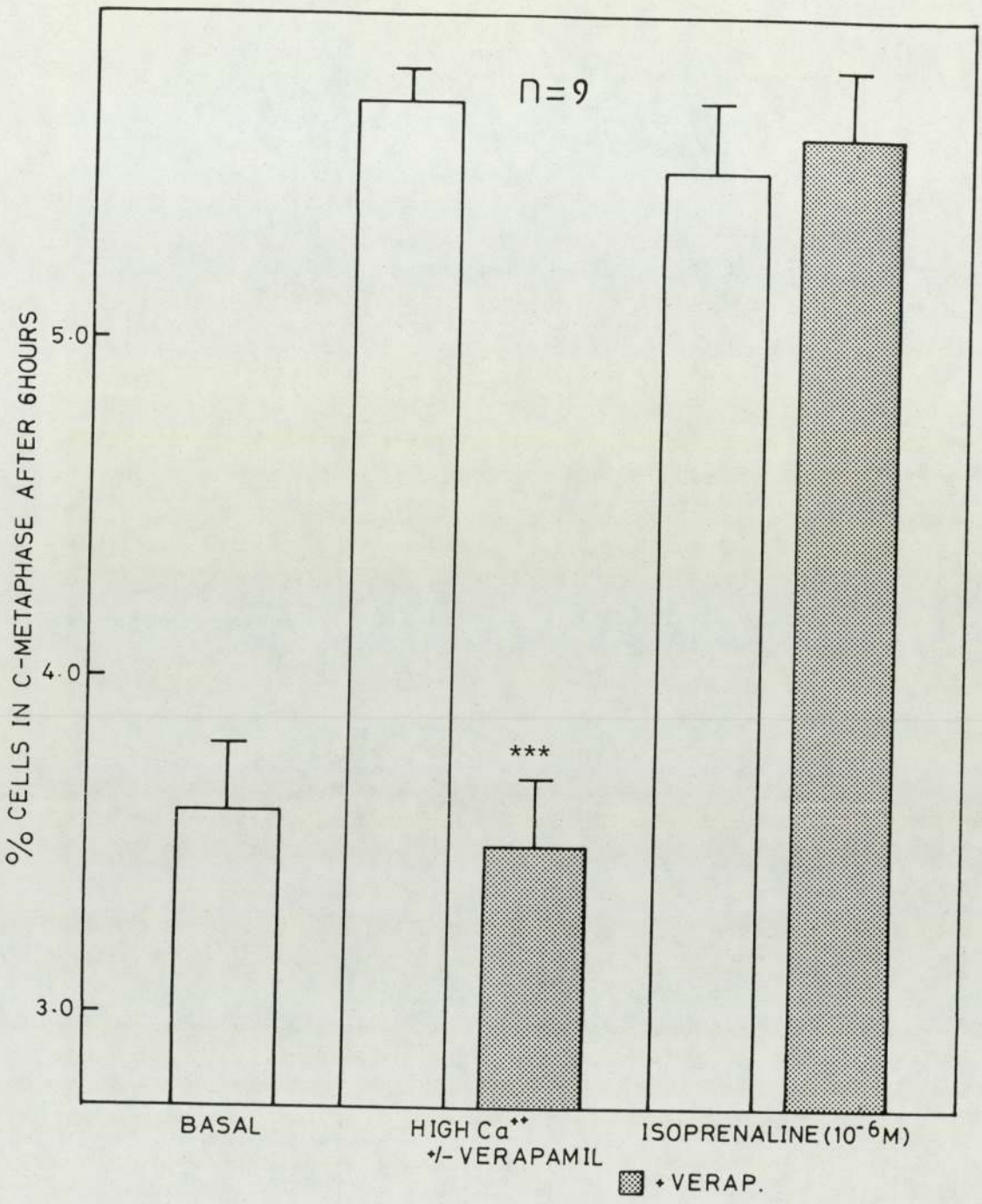


Figure 56 Effect of the Calcium Channel Blocker, Verapamil on High Ca^{++} - and Isoprenaline-Induced Mitogenic Stimulation.

High Ca^{++} is where extracellular calcium concentration is raised to 1.8 mM. Verapamil was employed at a final concentration of 5×10^{-6} gml^{-1}. Values significantly different from those of corresponding cultures incubated free of the calcium antagonist are indicated by asterisk(s).

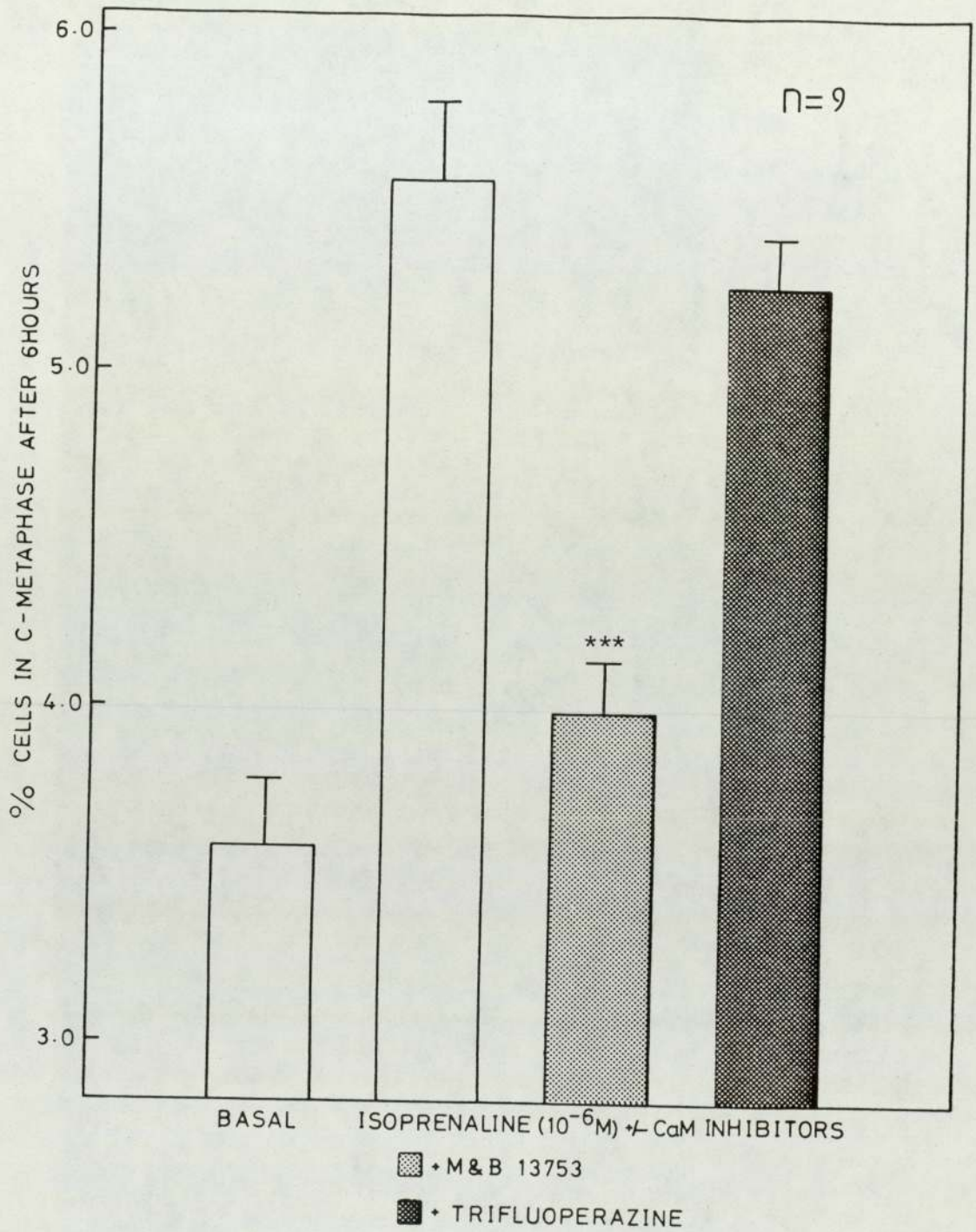


Figure 57 Effect of Calmodulin Inhibitors TFP and M & B 13753 on Isoprenaline-Induced Mitosis.

The calmodulin inhibitors TFP and M & B 13753 were employed at concentrations 10^{-6}M and 10^{-5}M respectively. Values significantly different from those of cultures incubated with isoprenaline in the absence of either calmodulin inhibitor are indicated by asterisk(s).

accordance with previous findings (Morgan, Hall and Perris, 1977; see also table (ii) of section 2.3), cAMP and cGMP exhibited biphasic actions. At high concentrations (10^{-7} M and 10^{-6}M) the cyclic nucleotides were magnesium dependent whereas low concentrations of cAMP and cGMP (10^{-12}M and 10^{-11}M) showed an absolute requirement for extracellular calcium ions (figures 58 and 59). These lower concentrations seemed to increase intracellular calcium as indicated by Quin 2 fluorescence (table 18).

Table 18 Effects of Low Mitogenic Concentrations of Cyclic Nucleotides (type 2 mitogens) on Intracellular Ca^{++} Concentration and the Influence of the Divalent Cationic Environment.

Cyclic Nucleotide (Concentration)	Medium Cation Concentration (mM) Calcium	Magnesium	Change in Intracellular Ca^{++} levels above basal ($\triangle$I) 20 mins	22 mins	27 mins
cAMP (10^{-12}M)	0.6	1.0	0.30		
cGMP (10^{-11}M)	0.6	1.0	0.28		
cAMP (10^{-12}M)	0	1.0	0	+0.19	+0.29
cGMP (10^{-11}M)	0	1.0	0	+0.17	+0.27

+ denotes I after external Ca^{++} replacement which occurred at time 21 minutes.

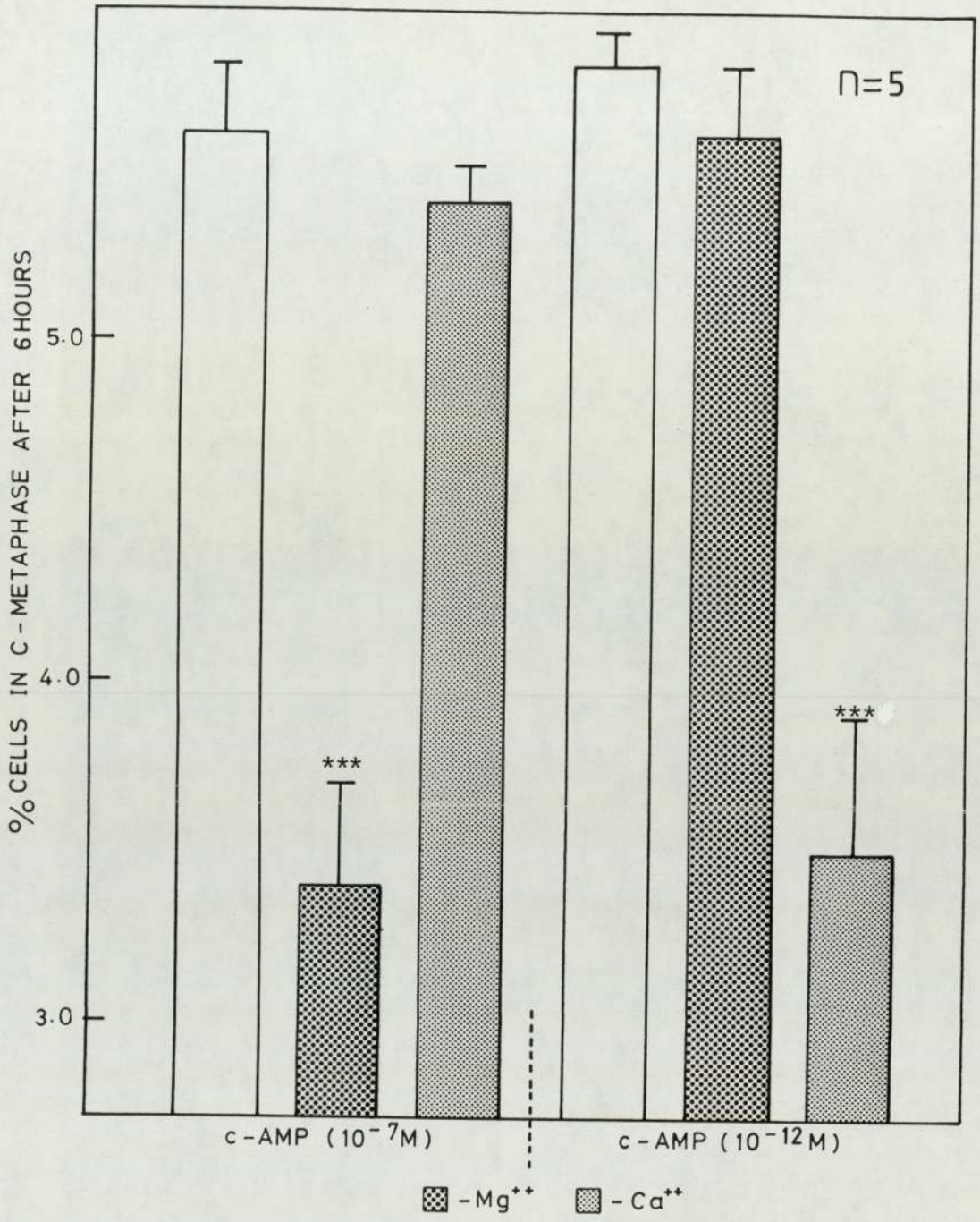


Figure 58 Ionic Dependencies of Mitogenic Concentrations of Cyclic AMP.

Values significantly different from those of corresponding cultures incubated in basal media (0.6 mM Ca^{++}, 1.0 mM Mg^{++}) are indicated by asterisk(s).

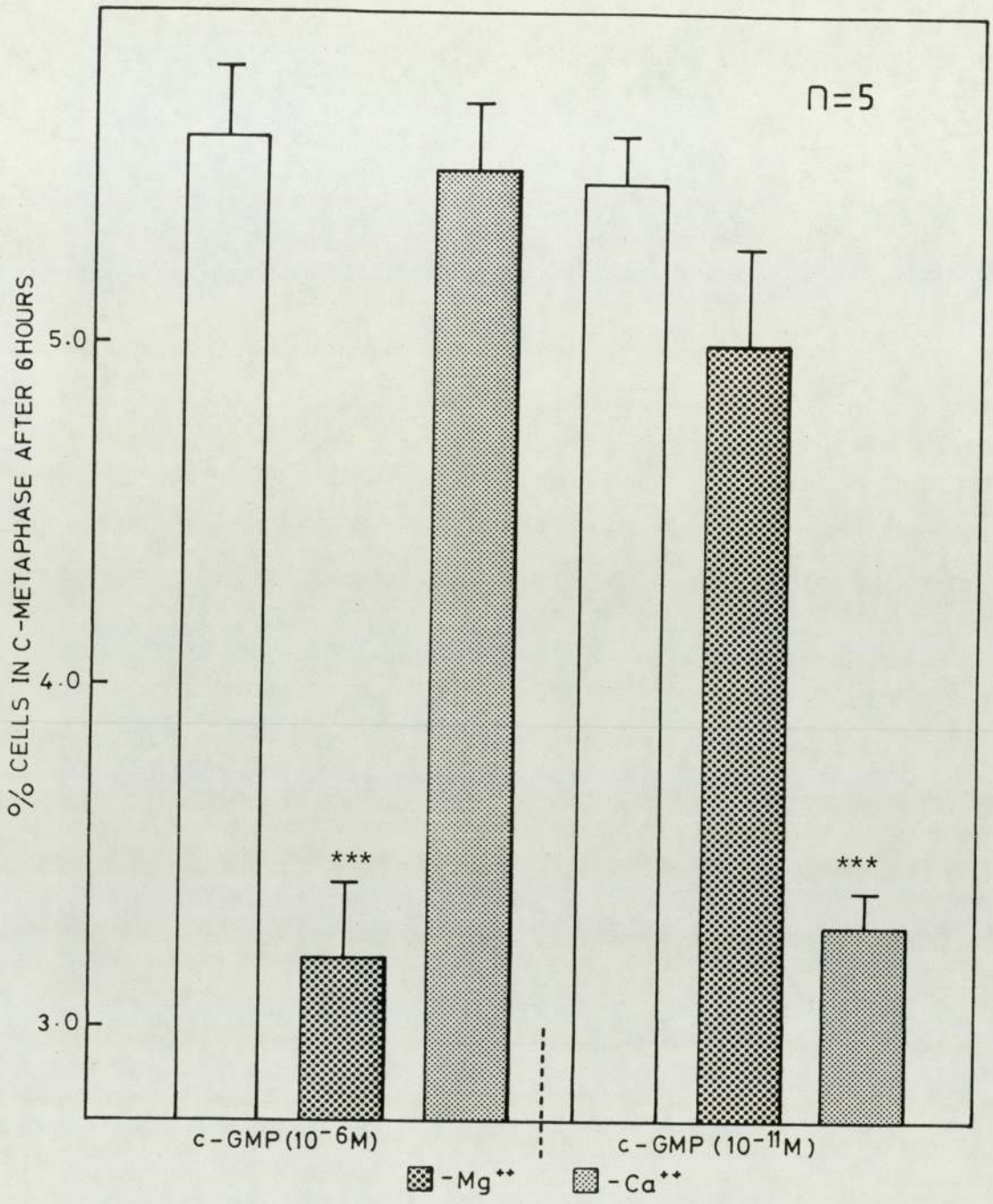


Figure 59 Ionic Dependencies of Mitogenic Concentrations of Cyclic AMP

Values significantly different from those of corresponding cultures incubated in basal media (0.6 mM Ca^{++}, 1.0 mM Mg^{++}) are indicated by asterisk(s).

Perhaps not surprisingly the calcium channel blocker verapamil, prevented the mitogenic action of the low concentrations of cAMP and cGMP which required extracellular calcium to be effective (figures 60 and 61). Indeed, only when extracellular Ca^{++} was available did the low concentrations of cyclic nucleotide raise the intracellular Ca^{++} content (table 18). Paradoxically however, the cyclic nucleotides did not promote Ca^{++} influx at these or any other concentration tested (figure 62). Nevertheless, the elevation of intracellular calcium concentration, however caused, appears to be critical for the activation process of cAMP and cGMP (at $10^{-12}M$ and $10^{-11}M$ respectively), since calmodulin inhibition (achieved by TFP or M & B 13753) prevented the mitogenic response to these nucleotides (figure 63 and 64).

Even though at high concentrations ($10^{-7}M$), cAMP induced a very slow rise in intracellular calcium content (table 19) this is probably not a vital feature of mitogen potency of high concentrations of cAMP or cGMP.

Table 19 Effect of High Mitogenic Concentrations of Cyclic Nucleotides (type 1 mitogens) on Intracellular Ca^{++} Levels

Cyclic Nucleotide (Concentration)	Medium Cation Concentration (mM)		Change in Intracellular Ca^{++} Concentration above basal. ($\triangle I$)				
	Calcium	Magnesium	at minutes				
			20	25	30	35	40
cGMP ($10^{-6}M$)	0.6	1.0	0	-	-	-	-
CAMP ($10^{-7}M$)	0.6	1.0	0.03	0.04	0.05	0.14	0.15

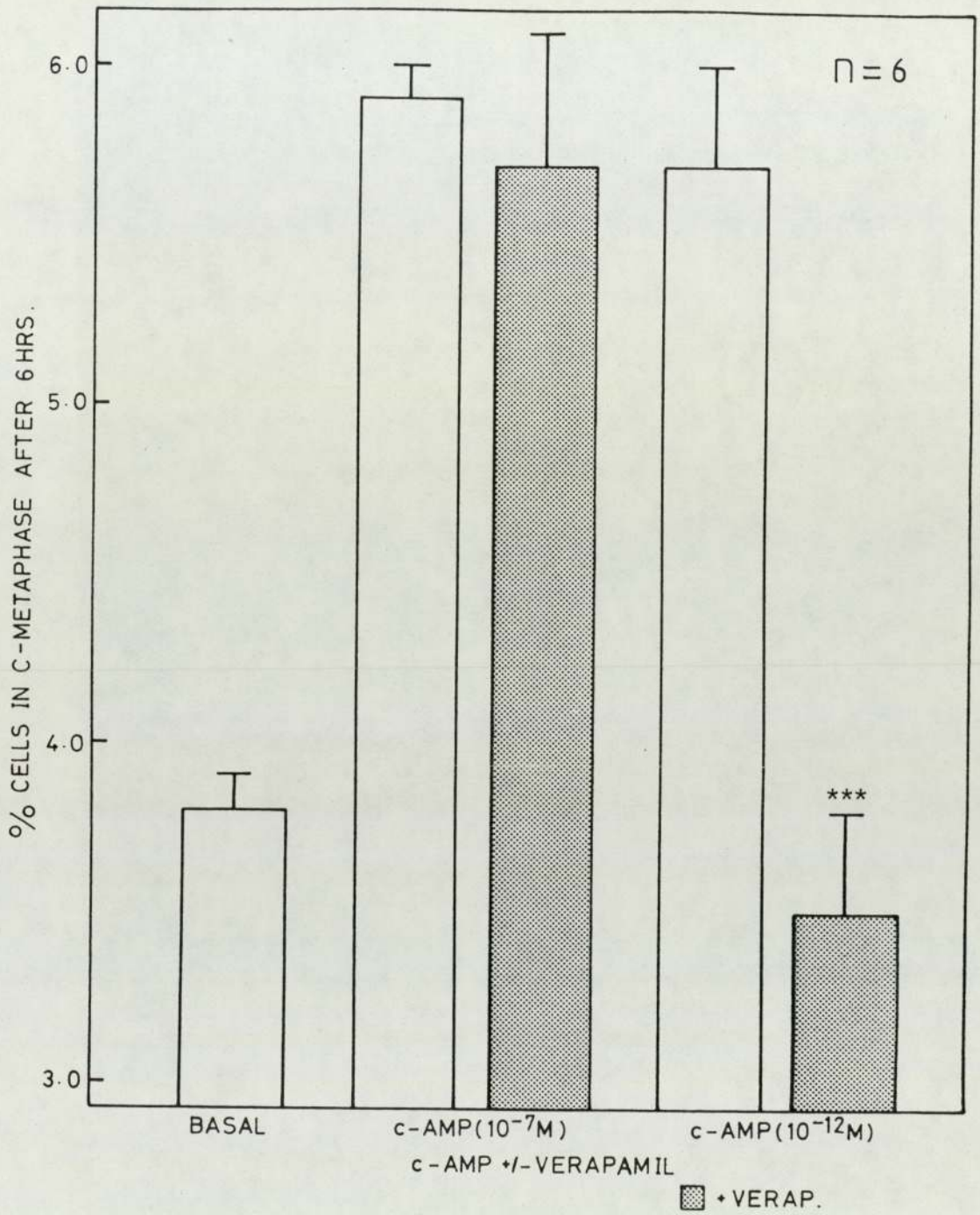


Figure 60 Effect of Calcium Channel Blockade on Cyclic AMP-Induced Mitogenesis in Basal Media.

Verapamil (verap.) was employed at a final concentration of 5×10^{-6} gml^{-1}. Values significantly different from those of corresponding cultures incubated in the absence of the calcium antagonist are indicated by asterisk(s).

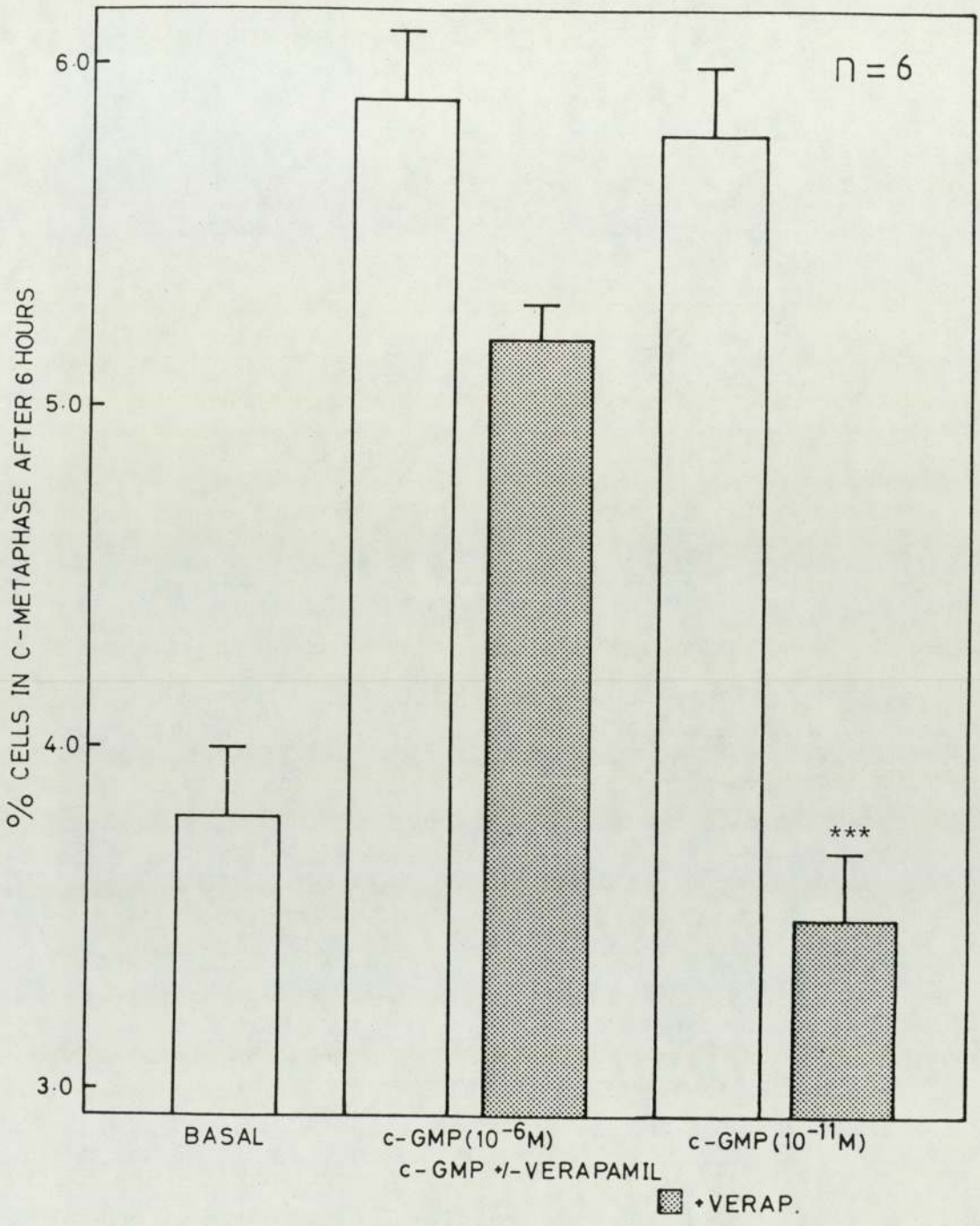


Figure 61 Effect of Calcium Channel Blockade on Cyclic-GMP Induced Mitogenesis in Basal Media.

Verapamil (verap.) was employed at a final concentration of 5×10^{-6} gml^{-1}. Values significantly different from those of corresponding cultures incubated free of the calcium antagonist are indicated by asterisk(s).

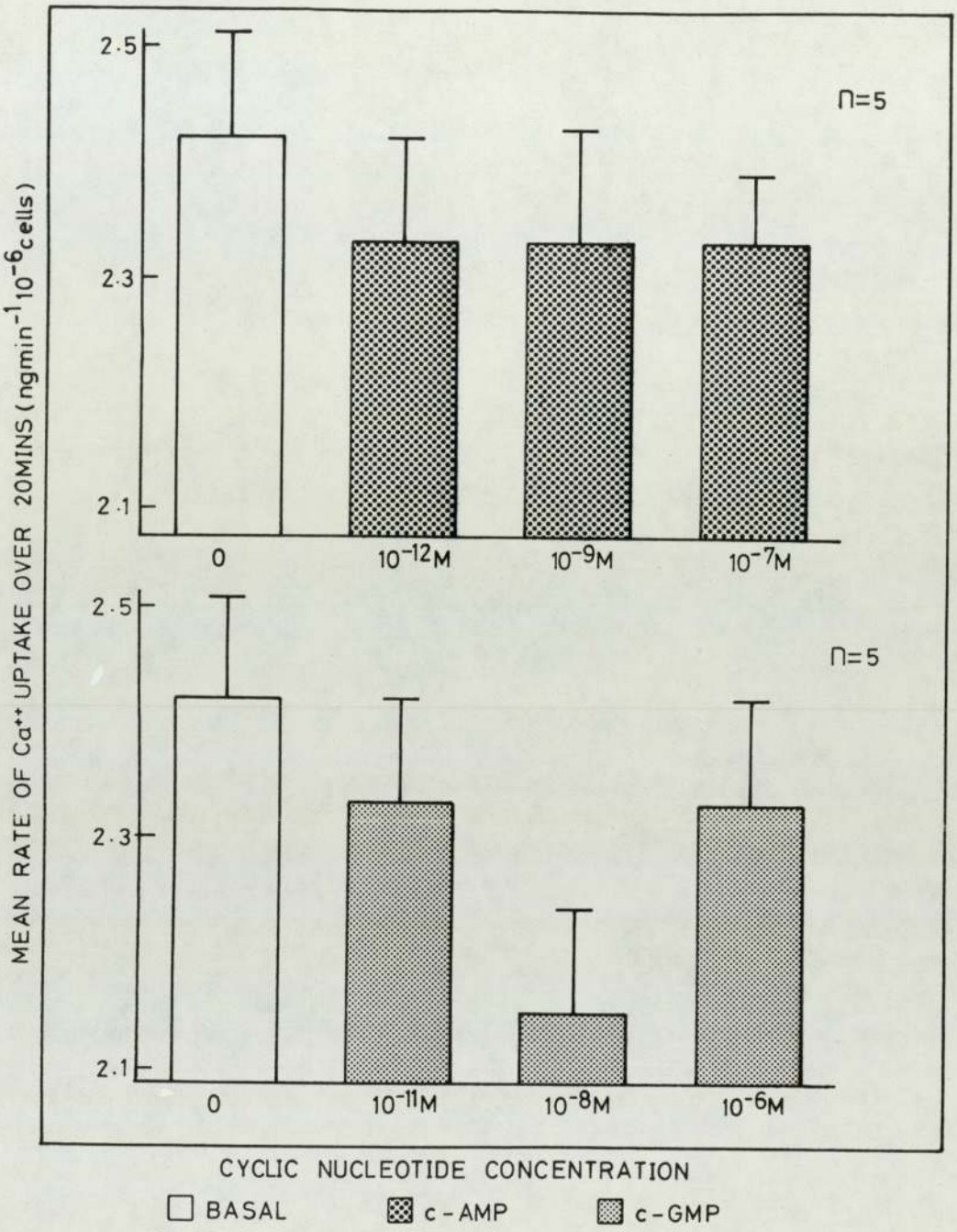


Figure 62 Effect of Cyclic Nucleotide Concentration on the Rate of Calcium Uptake into Thymocytes Cultured In Basal Media.

Basal represents 0.6 mM Ca^{++}, 1.0 mM Mg^{++}, incubated in the absence of any cyclic nucleotide concentration. Values did not differ significantly from those representing basal rates of calcium uptake.

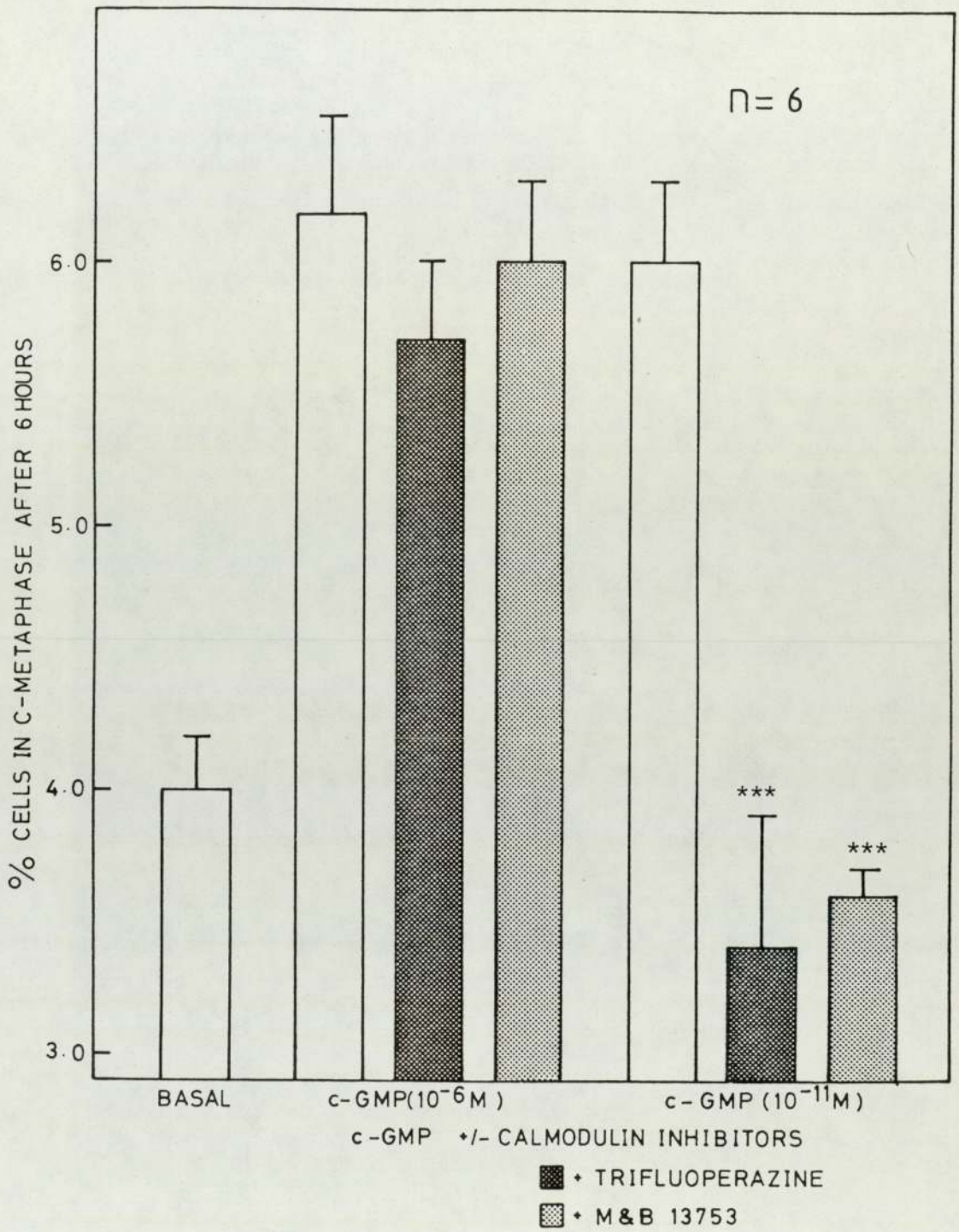


Figure 63 Effect of Calmodulin Inhibition on cGMP-Induced Mitogenesis in Basal Media.

Calmodulin inhibitors (TFP and M & B 13753) were employed at concentrations of 10^{-6}M and 10^{-5}M respectively. Values significantly different from those of corresponding cultures incubated free of these inhibitors are indicated by asterisk(s).

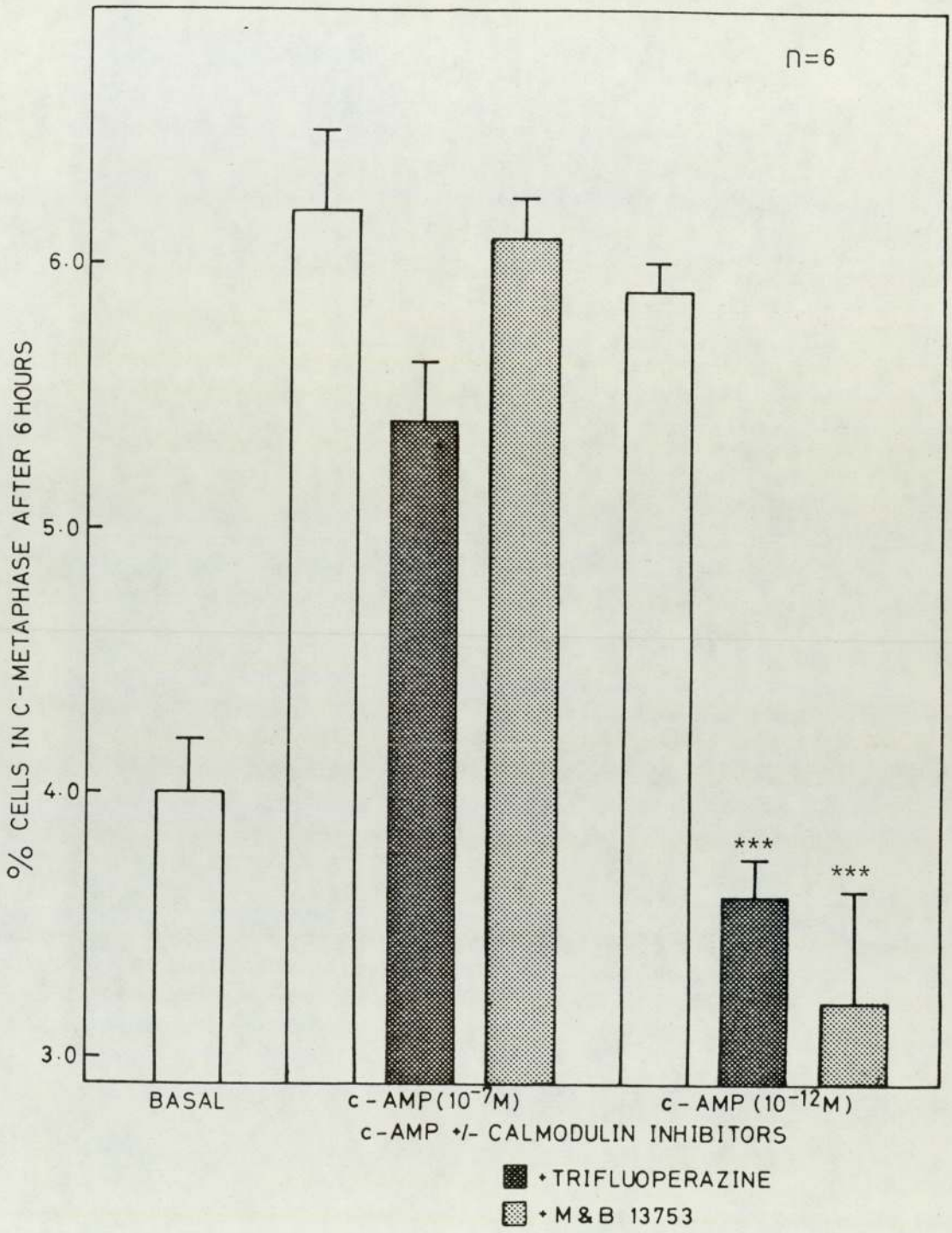


Figure 64 Effect of Calmodulin Inhibitors on Cyclic AMP-Induced Mitogenesis in Basal Media.

Trifluoperazine and M & B 13753 were employed at concentrations of 10^{-6}M and 10^{-5}M respectively. Values significantly different from those of corresponding cultures incubated in the absence of the calmodulin inhibitors are indicated by asterisk(s).

Consistent with their Mg^{++} dependence, verapamil did not affect the mitogenic propensity of either cyclic nucleotide at these levels (figures 60 and 61) and their failure to influence basal calcium uptake was also fully anticipated (figure 62). Similarly, the Mg^{++}-dependent concentrations were shown to initiate DNA synthesis by a process in which Ca^{++} or Ca^{++}-calmodulin played no part, since TFP and M & B 13753 were without effect (figures 63 and 64).

In view of the evidence presented here it is likely that there is a range of intracellular calcium concentrations which determine the mitotic status of the cell. For type 2 mitogens at least, it is possible that in a particular cell a certain low (or small range of) $[Ca^{++}]i$ corresponds to basal mitotic activity. If this level is rapidly raised above a specific threshold then enhanced cell division ensues. However, further elevation of cytosolic content beyond an upper limit results in inhibition of the stimulated mitogenic response. Whether or not type 1 mitogens exert their effect by similar modulation of intracellular magnesium concentration is still very unclear but these substances certainly appear to stimulate mitosis via a different (if not totally independent) mode of action. It is also unknown if the mitogens exert their effect on the divalent cation regulatory system(s) directly or via the intermediary action of the cyclic nucleotides.

The concept of stimulus-mitosis coupling by modulation of the internal divalent cation concentration and interplay between other putative messengers during the mechanism is discussed in the following section in the light of these experimental findings.

5.0 DISCUSSION

Although the thymus contains a high proportion (75%) of terminal proliferatively inactivated cells the entire lymphoid compartment is continuously renewed due to the high mitotic activity of large and medium lymphoblasts (Metcalf and Wiadrowski, 1966). Such proliferative events within the thymus were originally considered to be autonomous (Miller & Osoba, 1967). However, thymocyte turnover *in vivo* is now known to be influenced by intrathymic self regulatory processes. Several products released by the thymic epithelium are able to maintain lymphocyte development. A crude fraction isolated from the calf thymus gland termed thymosin fraction V contains a whole set of molecules and has proved biologically active in various *in vitro* assay systems e.g. the mixed lymphocyte reaction assay (Cohen, Hooper & Goldstein, 1975). Thymosin fraction V, also stimulates thymocyte proliferation and differentiation *in vivo* (Wara & Ammann 1975; Thurman & Goldstein, 1975). A further polypeptide factor isolated from the thymus is thymopoietin which induces the differentiation of prothymocytes into thymocytes and promotes several T cell functions (Goldstein, 1975). More recently another growth factor has been identified and partially purified. This thymocyte growth factor which is distinct from thymosin and thymopoietin acts directly on the immunologically immature proliferating thymocyte of the thymic cortex (Soder & Ernstrom, 1983). Inhibitory factors similarly may influence the development of the thymus. A thymus crude factor, TCF (which has yet to be completely purified and characterised but may contain a series of factors) has been labelled

as the most probable endogenous chalone of lymphoid cell proliferation (Rijke, Lempers & Ballieux, 1981). In addition to these substances the thymus and T-lymphocytes themselves produce and release a variety of stimulatory and inhibitory factors during the immune response. Collectively known as lymphokines these substances (which include the interleukins (Duff & Durum, 1983; Pauly et al., 1983)) influence the immune reaction rather than thymus development and are therefore not considered here.

Clearly throughout life the thymus gland is also sensitive to extrathymic factors which include many classical endocrine hormones. Initial development and maintenance of the tissue is dependent upon corticotropin (ACTH) and growth hormone (GH). Hypophysectomy or administration of antiserum raised to GH both induce thymic atrophy in young animals (Pierpaoli and Sorkin, 1972). In addition, GH significantly enhances the mitotic activity in rat thymocyte populations maintained in vitro (Whitfield, Perris & Youdale, 1969). A further pituitary-thymus axis is indicated by the observation that thyroid stimulating hormone (TSH) can initiate the restoration of thymic growth in congenitally hypopituitary mice (Pierpaoli, Baroni, Fabris & Sorkin, 1969). Thyroxine (TH) itself and GH can similarly restore the mitotic status of thymic lymphocytes in these mice (Pierpaoli et al., 1969). Parathyroid hormone also influences intrathymic cell production. Surgical removal of the parathyroid glands induces hypocalcaemia and also hypoplasia of the thymus (Perris, Weiss & Whitfield, 1970; Perris, 1971). This hormone also promotes mitotic activity in cultured thymocytes (Whitfield, Perris & Youdale, 1969). In contrast to these positively acting hormones, calcitonin and steroid hormones appear to exert an inhibitory effect on thymocyte

proliferation (Whitfield, MacManus & Gillan, 1971; Dougherty, Berliner, Scheebeli & Berliner, 1964). Sex steroid administration clearly produces thymic atrophy whilst their removal by castration increases the size of the gland (Scheiff & Haumont, 1979). Similarly, adrenalectomy may promote thymic mitotic activity. This may be the result of the removal of lymphotoxic corticosteroids (Kinoshita, Kimura & Fukamizu, 1974) or the enhancement of the circulating levels of ACTH. However, in marked contrast to its lethal action on thymocytes at high concentrations, cortisol at low more physiological levels actually stimulates thymocyte proliferation (Whitfield, MacManus & Rixon, 1970). Thus, it is clear that the mitotic activity of the lymphoid population of the thymus gland in vivo is regulated by both endogenous and exogenous 'hormones'. Studies employing short term cultures of isolated thymic lymphocytes obviously do not reproduce the precise intrathymic environment and therefore may not reflect the whole co-ordinated response occurring in vivo. Nevertheless, the intracellular regulatory mechanisms should remain essentially intact providing a powerful tool for the investigation of processes involved in mitotic recruitment. The physiological relevence of in vitro studies is further supported by the observation that mitogenic stimuli (which include many hormones) show comparable abilities to initiate a proliferogenic response in the native tissue and in cultured cell suspensions (Morgan, Hall & Perris, 1975).

Cellular functions as diverse as nerve excitability muscular contraction and glandular secretion are influenced by transient elevations in the free intracellular calcium concentration (Putney, 1977; Williams, 1980a,b; Blinks, et al., 1976). In view of the observations that hormones such as PTH and calcitonin which have

physiological roles in calcium homeostasis also influence the development of the thymus gland (MacManus, Youdale, Whitfield & Franks, 1972) it is not unreasonable to assume an additional role for this divalent cation in the process of proliferation. Certainly calcium has been implicated in the arousal from quiescence in a variety of cell types (see section 2.3.3a). Significantly under a variety of physiological and artificial conditions, hypercalcaemia is invariably accompanied by elevated numbers of dividing cells within the thymus gland (Perris, et al., 1967; Perris et al., 1968; Perris, 1971; Edwards et al., 1981). This effect can also be mimicked in culture by raising the extracellular Ca^{++} concentration (Perris et al., 1967). Such calcium-induced mitosis may represent an important aspect of the phenomenon of stimulus-mitosis coupling. In a similar manner raised extracellular Mg^{++} concentrations in vivo and in vitro also stimulate the mitotic recruitment of thymic lymphocytes (Perris, 1971; Perris et al., 1967). Although hyper magnesaemia (unlike hypercalcaemia) is not an accompaniment of mitotic recruitment in vivo under physiological circumstances valuable information may be gained by studying magnesium-dependent recruitment mechanism(s). Previous investigations have shown that mitogens for thymic lymphocytes are clearly divisible into two distinct categories based upon their cationic dependencies. Type 1 mitogens have an absolute requirement for extracellular magnesium ions. Type 2 mitogens on the other hand proved to be calcium-dependent. In support of two discrete mechanisms of action the mitogenic propensity of type 1 mitogens (which include elevated extracellular levels of magnesium ions) is blocked by testosterone whereas the actions of type 2 mitogens (including raised extracellular calcium concentrations) are compromised by oestradiol.

The present study has served to investigate further the modulatory effects of the divalent cationic environment upon mechanisms involved in the recruitment of cells from "GO" into the cycle and which lead ultimately to mitotic division.

In accordance with other studies a sudden elevation in the concentration of calcium or magnesium ions in the culture medium induced a normally quiescent population to enter the cell cycle (figure 1). This activation process in thymic lymphocytes was completed in less than 20 minutes since removal of the triggering stimuli after such a time did not influence the proliferogenic response (figure 4). The rapidity with which these cells became committed to DNA synthesis and mitosis is not necessarily inconsistent with the view that the mitogenic signal is required at 2 temporally distinct points, one for recruitment from "GO" and the other required for cells to surmount the G1/S boundary, a control point through which all normal cells are thought to obligatorily pass (Rixon & Whitfield, 1982; MacManus et al., 1972; Anathakrishnan et al., 1981). Rather, it supports the contention that the "GO" compartment of the thymic cells recruited in these studies is situated at or very close to the G1/S boundary in such a way that a single mitogenic trigger serves to simultaneously (or at least very rapidly) provoke "GO" exit and passage through the G1/S transition point (Whitfield et al., 1979; Edwards et al., 1981).

Calcium-induced mitosis as expected was inhibited by the administration of the female sex steroid oestradiol (at 0.1 $\mu g ml^{-1}$) whereas the response to magnesium ions was abolished by testosterone at the same concentration (figure 1). The inhibitory capacity of each of the sex steroids (oestradiol and testosterone) was specific

for the calcium or magnesium-dependent processes respectively. The two mechanisms of recruitment thus appear to be independent. However, they must at some stage interact with a common series of events leading ultimately to activation of the mitotic process. Although it is clear that the steroids must exert their inhibitory effect prior to this common pathway their precise mechanism or point of action remains unknown.

Extracellular calcium ions only promote cell division in the rat thymocyte or chick embryo fibroblasts if they are available in the freely diffusable non chelated form (Atkinson, personal communication, 1981; Moscatelli, Sanui & Rubin, 1979). These free calcium ions must also have access to the intracellular environment since verapamil which presumably blocks the calcium channels of the plasma membrane (Fleckenstein, 1977; Janis, 1981) prevented the mitotic response induced by high Ca^{++} in a dose dependent manner (figure 2). This suggests that it is the free calcium ions entering the cell which serve to promote mitosis. Verapamil did not inhibit basal mitotic activity or the mitogenic capacity of raised extracellular magnesium concentrations (figure 3). This suggests that a) magnesium does not stimulate proliferation via enhanced Ca^{++} influx and b) if magnesium enters the cell down its concentration gradient as a prelude to its stimulatory action it is not doing so via the calcium channels in the plasma membrane. In an attempt to verify that verapamil is indeed inhibiting Ca^{++}-induced mitogenesis by blocking the entry of the ion into the cell calcium-45 tracer studies were initiated. Although many aspects of cellular calcium metabolism remain concealed during such studies several conclusions may be drawn from the results achieved. Following a three-fold increase in the external calcium concentration

(corresponding to the mitogenic dose) the membrane associated calcium pool expanded (figure 5). The increase was probably the result of displacement (by calcium) of sodium ions bound to the negative phospholipid residues present in the plasma membrane (Hauser, Finer & Darke, 1977). The precise function (if any) of the membrane binding in mitotic activation is unclear. Calcium uptake recorded at 4°C was assumed to correspond to rapid exchange of the labelled material with the cations adsorbed on the cell surface. After 20 minutes in basal or high Ca^{++} conditions the membrane associated calcium represented approximately 71% ($\pm$ 3 for n = 20) of the total calcium taken up by the cell during this time. Administration of mitogenic agents did not influence this value, inferring that membrane bound calcium probably has no specific function in the mitogenic response of thymic lymphocytes. Since verapamil similarly had no effect on Ca^{++} association at 4°C the expanded membrane bound calcium reservoir of the thymocyte incubated in a high Ca^{++} environment may not contribute calcium directly to the cytosol. Indeed, kinetic studies performed in HeLa cells and rat kidney slices suggest that the membrane associated calcium pool is not in direct equilibrium with the cytosol (Uhikawa & Borle, 1978; Borle, 1981). Following the sudden calcium elevation the calcium uptake which represented transmembranal influx into the cytosol increased from the basal rate of 2.0 ng $min^{-1}10^{-6}$ cells (after subtraction of membrane Ca^{++}) to 6.3 ng $min^{-1}10^{-6}$ cells (table 1). Verapamil had no effect on the basal calcium influx but significantly reduced that promoted by high extracellular calcium concentrations (figure 5). The mean rate of Ca^{++} influx (after subtraction of surface calcium) was depressed from 6.3 ng $min^{-1}10^{-6}$ cells to levels approximating basal influx rates (2.2 $min^{-1}10^{-6}$ cells).

These observations support the contention that calcium antagonists (channel blockers) prevent the flow of calcium ions through activated calcium channels but do not significantly influence the resting membrane permeability to these cations (Rosenberger et al., 1979). It is probable that type 2 mitogens (i.e. those which show an absolute requirement for extracellular Ca^{++}) and substances which are able to interfere with Ca^{++}-induced mitogenic responses do so via the modulation of such calcium translocation into the cell. It was predicted therefore that oestradiol (at 0.1 $\mu g ml^{-1}$) exerted its inhibitory action by a topical action at the membrane preventing the influx of calcium provoked by the elevated transmembrane calcium ion gradient.

Certainly, the antimitotic activity of oestradiol is unlikely to be mediated via normal steroid-genome interaction since it can cause inhibition of a mitogenic response within ten minutes (Morgan, 1976). In fact, in the whole animal and in vitro oestradiol had to be present at the time the mitotic stimulus was applied in order to exert its inhibitory effect (Smith et al., 1975). This must favour an immediate cell surface phenomenon. Moreover, the alpha form of the steroid which is relatively rare and physiologically inert and does not bind to cytosolic oestrogen receptors in target tissue (Noteboom & Gorski, 1965) is an equally effective inhibitor of calcium-induced mitosis (Bramhall, Morgan, Britten & Perris, 1976). Oestradiol also inhibits the division of 3T3 fibroblasts which have no cytosolic oestradiol receptors (Breslow, Epstein, Forbes and Fontaine, 1979).

It is conceivable that the many effects of oestrogens on growth and development in several tissues could be partially explained by such a topical action on the plasma membrane rather than or in

addition to a classical gene derepression mechanism. Thus female rats and mice are generally smaller and grow less rapidly than males of the same age (Santisteban, 1960). The marked diurnal fluctuations in mitotic bone marrow and thymus tissues of the male rat are absent in the female rat, but can be restored by ovariectomy (Hunt, 1973). Calcium-induced (by injection of $CaCl_2$) cell proliferation in rat bone marrow and thymus is sensitive to oestradiol when administered to male rats by injection or when present naturally in the female rat circulatory system (Smith et al., 1975; Dawson & Perris, 1972). Oestradiol is also known to inhibit the restoration of normal erythrocyte numbers after haemorrhage and thus to induce anaemia, which may be related to its ability to suppress erythropoietin production (Mirand and Gordon, 1962) or to promote the release of inhibitors of erythroid stem cell development (Pololi-Anagnostou, Schade & Anagnostou, 1981). The inhibitory actions of the steroid are extended to include modulation of the cell mediated immune system and other lymphoid proliferative activity. For example, oestradiol administration depresses skin reactivity in guinea pigs (Kappas, Jones & Roitt, 1963), delays skin graft rejection (Waltman, Burde & Berrios, 1971) and suppresses the response of lymphocytes to polyclonal activators (Ablin, Bruns, Guinan & Bush, 1974) while reduction in circulating steroid levels substantially enhances tissue rejection in female rats (Graff, Lappe, Snell, 1969). Similarly, bovine immune status is attenuated during pregnancy. In fact, sera from such pregnant animals will suppress the mitotic response of lymphocytes from ovariectomized heifers (Manak, 1982). It has been suggested that oestradiol influences the function of the immune system by modulating the production and release of serum factors from the

thymus to which thymocytes are sensitive (Grossman, Sholiton & Roselle, 1982).

Other non-mitotic processes are also susceptible to oestradiol inhibition. For example, oestrogen reduces the mechanical response of uterine smooth muscle. Recent studies have clearly shown that this sex steroid in vitro has an inhibitory effect on calcium entry into the myometrial cell which may explain its mechanism of action (Batra & Bengstrom, 1978).

In an attempt to determine whether a similar effect on calcium transport accounts for the inhibition by oestradiol of the thymocyte mitogenic response, $^{45}Ca^{++}$ uptake was monitored in resting and stimulated cells in the presence of oestradiol. The steroid failed to influence basal ion uptake over the twenty minute incubation period (figure 6). However, in complete contrast to the aforementioned hypothesis, oestradiol did not compromise the calcium-induced influx into mitotically activated cells but surprisingly appeared to potentiate the cationic uptake (figure 6). Thus, it appears that oestradiol may exert its antimitotic action by elevating the cell calcium to inhibitory levels. Certainly, if thymocytes are exposed to external calcium concentrations above 3 mM, the mitotic activity is no longer stimulated above basal levels. Glucocorticoid steroid hormones (in a similar manner to the sex steroid) exert immunosuppressive effects in vivo and inhibit Con A-induced transformation of thymocytes in vitro (Homo, Picard, Durant, Gagne, Simon, Dardenne & Duval, 1980). The glucocorticoid-induced cell lysis underlying these inhibitory effects may also be mediated by elevating cell calcium to cytotoxic levels (Homo & Simon, 1981).

Since intracellular free calcium is subjected to strict homeostatic control namely, cytosolic and microsomal buffering, it is essential to validate the experimental interpretation of $^{45}Ca^{++}$ flux studies by monitoring a rise in the internal active calcium pool. Intracellularly located Quin 2 fluorescence was employed as a direct indication of free calcium concentration. Elevation of the external calcium content as predicted served to raise the free intracellular calcium pool (figure 7 and table 8). Oestradiol at (0.1 $\mu g\ ml^{-1}$) also increased intracellular Ca^{++} concentrations confirming the isotopic calcium influx studies. Thus, oestradiol appears to be able to inhibit mitosis by promoting movement of calcium into the thymocytes serving to elevate the internal content beyond the upper threshold limit required for mitogenic activation.

Because oestradiol at this high concentration was able to promote calcium uptake in a high Ca^{++} environment but not in basal conditions it was postulated that the steroid at some other concentration may enhance basal rates of calcium influx. Indeed, oestradiol at lower more physiological concentrations (1 $pgml^{-1}$ and 10 $pgml^{-1}$) clearly enhanced calcium uptake whereas higher levels failed to influence the basal influx rates (figure 8). The enhanced uptake served to raise the intracellular calcium concentrations (table 3) which suggested therefore that the steroid at these lower concentrations may be mitogenic. Certainly, in complete contrast to its inhibitory actions, described above, oestradiol appears also to have a positive regulatory action on growth and development in some tissues. In addition to its general trophic action in promoting secondary sex gland development in vivo (Conti, Giminez-Conti, Zerbe & Gerschenson, 1981; Kimura, Obata &

Okada, 1978; Sonnenschein & Soto, 1980; Rao, Midgley & Richards, 1978; Kirkland, LaPoint, Justin & Stancel, 1979) oestradiol will stimulate proliferation of non target tissue in vivo and in vitro and of target tissue in vitro. For example, oestrous-cycle-controlled cell proliferation is apparent in the adrenal cortex of female rats (Pappritz, Meazor & Ueberberg, 1977) and the steroid promotes DNA synthesis and mitosis in cultured decidual cells (Peleg & Lindner, 1980), rat hepatocytes (Pietras & Szego, 1979a) and erythroid stem cells in spleen (CFU-E) cells (Anagnostou, Zander, Barone & Fried, 1976). Oestradiol may also induce and maintain tumour growth of human breast cancer cells in vitro (Darbre, Yates, Curtis & King, 1983; Coosen, DeJong & Schwartz, 1982). In contradiction to these latter observations however, some authors have been unable to detect direct oestrogenic effects on cultured tumour lines from mammary pituitary and kidney tissue (Sirbasku, 1978; Leung, et al., 1981) and have thus proposed that oestrogen mediates its stimulatory effect via specific growth factors. Three possible mechanisms of action for these 'oestromedins' on tumour growth have been suggested namely autocrine, paracrine and endocrine methods (Ikeda et al., 1982). Although the mitogenic effect of oestrogen may be related to its ability to provoke tumour appearance it is evident that progression towards neoplasia is a somewhat more complex mechanism which is as yet poorly understood. Interestingly, the positive effect of oestrogen on uterine smooth muscle contraction is thought to be mediated by increased myometrial membrane permeability to calcium ions (Batra & Sjogren, 1983). Similarly the rapid oestrogenic effects on calcium exchange in endometrial cells is thought to have significant bearing on the processes involved in metabolic and mitogenic responses to

this hormone (Pietras & Szego, 1975). Thus, it was not unreasonable to predict that the lower oestradiol concentrations would be mitogenic for thymic lymphocytes.

Indeed, oestradiol at concentrations which induced maximum calcium uptake (1 $pgml^{-1}$ and 10 $pgml^{-1}$) provoked a mitogenic response whereas higher levels were without effect (figure 9). Therefore oestradiol appears to have contrasting actions; stimulatory at low and inhibitory at high concentrations. Such a phenomenon however, is certainly not unique for this tissue. Prolonged exposure to oestrogen causes uterine cells to become metabolically refractory whereas intermittent administration was ineffective in this way (Stormshack, Leake, Wertz & Gorski, 1976). Similarly, oestradiol was found to promote growth of GH cells (a clonal line of rat pituitary tumour cells) at low concentrations (10 $pgml^{-1}$) and have no stimulatory effect at all at higher concentrations of 1 $ngml^{-1}$ and above (Amara & Dannies, 1983). These latter results are comparable to those obtained in this study.

The speed of the response to oestradiol application in this investigation, and the observation that the alpha isomer also enhanced calcium uptake and initiated a comparable level of stimulated mitotic activity (figures 11, 12, and table 2) infers a non-genomic mode of action. In support, neither the inhibition of messenger RNA synthesis by actinomycin D nor the abolition of protein synthesis by cycloheximide reduced the calcium movement induced by beta-oestradiol (figure 10). Thus, despite the presence of specific cytosolic oestradiol receptors in rat thymocytes (Reichman & Villee, 1978; Malacarne, Piffanelli, Indelli, Fumero, Mondino, Gionchiglia & Silvestri, 1980) the mitogenic capacity of the steroid for these

lymphocytes may be mediated via binding to receptors at the plasma membrane or via a non-specific membrane effect.

Recently, evidence for a process of cell surface recognition for the steroid hormones has become available. In this way oestrogen receptors have been partially purified and characterized from subfractions of hepatocyte plasma membranes (Pietras & Szego, 1979b). Studies employing ligands immobilized by covalent linkage to an inert support have indicated the feasibility of selecting, by affinity binding, cells equipped with recognition sites at the external surface (Edelman, Rutishauser & Millette, 1971). To this end, oestrogen covalently linked to nylon fibers have been used for selection of hormone responsive cells. It has been demonstrated that fiber-binding endometrial cells respond to mitogenic challenge of subsequently administered oestradiol with far greater intensity then do those that fail to associate with immobilized oestradiol during the initial selection process (Pietras & Szego, 1981). Metabolic and proliferative responses to oestrogen have also been reported for hepatocytes similarly selected for plasma membrane binding sites specific for the steroid (Pietras & Szego, 1979a). Membrane binding is generally associated, in a variety of cell types, with an array of 'indicators' that are not necessarily peculiar to any given cell. These may include direct modification of ionic fluxes, and inhibition or stimulation of the activities of membrane bound enzymes such as nucleotide cyclases and certain ATPases, functional alterations of which are likewise critically associated with shifts in the ionic environment (see section 1 and 2). Although such effects are frequently encountered with peptide agonists such allosteric modifications are not usually thought to be invoked for lipid soluble agonists. Nevertheless, there are now examples of such effects

initiated by steroid hormones. Indeed, steroids have been linked with increased or decreased cAMP production (Tang, Martellock & Tang, 1982; Flandroy & Galand, 1978), cGMP accumulation (Vesely & Hill, 1980; Flandroy & Galand, 1978;1979; 1980; Kuehl, Cirillo, Zanetti, Beveridge & Ham, 1976), activation and inhibition of the (Na^+/K^+)-ATPase (Knudsen, 1976) and as demonstrated in this investigation modification of the cellular calcium homeostatic system (Batra & Sjogren, 1983; Pietras & Szego, 1975; for general review see Duval, Durant & Homo-Delarche, 1983).

Because of the exact parallelism demonstrated between Ca^{++} uptake and mitosis provoked by oestradiol it was totally unanticipated that the ommission of calcium ions from the culture medium did not inhibit the oestradiol induced mitotic response nor prevent the elevation of cytosolic calcium concentration (figure 13, table 2). The latter observation is consistent with the premise that the increase in cellular Ca^{++} content may (at least in part) be attributed to Ca^{++} release from cytosolic sequestration sites, suggesting that oestradiol may diffuse through the plasma membrane and subsequently exert an effect at the endoplasmic reticular or mitochondrial membranes. Certainly there is evidence available to demonstrate that oestrogens can inhibit the Ca^{++} uptake processes of isolated mitochondria and endoplasmic reticulum of myometrial and hepatic tissues (Batra, 1973; Moore, Knapp & Landon, 1977). Certain oestrogens (e.g. diethyl stibestrol) also promote the release of calcium from mitochondrial stores (Batra, 1973).

Surprisingly, the removal of external magnesium ions significantly inhibited the mitogenic response to either of the oestradiol epimers (figures 13 and 14). Furthermore, in accord with properties of other type 1 mitogens (i.e. magnesium dependent-substances)

the male sex steroid, testosterone abolished the mitogenicity of alpha- and beta-oestradiol (figures 15 and 16). Interestingly the normally anti-proliferative concentration of beta-oestradiol (0.1μ gml^{-1}) did not compromise the cells mitogenic response to its alpha counterpart at 10 $pgml^{-1}$ (figure 15). The removal of magnesium ions from the extracellular environment reduced the oestradiol-induced Ca^{++} influx rates for 1 $pgml^{-1}$ and 10 $pgml^{-1}$ concentrations (figure 17) suggesting that oestradiol action at the plasma membrane may be facilitated by magnesium ions. Similarly the elevation of cytosolic calcium was a magnesium dependent process (table 3). It is possible thus that the affinity of any binding sites for the steroid at the plasma membrane and organelle membranes is modulated by the magnesium ion. Magnesium removal may confer a low affinity state of the oestradiol receptor or reduce the numbers of receptors available to the steroid. Certainly Mg^{++} can induce an increase in the numbers of functional surface receptors in mammary gland membranes (Pearlmutter & Soloff, 1979) and modify the affinity of beta-receptors for specific agonists in a variety of cellular systems (Turlapaty & Altura, 1982; Maguire & Erdos, 1978; Osa & Ogaswara, 1979). Alternatively, the release of Ca^{++} from subcellular sequestration sites may be induced by an elevation of the intracellular magnesium concentration achieved by a magnesium uptake process. The removal of external magnesium would obviously compromise such a phenomenon. Indeed, data are available consistent with the premise that intracellular Mg^{++} can modulate cytosolic Ca^{++} levels by influencing binding of calcium within a cell (section 1.2.2a). High intracellular Mg^{++} concentrations may competitively inhibit Ca^{++} uptake by the endoplasmic reticulum (E.R.) and mitochondria (Chiesi & Inesi, 1981; Akerman, 1980; Fiskum & Lehninger, 1980) and/or promote

Ca^{++} extrusion from such sites (Nicholls, 1978). The hypothesis that magnesium influences binding sites at the ER and mitochondria or that the ion itself modifies Ca^{++} homeostasis at this level, requires that removal of external magnesium ions would reduce the internal free magnesium content. A recent report does suggest that transmembrane equilibrium of this ion is achieved within five minutes (Ling, Walton & Ling, 1979) and furthermore the magnesium content of chick embryo fibroblasts and murine lymphoblasts certainly appears to be reduced by drastic reduction in the external magnesium levels (Brennan & Lichtman, 1973; Sanui & Rubin, 1977). The Mg^{++} deprivation could thus, in addition to reducing oestradiol binding at specific internal and external sites and preventing any Mg^{++} induced Ca^{++} release, inhibit the proliferation process by loss of intracellular Mg^{++} such that Ca^{++} binds to the sites formerly occupied by this ion. This would reduce the availability in the cytosol of calcium ions which are pertinent for triggering of the mitogenic response.

Such theories demand that oestradiol acts at the plasma membrane to promote Mg^{++} influx, but the prevailing transmembrane gradient (presumed to be 1:1) make it unlikely that entry of magnesium can occur simply by increased membrane permeability to this ion. However, a specific inwardly directed Mg^{++} pump has been proposed by Sanui & Rubin (1982a) and a Mg^{++} carrier protein closely associated with the beta-receptor cyclase complex has also been suggested (Maguire & Erdos, 1980; Cech et al., 1980). Certainly, many hormones can modify Mg^{++} transport (e.g. Elliot & Rizack, 1974; Cech et al., 1980). Unfortunately, Mg^{++} flux was not considered in this experimental study; it is therefore still unclear as to whether oestradiol does indeed act via enhanced Ca^{++} and Mg^{++} uptake.

Of particular interest was the observation that when oestradiol concentrations were raised (to 100 $pgml^{-1}$ and 1 $ngml^{-1}$) calcium influx was significantly enhanced in the absence of external magnesium ions (figure 17). This heightened influx also resulted in an elevated intracellular calcium content (table 3). Therefore, if it is to be accepted that the proliferogenic process revolves around the modification of cytosolic calcium levels (as proposed above) these higher oestradiol concentrations should prove mitogenic. Indeed a parallel increase in mitotic activity accompanied the calcium uptake induced by oestradiol at 100 $pgml^{-1}$ and 1 $ngml^{-1}$ in the absence of extracellular magnesium ions. In such conditions the steroid's mitogenicity at 100 $pgml^{-1}$ was not compromised by testosterone administration (figure 16) but showed an absolute requirement for external calcium ions. Therefore, at this concentration it appears that oestradiol does not release sufficient (if any) calcium from intracellular sequestration sites to provide an appropriate trigger. The mitotic action of oestradiol on thymic lymphocytes thus appears to be rather a complex phenomenon changing from antimitotic at 'high' concentrations, calcium-dependent at 'moderate' concentrations (provided there is no extracellular magnesium) to magnesium-dependent at 'low' concentrations. Nevertheless the common feature of all effects seems to be ultimately a raised intracellular calcium concentration. As anticipated verapamil significantly impaired beta-oestradiol (10 $pgml^{-1}$)-induced calcium uptake (figure 19), and alpha- and beta-oestradiol-induced mitosis at the mitogenic concentrations (10 $pgml^{-1}$, 10 $pgml^{-1}$ and 100 $pgml^{-1}$ in Mg^{++} free medium) as depicted in figures 21 and 22. Subsequent experiments have shown that verapamil exerts its inhibitory action on oestradiol at 10 $pgml^{-1}$ even when Ca^{++} is removed from the extra-

cellular environment (table 4) which suggests that this calcium antagonist in addition to acting by blocking Ca^{++} channels in the plasma membrane may also influence calcium movement across the membranes of subcellular organelles. This is consistent with recent evidence that verapamil can traverse the plasma membrane and accumulate within muscle cells (Pang & Sperlakakis, 1983). Verapamil has also been shown to influence calcium transport in sarcoplasmic reticulum and mitochondria (Colvin, Pearson, Messineo & Katz, 1982; Dan & Gemba, 1980; Frey & Janke, 1975), although in most of these studies extremely high concentrations of verapamil were generally employed. If indeed it is the rise in $[Ca^{++}]i$ which initiates the proliferogenic response to oestradiol it is not unreasonable to assume that the next step in the sequence of events, leading to DNA synthesis and ultimately mitosis, is the association of Ca^{++} with its specific binding protein, calmodulin. The calmodulin molecule contains four calcium binding domains which may also bind Mg^{++} with a lower affinity (Demaille, 1982). The physiological relevance of such Mg^{++} association (if it does exist) however, is still a matter of controversy (Section 1.2.1b). Upon binding calcium ions, calmodulin presumably undergoes a conformational change(s) which exposes a specific site for binding and activation of certain response elements (Lin, 1982) which in this case probably correspond to proliferogenic related enzymes. In order to test the feasibility of a role for calmodulin in oestrogen-induced mitosis, specific calmodulin inhibitors were employed. Several types of antipsychotic agents have been shown to inhibit the biological actions of the calmodulin molecule (Weiss, Prozialeck & Wallace, 1982), although the phenothiazine antipsychotics are probably the most potent class of calmodulin inhibitors (Prozialeck

& Weiss, 1982). These drugs are known to selectively bind to and inactivate the calcium-calmodulin complex, the hydrophobicity of the drugs being the major determinant of antagonistic potency (Roufogalis, 1983). Although it is still not clear to which specific sites on the calmodulin molecule the phenothiazines and related drugs bind, it is likely to be a region involved in the association with receptor proteins i.e. response elements (Klevit, Levine & Williams, 1981). Such regions presumably become available when calcium ions bind to calmodulin (Klee, et al., 1980). Trifluoperazine (TFP) is one such phenothiazine antipsychotic agent which selectively binds to and inactivates calmodulin and subsequently inhibits the activity of a number of enzymes involved in a variety of Ca^{++}-CaM dependent processes. These include granulocyte chemotaxis, lysosomal enzyme secretion, phospholipid metabolism and cell migration (Elferink, Deierkauf & Riemersma, 1982; Takenawa, Homma & Nagai, 1980; Connor, Levin & Brownstein, 1982).

Thus if calmodulin is activated during the proliferogenic response of thymocytes then mitotic activation should be compromised by TFP application. Indeed, the enhanced mitotic activity, generally achieved by elevating the transmembrane calcium gradient was inhibited by the presence of TFP in a dose dependent manner (figure 22). Concentrations of TFP between 10^{-6} and 10^{-4} M were consistently effective as inhibitors of mitosis whereas higher levels proved cytotoxic. Significantly, elevation of external magnesium concentrations promoted a mitotic response in the presence or absence of the calmodulin antagonist (figure 23). A further calmodulin inhibitor M & B 13753, a phenoxypropanolamine compound (Brown, personal communication, (May & Baker Limited)) provided similar results. M & B 13753 (at

10^{-5} and 10^{-6} M) prevented the Ca^{++} induced but not the Mg^{++} induced mitotic response (figure 24). These inhibitors when added to thymic lymphocyte cultures treated with beta-oestradiol ($10pgm^{-1}$ in basal ionic conditions and 100 $pgml^{-1}$ in Mg^{++} free medium) or alpha-oestradiol, both prevented the normal mitotic responses (figure 25, 26 and 20). At present it is not possible to provide direct evidence that the intracellular receptor(s) for TFP and M & B 13753 in this system is calmodulin. However, since both compounds inhibit Ca^{++}- but not Mg^{++}-induced mitogenesis by a mechanism which does not include an impairment of the Ca^{++} uptake process (of cells stimulated to divide by high Ca^{++} (table 5) or a mitogenic concentration of oestradiol (figure 27)), it does not seem to be an unreasonable assumption. Therefore, despite the magnesium dependence, both beta- and alpha-oestradiol (at 10 $pgml^{-1}$) appear to raise the intracellular concentration of free Ca^{++} and via some calmodulin dependent event (or series of events) serve to initiate DNA synthesis and the ultimate mitotic response. Magnesium may facilitate oestradiol association with the plasma membrane and possibly with internal membranes or alternatively induce calcium mobilization from intracellular stores.

In view of its antioestrogenic propensity it was of particular interest to examine the effects on thymic lymphocyte mitotic activity of a triphenylethylene compound tamoxifen. Tamoxifen has been shown to prevent oestrogen-stimulated cell division of human breast cancer cells in vitro (Iacobelli, Natoli, Sica & Gaggina, 1982) and has thus been widely employed in the treatment of advanced breast cancer (Wakeling & Slater, 1980). Many of the actions of this compound stem from its ability to effectively compete with oestrogen for specific cytosolic receptors (Patterson, Furr, Wakeling

& Battersby, 1982). Its metabolites (which appear to have enhanced affinity for the oestrogen receptors) are selectively accumulated in the nuclear receptor in target tissues and thus appear to play an important role in mediating the actions of tamoxifen in vivo (Robertson, Katzenellenbogen, Long, Rorke & Katezenellenbogen, 1982). It has recently been shown that tamoxifen at high concentrations can rapidly influence the responsiveness of rat myometrial strips to stimulation by oxytocin and prostaglandin in vitro (Lipton & Martin, 1982). The antioestrogen inhibited the contraction in a dose dependent manner within ten minutes of application, which tends to implicate a membrane effect of tamoxifen. Since oestradiol has also been shown to have similar inhibitory effect on contractile responses of smooth muscle (Batra & Bengtsson, 1978), it appears that this antioestrogen may also show oestrogenic propensities. Indeed tamoxifen appears to exert a range of effects dependent on species. In the chick and man the compound is predominantly antioestrogenic, whereas in mouse it is in most instances purely oestrogenic although a few studies have documented antioestrogen properties (Sutherland, Mester & Baulieu, 1977; Jordan, Allen & Dix, 1980). In the rat tamoxifen is a partial oestrogen agonist with antioestrogen properties (Okamoto & Imamura, 1982; Jordan & Dix, 1979).

Thus, in rat thymic lymphocyte cultures it was not surprising to find that high concentrations of tamoxifen (0.1 and 0.01 $\mu g ml^{-1}$), like oestradiol at these concentrations, blocked the mitogenic effect of raised extracellular calcium concentrations (figures 28 and 29). However, in contrast to the steroid, tamoxifen also abolished the mitogenic response to magnesium ions (2.5 mm). Thus, in a sense tamoxifen acts in a similar manner to anti-mitotic concentrations of

oestradiol and testosterone. Whether such actions are related in any way to its anti-tumour properties remain to be demonstrated.

Tamoxifen enhanced the rate of calcium uptake in a dose related fashion which was identical to the effect of beta-oestradiol. Lower concentrations (1 $pgml^{-1}$ and 10 $pgml^{-1}$) increased Ca^{++} uptake and consequently elevated the intracellular calcium content of the cell (figure 3 and table 6) although the higher tamoxifen concentration (10 $pgml^{-1}$) despite promoting a greater $^{45}Ca^{++}$ influx did not appear to raise intracellular Ca^{++} to levels comparable with the 1 $pgml^{-1}$ concentration. Thus, tamoxifen may also directly (and rapidly) influence the cellular calcium homesotatic system and by enhancing Ca^{++} extrusion or promoting sequestration of Ca^{++} by the the E.R. and mitochondria, serve to limit the size of the calcium signal. The physiological significance of such a mechanism is however, unclear. Regardless of these observations both tamoxifen concentrations (1 and 10 $pgml^{-1}$) were equally effective as mitogenic stimuli (figure 31). Higher tamoxifen concentrations (0.01 and 0.1μ gml^{-1}) were unable to influence basal mitotic activity. In spite of the obvious similarities between tamoxifen and beta-oestradiol as mitogens, there appears to be a subtle difference in ionic dependency. Although the antioestrogen at 10 $pgml^{-1}$ was identical to the steroid in that it showed an absolute requirement for magnesium ions, was inhibited by testosterone but unaffected by oestradiol or verapamil (figure 33 to 35), the lower concentration (1 $pgml^{-1}$) was calcium dependent and was inhibited by oestradiol and by calcium channel blockade but not testosterone (figures 32 to 34). Removal of external calcium ions resulted in a delayed but moderate elevation of intracellular calcium content provoked by this lower concentration

(1 $pgml^{-1}$), presumably achieved by mobilization from internal calcium binding sites. The time factor however, makes it unlikely that this calcium rise would trigger recruitment from quiesence and commit cells to the division cycle. Significantly, replacement of calcium ions in the extracellular environment resulted in an immediate influx of Ca^{++}, raising internal calcium to those levels normally reached with tamoxifen in basal conditions. These observations indicate that tamoxifen (1 $pgml^{-1}$)-induced mitosis is dependent almost exclusively upon the influx of extracellular calcium ions. The ommission of magnesium from the culture medium significantly depressed the Ca^{++} elevation in response to tamoxifen at 10 $pgml^{-1}$ (table 6) which implicates calcium in the activation process of this magnesium dependent concentration. Indeed both mitogenic tamoxifen concentrations appear to act via the activation of the calmodulin molecule since TFP and M & B 13753 severely restricted the mitogenic responses to the antioestrogen (figure 36). There must exist however, fundamental differences between the mechanisms of activation. Since the prevention of calcium influx by verapamil did not compromise the mitotic response to tamoxifen at 10 $pgml^{-1}$ (figure 34) the measured tamoxifen-induced increase in Ca^{++} uptake rates in basal conditions is effectively redundant. In view of the fact that intracellular Ca^{++} levels in cells stimulated by 10$pgml^{-1}$ tamoxifen were lower than the levels achieved by 1 $pgml^{-1}$ (table 6) it appears that tamoxifen may be able to activate compensatory mechanisms. If the compound can promote membrane pump activity and/or stimulate sequestration mechanisms at the same time as it promotes influx then the free $[Ca^{++}]i$ elevation would be limited. It has been suggested that high oestradiol concentrations inhibit mitosis by elevating cell calcium concentration beyond an upper threshold. If tamoxifen (as

predicted above) can rapidly compensate, for such elevations, this would explain why the anti-mitotic concentration of oestradiol was unable to inhibit the mitotic response to tamoxifen at 10 $pgml^{-1}$ (figure 34). Calcium efflux studies however, were not included in this study to test such possibilities.

In conclusion therefore, tamoxifen appears to increase cellular Ca^{++} content by enhancing calcium influx across the plasma membrane or via a magnesium-dependent mobilization of sequestered calcium ions from subcellular organelles. For tamoxifen (10 $pgml^{-1}$ at least) it is possible that a precise Mg^{++}:Ca^{++} ratio is required for activation of calmodulin or some later process which may provide the necessary impetus for the initiation of DNA synthesis and mitosis. Certainly, fractional occupancy of the Ca^{++} binding sites of calmodulin as a specific signal has been suggested. For example the active species for stimulation of phosphodiesterase is thought by some to be the tricalcium-monomagnesium calmodulin complex (Wolff & Brostrom, 1979). However, due to technical difficulties in monitoring Mg^{++} fluxes the effects of magnesium-dependent mitogens on such movements are unknown in this system. It can only be hypothesised that these agents can modify Mg^{++} uptake. In this way tamoxifen at 10 $pgml^{-1}$ may by some complex mechanism modulate the intracellular concentrations of both divalent cations to create the required ratio to specifically control the proliferative response. It is possible that the anti-tumour capacity of tamoxifen is related somehow to its ability to modulate cation fluxes.

The effect of oestradiol on Ca^{++} translocation was an unexpected phenomenon but another steroid hormone which functions physiologically to maintain constant plasma calcium levels by enhancing

absorption of calcium by the small intestine (Omdahl at al., 1971), stimulating bone resorption (Tanaka & DeLuca, 1971) or decreasing the excretion of calcium (Steele, Engle, Tanaka, Lorenc, Dudgeon & DeLuca, 1975) might be expected to influence the cellular calcium homeostasis of these thymus derived cells. Vitamin D_3 undergoes hydroxylation at the liver and kidney levels to its active metabolites, 25-hydroxycholecalciferol ($25(OH)D_3$) and 1,25 dihydroxycholecalciferol ($1,25(OH)_2D_3$), the latter of which is thought to be the major active 'hormone'. The mechanism of action of the hormone involves binding to a high affinity cytosolic receptor after which it is transferred to a nuclear receptor followed by gene derepression, messenger RNA synthesis and de novo protein synthesis. In the case of the action at the small intestine, the protein 'calcium binding protein' is produced and this subsequently promotes calcium absorption (Norman, Roth & Orci, 1982; Morrisey, Zolock, Bikle, Empson & Bucci, 1978). Such genomic effects of steroids are complex and protracted. In contrast, vitamin D_3 and its hydroxylated derivatives enhanced the rate of calcium uptake and cytosolic accumulation by rat thymocytes within twenty minutes of administration (figure 37). This effect was not sensitive to inhibitors of transcription or translation (figure 10) and therefore probably was not mediated by the general mechanism of steroid induced protein synthesis. It is reasonable to suggest (as appears to be the case for oestradiol) that vitamin D_3 and its metabolites are exerting a direct effect at the plasma membrane. Other authors have recently demonstrated similar direct membrane actions of itamin D_3 metabolites at physiological concentrations serving to enhance Ca^{++} translocation and accumulation in primary cultures of bone cells cells (Eilam, Szydel & Harell, 1980) and in isolated epithelial cells from rat intestine (Nemere & Szego, 1981). The absorption of phosphate by

rat intestine which increases within a few minutes of 1,25 $(OH)_2D_3$ application may also be a membrane mediated event (Bachlet, Lacour & Ulmann, 1982). The induced Ca^{++} uptake in this system was exactly paralleled by the mitogenic potential of vitamin D_3 and its mono- and di- hydroxylated metabolites (figure 38). The proliferogenic process showed a calcium ion requirement, was oestradiol blockable and dependent on calcium influx from an extracellular source since verapamil clearly prevented the action of all three compounds (figure 39 to 43). The effective molar concentrations required for Ca^{++} uptake and mitosis were comparable to the relative potencies in other tissues (Stern, 1981), with $1,25(OH)_2D_3$ having greatest biological activity. In keeping with the other results of this study calcium is the primary signal and appears to be stimulating the mitogenic response via calmodulin activation, (figures 43 to 45). Thus, vitamin D_3 appears to be able to influence the mitotic activity of thymic lymphocytes by a direct action at the plasma membrane and subsequent modulation of cellular Ca^{++} homeostasis. Whether or not the hormone and its active metabolites have physiological roles as regulators of growth and development in any specific tissues however remains to be seen. Recently, evidence has been presented to suggest that $1,25(OH)_2D_3$ might play a role in blood cell differentiation. A specific high affinity receptor for this metabolite has been described in a human monocyte cell line U937 and human peripheral blood monocytes/macrophages. Since $1,25(OH)_2D_3$ promotes maturational changes in U937 cells and in conjunction with a T-lymphocyte factor augments monokine production, the presence of receptors in normal monocytes certainly suggests a similar role for $1,25(OH)_2D_3$ in normal cellular differentiation (Bhalla, Amento, Clemens, Holick &

Krane, 1983). The metabolite has also been demonstrated to exert a biphasic effect on the replication of human breast cancer cells, inhibitory at higher concentrations and stimulatory at lower levels (Eisman, Frampton & Omond, 1983) which is analogous to the effect of oestrogens on thymic cells in vitro. A physiological role for vitamin D_3 and it metabolites in growth regulation must therefore be considered.

Parathyroid hormone (PTH) is an additional hormone with the primary function of maintenance of calcium homeostasis. Toward this end, PTH interacts directly with bone to stimulate resorption and also influences tubular reabsorbance of calcium at the level of the kidney (Hunt, Atkins & Martin, 1976). It was predicted therefore, that this peptide hormone may modulate the calcium fluxes of thymic lymphocytes and subsequently influence mitotic activity. Certainly increased calcium uptake is seen in a variety of cell types treated with PTH in culture. PTH rapidly (thus suggesting binding of the hormone at the cell surface) enhances Ca^{++} accumulation in isolated osteocytes (Dziak & Stern, 1975) and kidney cells (Borle, 1968a; 1970; Borle & Uchikawa, 1978). This peptide has a similar effect in 'non-target' tissues promoting Ca^{++} uptake in for example, isolated intestinal cells (Nemere & Szego, 1981) and HeLa cells (Borle, 1968b). PTH administered in vivo to parathyroidectomized rats also increases calcium influx into liver slices (Chausmer, Sherman, & Wallach, 1972).

In the present study, the NH_2-terminal fragment (PTH 1-34), which has been shown to mimick the in vivo biological activity of the native hormone (Herrmann-Erlee, Heersche, Hekelman, Gaillard, Tregear, Parsons & Potts, 1976), enhanced the accumulation of calcium in the cytosol by promoting calcium uptake in a dose related manner (figure 46, table 8). It was therefore predicted that PTH (1-34)

at 10^{-8} gml^{-1} should prove mitogenic for rat thymocytes which would then implicate intracellular calcium as a mediator for PTH fragment mitogenicity. Certainly PTH (1-34)-induced histamine release from rat mast cells is dependent on intracellular calcium levels, (Tsakalos, Theoharides, Kraeuter Kops & Askenase, 1983). Parathyroid hormone has been shown to exert anabolic activity both *in vivo* and *in vitro* in a variety of tissues. Recent evidence suggests that such effects of PTH on growth may be intrinsically related to dosage since low levels of the hormone (approx. 10^{-10} M and below) enhance DNA synthesis in osteosarcoma cells whereas high concentrations become inhibitory (Majeska & Rodan, 1981). A similar biphasic phenomenon has been described for effects of PTH on erythropoiesis. Small amounts of PTH stimulate mitogenesis and RNA and heme synthesis by erythroid precursors (Levi, Bessler, Hirsch & Djaldetti, 1979) while large amounts inhibit the erythropoietic process (Levi, *et al.*, 1979; Meytes, Bogin, Ma & Dukes, 1981). The positive effect of PTH on erythropoiesis has also been demonstrated in aparathyroid rats. Parathyroidectomy dramatically reduces reticulocyte levels in bone marrow and peripheral blood and inhibits incorporation of ^{59}Fe into erythrocytes but PTH injections were able to completely restore the reticulocyte status and the ^{59}Fe uptake levels to normal (Perris, 1971). Thus, such proliferative activity seems to be closely linked to parathyroid hormone levels. As mentioned previously (section 2.4) PTH also appears to modulate growth and development of the thymus gland and stimulate mitosis of thymocytes *in vitro* (Whitfield, Perris & Youdale, 1969). Surgical removal of the parathyroid glands results in hypocalcaemia and subsequent hypoplasia which can be reversed by supplementation with PTH or calcium (Perris, 1971).

In this investigation the PTH (1-34) fragment stimulated mitosis of isolated thymic lymphocytes in a dose dependent manner. The stimulatory dose corresponding to that promoting Ca^{++} influx in this same system (figures 46 and 47). In view of the current data available on PTH action it was not surprising to find that PTH (1-34) at 10^{-7} and 10^{-8} gml^{-1} exhibited a calcium- but not magnesium-dependence (table 9). Inhibition of calcium influx prevented the mitotic response to the hormone at these concentrations, suggesting that PTH is unable to direcly influence intracellular mobilization but may do so by facilitating Ca^{++}-induced Ca^{++} release from endoplasmic reticulum (ER) Certainly, intracellular calcium concentration (table 8) is increased to a level comparable with that achieved with beta-oestradiol at 10 $pgml^{-1}$ (table 3) despite the fact that PTH-induced Ca^{++} influx (figure 46) appears to be somewhat lower than oestradiol promoted uptake (figure 8). This supports the above theory that PTH elevates internal Ca^{++} levels by some other mechanism in addition to the Ca^{++} uptake process. In accord with other studies the female sex steroid oestradiol (at high concentrations) inhibited the mitotic effect whereas testosterone was without effect (table 10). Calcium ions mediate the proliferogenic action of PTH (1-34) by activation of the calmodulin molecule (figure 48) which in someway subsequently leads to a sequence of events resulting ultimately in mitosis.

Thus far it has been shown that an elevated intracellular calcium concentration triggers mitosis. Such an increase may result from enhanced calcium influx or alternatively from displacement of the ion from intracellular binding sites and stores. A similar increase in cytosolic calcium could also conceivably be achieved by the inhibition of calcium extrusion. Ca^{++} export from the cell may be directly linked

to inflow of Na^+ down its concentration gradient (Blaustein, 1974). A decrease in the transmembranal sodium gradient should therefore restrict Ca^{++} exit and allow its accumulation within the cell. It was reasoned that moderate increases in intracellular Na^+ levels would be insufficient to activate the (Na^+/K^+)-ATPase at the plasma membrane and hence serve to reduce the sodium gradient. Provided Ca^{++} entry was not restricted this process would subsequently favour Ca^{++} accumulation and perhaps cause mitotic activation. Accordingly, the external sodium ion concentration was raised progressively from the basal 145 mM to 185 mM. Sodium increments of between 5 and 20 mM induced a mitotic response in the thymic lymphocytes (figure 49). Greater increments were ineffective, which is consistent with the premise that extracellular sodium increments above 20 mM, rapidly raise intracellular levels to those which would certainly stimulate the membrane pump. The sodium gradient would then be restored and a normal Na^+/Ca^{++} exchange process could resume, maintaining a low intracellular Ca^{++} concentration. Equiosmolar sucrose increments were unable to mimic the actions of Na^+ (table 11) inferring that the mitotic signalling mechanism could not be attributed to alterations in osmotic potential.

Ouabain is a cardiac glycoside which competitively inhibits the (Na^+/K^+)-ATPase, causing the cells to lose K^+ and gain Na^+ (Negendank & Collier, 1976). Certainly the Na^+ efflux process of thymocytes was compromised by its presence in the culture medium (table 12). Ouabain thus, reduces the transmembrane gradient and should therefore reduce Ca^{++} exit and as a consequence raise intracellular Ca^{++} levels. Such a glycoside-induced increase in the cellular calcium content of cardiac tissue has been observed (Wood &

Schwartz, 1978; Bierdert, Barry & Smith, 1979). Non-cardiac tissue have also proven susceptible to ouabain-induced alterations in cytosolic calcium transport (Lamb, & McCall, 1972). Indeed an elevation in $[Ca^{++}]i$ (as measured by Quin 2 fluorescence) was also demonstrated in thymic lymphocytes in response to ouabain (table 15). This rise would be expected to invoke a mitogenic response in these cells. Certainly, ouabain has been reported to stimulate cell division in a variety of tissues. These include embryonic neural retinal cells, mouse splenic lymphocytes and haemopoietic progenitor cells (Kaplowitz & Moscona, 1976; Ryser & Politoff, 1977; Spivak, Misiti, Stuart, Sharkis & Sesenbrenner, 1978; 1980). In the present study ouabain promoted mitosis, at two discrete concentrations 10^{-7} and 10^{-11} M, (figure 50). The biphasic action suggested a mechanism more complex than a simple inhibition of the membrane pump. This appeared to be the case since the higher concentration (10^{-7} M) exhibited type 2 mitogenic properties whereas the lower concentration may be considered a type 1 mitogen. The calcium-dependent concentration (10^{-7} M) induced a response which was inhibited by oestradiol and the magnesium-dependent concentration (10^{-11} M) provoked mitosis which was prevented by testosterone administration (table 13). In contrast, ouabain has also been reported to exhibit anti-mitogenic properties. For example it blocks nucleic acid synthesis in stimulated lymphocytes (Quastel & Kaplan, 1968; Szamel et al., 1980) and fibroblasts (Tupper et al., 1977; Rozengurt & Heppel, 1975). However, the concentrations employed in such studies were generally much higher than the mitogenic levels demonstrated in this study. Although ouabain at comparable high concentrations (10^{-4} M) failed to influence the basal mitotic activity of rat thymocytes it was able to inhibit high Ca^{++}- and high Mg^{++}-

induced mitosis (figure 51). A similar biphasic effect (suppression and potentiation of mitosis) of ouabain has been reported for haemopoietic precursors (Spivak et al., 1978; 1980). The anti-proliferative action of high glycoside concentrations is probably due to reduced potassium ion content as it can in other systems be overcome by an elevated extracellular (and by inference intracellular) potassium concentration (Kaplan, 1978). Certainly, high levels of cytosolic K^+ are required for the synthesis of proteins pertinent for DNA synthesis and cell cycle progression (Cahn & Lubin, 1978; Brooks, 1977). A reduced potassium content produced by an alteration in the membrane lipid composition of L-cells will likewise inhibit mitosis (Chen, Heiniger & Kandutsch, 1978). Interestingly, ouabain at 10^{-4} M has also been shown to enhance Ca^{++} uptake in these cells (Atkinson, personal communication) which may be related in some way to its inhibitory actions (cf. oestradiol at 0.1 $\mu g ml^{-1}$). If the primary mitogenic action of ouabain was to inhibit the activity of the membrane situated (Na^+/K^+)-ATPase and consequently increase intracellular sodium levels it follows that the mitogenic sodium increments should exhibit similar ionic dependencies. Indeed the mitogenic response to the 10 mM increment required extracellular calcium ions whereas the 20 mM increment was magnesium-dependent (table 14). It appears therefore that inhibition of the sodium pump by ouabain at 10^{-7} M prevents active extrusion of sodium from the cell and gradually reduces the transmembrane sodium gradient. Thus provided extracellular calcium was available, its passive diffusion into the cell would no longer be counteracted by the Na^+/Ca^{++} antiport exchange system. Calcium would accumulate within the cell and provide a mitogenic stimulus. In support of this theory, the observed elevation in the intracellular Ca^{++} concentration following treatment

with ouabain was not the result of enhanced Ca^{++} influx as basal influx rates were retained in the presence of the glycoside (table 16). Calmodulin activation appears to be the next step in the sequence of events leading to cell division since calmodulin inhibitors abolished the response (figure 52).

Clearly a different mechanism of action must be sought to explain the magnesium dependency of the lower mitogenic concentration (10^{-11} M). It is possible that at this concentration ouabain would only partially inhibit the ATPase activity and thus cause a smaller increment in intracellular sodium concentration. Fractional inhibition of the pump by ouabain has also been proposed in Friend erythroleukemic cells (Bernstein, et al., 1976). A modest increase in sodium might subsequently liberate calcium from mitochondrial stores (Nicholls, 1978; Crompton, Kunzl & Carafoli, 1977). It should be noted however, that attempts to assess the degree of ATPase inhibition via the measurement of radioactive sodium efflux revealed that both high and low concentrations of ouabain (10^{-4} M, 10^{-7} M and 10^{-11} M) all impaired sodium efflux to a similar extent. Whatever the mechanism, an elevation in cytosolic calcium which was not a result of enhanced Ca^{++} influx, was measured in response to ouabain at low levels. Significantly, removal of external magnesium reduced intracellular calcium content suggesting that the presumed calcium mobilization process is dependent on an 'appropriate' magnesium concentration intracellularly. An alternative mechanism might be that 10^{-11} M ouabain could activate thymocyte adenylate cyclase generating cAMP which itself is established as a magnesium dependent, testosterone-blockable thymocyte mitogen at physiological concentrations (Morgan, Hall & Perris, 1977; see table ii). In keeping with this possibility, ouabain application promotes increased cAMP formation in epithelial cells and fibroblasts (Le Lievre,

et al., 1977). Whichever mechanism is involved it appears that Ca^{++}-calmodulin association is required for the activation process to proceed. Since it is unknown if the sodium increments used in this study are sufficient or insufficient to activate the ATPase it is unclear whether or not the Na^{+} gradient diminishes as predicted. Further studies are required to evaluate the role of Ca^{++} in the mediation of the mitogenic action of sodium increments. Sodium thus, may act via the modulation of the cytosolic free calcium concentration or alternatively act directly as a primary mitogenic signal itself. The sodium ion is thought by many to act as a proliferogenic trigger (e.g. Deutsch et al., 1981; Rozengurt & Mendoza, 1980; Koch & Leffert, 1979; Deutsch & Price, 1982b) and indeed many mitogenic substances are able to provoke sodium influx in a variety of cell types (Koch & Leffert, 1979; Epel, 1980; Rozengurt & Mendoza, 1980; Owen & Villereal, 1983). The role of sodium in cell proliferation has been considered in detail in section 2.3.3b.

Despite the many observations in this study and elsewhere which suggest that $[Ca^{++}]i$ is the major factor determining the proliferative status of the cell, it is possible that at least in some cases magnesium ions play the primary role in the regulation of mitogenesis. Some authors have certainly demonstrated increases in cell Mg^{++} content which parallel the stimulated rates of DNA synthesis and proliferation (Sanui & Rubin, 1982, Rubin, 1977). In fact, it is believed by such authors that mitogens act via a single 'magnesium' axis, rather than a calcium axis. Thus it has been suggested that effects of extracellular calcium on the proliferative process are achieved indirectly by an ultimate modification of intracellular magnesium activity (Rubin, 1975; 1977). Others believe that cells can utilize temporally distinct changes in intracellular Ca^{++} and

Mg^{++} levels as specific cues for cell cycle recruitment and progression (McKeehan & McKeehan, 1980; McKeehan & Ham, 1978).

The elevation of Mg^{++} concentration in the external medium (and by inference the internal environment), which promoted a mitogenic response in thymic lymphocytes did not influence the Ca^{++} uptake process (figure 53). However, Ca^{++} content did appear to rise (albeit to a lower level than that achieved with high Ca^{++}) in response to the magnesium, (table 17), presumably as a result of mobilization from intracellular Ca^{++} stores. Since Mg^{++}-induced mitosis was not compromised at all by calmodulin inhibitors it is unlikely that the released calcium ions have a triggering role in this activation process. It is possible that Ca^{++} displacement is a non-specific effect of high $[Mg^{++}]i$ which is overridden by some Mg^{++}-initiated process related to DNA synthesis and cell division. Paradoxically perhaps, omission of calcium ions from the extracellular environment accentuated the high Mg^{++}-induced elevation in cell calcium content presumably as a direct result of increased membrane permeability to magnesium ions and hence further Ca^{++} liberation from internal stores. Ca^{++} in the extracellular fluid has long been thought to have a 'stabilizing' effect on the plasma membrane (Shanes, 1958). Indeed external Ca^{++} chelation leads to an increase in membrane permeability and cation leak in human lymphocytes (Quastel, Segel & Lichtman, 1981). However, since neither the high concentration of oestradiol nor calmodulin inhibitors block Mg^{++}-induced mitosis, the cytosolic calcium increment induced by exposure to high extracellular Mg^{++} concentrations is of no consequence in this mitotic induction. Presumably magnesium itself triggers the activation mechanism.

The effect of magnesium on intracellular calcium mobilization may nevertheless however help to explain the inhibitory action of testosterone on Mg^{++}-induced mitogenesis and Mg^{++}-dependent mitogens. This steroid can enhance Ca^{++} influx and possibly Ca^{++} mobilization in mouse kidney cortex slices via a mechanism which involves polyamines (Goldstone, Koenig & Lu, 1983; Goldstone & Chung, 1983). The net effect in Mg^{++}-stimulated thymocytes therefore might well be to raise intracellular calcium to inhibitory or toxic levels. Unfortunately time did not permit an examination of the effects of testosterone on calcium metabolism in the thymic lymphocyte. When cells were exposed to high (1.8 mM) calcium concentrations, the raised intracellular concentrations were further increased when magnesium was omitted from the culture medium (table 17). It must be presumed that the inhibitory threshold for intracellular calcium was not exceeded in this case, for mitotic activation still occurred (results not shown). The increase in calcium content of the cell in this instance may be a direct consequence of diminished activity of the plasma membrane (Ca^{++}-Mg^{++})ATPase since Mg^{++} is required for maximum efficiency of this pump (Schatzman & Burgin, 1978). Significantly a similar phenomenon has been reported in both normal and transformed BALB/c 3T3 cells (Rubin, Vidair & Sanui, 1981).

When thymocytes were incubated in the presence of normal calcium concentrations (0.6 mM) an acute reduction in extracellular magnesium appeared to heighten Ca^{++} influx (figure 53) yet intracellular calcium levels remained unaffected (table 17). Thus, if Mg^{++} deprivation does inhibit a Ca^{++}-extrusion pump it must be assumed that it can still function sufficiently to export the excess calcium or that this ion is rapidly sequestered elsewhere. Calcium may simply be bound to sites formerly occupied by magnesium ions

or the low intracellular Mg^{++} level may facilitate an active uptake of Ca^{++} into the endoplasmic reticulum A comparable stimulation of the Ca^{++} uptake process has certainly been described in sarcoplasmic reticulum of cultured muscle cells (Chiesi & Inesi, 1981).

Thus although the mitogenic action of high extracellular Mg^{++} does not seem to depend on a Ca^{++}-CaM activation step, magnesium undoubtedly can modulate the cellular homeostatic processes governing intracellular calcium levels and under some circumstances this could be of some importance in mitotic induction or inhibition. Since acute increases or reductions in extracellular magnesium are unlikely to occur under physiological circumstances, attention was therefore focussed upon the magnesium-dependent hormonal mitogens to try and clarify the role of this divalent cation in the mitotic activation process. In accord with previous studies in this laboratory isoprenaline, a synthetic beta-adrenergic agonist was found to stimulate mitosis in thymocytes at a concentration of 10^{-6} M (figure 54). Consistent with a mitogenic propensity of beta-agonists, isoprenaline also stimulates DNA synthesis in quiescent cultures of 3T3 fibroblasts (Rozengurt, 1982) and parotid gland cells (Tsang, Whitfield & Rixon, 1981). At any of the concentrations tested (including the mitogenic 10^{-6} M) isoprenaline was unable to influence the calcium uptake process or indeed intracellular calcium homeostasis (figure 55). Hence the cytosolic free calcium concentration is maintained at a basal level and thus, (presumably) has no specific role in the mitogenicity of this beta agonist. Similarly, in smooth muscle there is no increase in intracellular calcium content in response to beta-agonist action. In fact beta-receptor occupation in this tissue has been associated with enhanced calcium extrusion and/or seques-

tration within the cell (Bolton, 1979). Whether or not local redistribution of intracellular Ca^{++} can occur in thymic lymphocytes is unknown. However, in support of calcium independence in the mitogenic action of isoprenaline in thymocytes, the response to its administration (at 10^{-6} M) was not compromised by calcium channel blockade nor (probably more significantly) the calmodulin inhibitor, trifluoperazine (figures 56 and 57). Thus, whatever, mechanism(s) is involved in the mediation of isoprenaline's action it can be clearly distinguished from the other agents considered in this investigation (with the exception of raised extracellular Mg^{++} itself) in that Ca^{++}-induced calmodulin activation is not involved. Such a conclusion is not necessarily contradicted by the observation that the novel calmodulin inhibitor, M & B 13753 inhibited the isoprenaline-induced mitogenic response of thymic lymphocytes (figure 57). The compound is in fact a phenoxypropanolamine and is considered to be a beta-blocker (Brown, personal communication) and in this way may serve to block the drug action at the level of the specific membrane beta-receptor. Certainly, propanolol an established beta-antagonist will inhibit the mitogenic actions of isoprenaline in thymocytes (Morgan, 1976). Thus it appears that isoprenaline exerts its mitogenic potential through a specific plasma membrane beta-adrenergic receptor. The magnesium dependence of isoprenaline may be attributed to the capacity of Mg^{++} to induce an increase in numbers of functional surface receptors (Pearlmutter & Soloff, 1979) or (of specific relevance here) induce a high affinity state of beta-adrenergic receptors in many cellular systems (Turlapaty & Altura, 1982; Maguire & Erdos, 1978). Alternatively, or in addition, the beta-agonist may promote magnesium uptake into the intracellular environment from the external

medium. Certainly in adipocytes such beta-stimulants enhance magnesium translocation into the cytosol (Elliot & Rizak, 1974). It should be reiterated here that a high extracellular magnesium concentration which by inference also enhances Mg^{++} influx has identical properties, in that the effect is testosterone blockable and totally independent of increased calcium uptake and calmodulin activation. Thus although the mechanism(s) are unknown it appears that magnesium ions are the primary mediators of the mitogenic response to a certain class of thymocyte mitogens (classical type 1 mitogens).

Since some of the type 1 and type 2 mitogens (table ii) are physiological antagonists which in 'target' tissues are assumed to exert their specific effects via contrasting actions of the secondary messengers cAMP and cGMP, a recent hypothesis (Perris & Morgan, 1976) suggested that as a general phenomenon the mitogenic actions of calcium- and magensium-dependent agents may be mediated by cGMP and cAMP respectively. The cyclic nucleotides were thus considered in this study. As previously shown (Morgan, Hall and Perris, 1977), cAMP and cGMP when added exogenously to isolated thymocytes proved to be mitogenic, exhibiting a biphasic action. At low physiological concentrations (10^{-11} M), cGMP showed an absolute requirement for extracellular calcium ions whereas high concentrations (10^{-6} M) were magnesium dependent. In contrast (as would be expected) high, physiological concentrations of cAMP (10^{-7} M) were dependent upon the presence of extracellular magnesium ions but the unphysiological concentrations (10^{-12} M) required external calcium (figures 58 and 59).

The low concentrations of the cyclic nucleotides were unable to stimulate the cells to divide in the presence of the calcium channel blocker verapamil (figures 60 and 61). Because of the size and water solubility of cAMP and cGMP molecules it is unlikely that they would rapidly traverse the plasma membrane and enter the cells to exert their effects. Binding to a membrane associated protein kinase must therefore be considered. Kinase activation may promote Ca^{++} influx and subsequently the initiation of the mitogenic response. Certainly, a cAMP-dependent phosphorylation at the cytosolic side of the channel has been proposed to facilitate the opening of specific gating mechanisms and thus allow Ca^{++} channel conduction (Reuter, 1979; Cachelin, dePeyer, Kokubun & Reuter, 1983; see diagram 3). Whether or not a similar phosphorylation site exists at the external surface remains to be seen. Consistent with channel activation however, both cGMP (at 10^{-11} M) and cAMP (at 10^{-12} M) raised intracellular calcium concentrations in the presence but not in the absence of extracellular calcium ions (table 18). Furthermore, replacement of external Ca^{++} (following its removal) promoted an immediate increase in cell calcium content. This was presumably a result of calcium movement down its renewed concentration gradient through the activated (open) calcium channels in the plasma membrane. Paradoxically however, $^{45}Ca^{++}$ influx studies failed to substantiate these findings (figure 62). No feasible explanation for such an obvious contradiction can be offered at present. In contrast the inability of the cyclic nucleotides at high concentrations to influence basal calcium uptake (figure 62) was anticipated since calcium channel blockade did not prevent the mitogenic response to these higher concentrations (figure 60 and 61). Similarly, neither nucleotide raised the cytosolic free calcium content of thymocytes during a twenty minute incubation period.

Cyclic AMP at 10^{-7} M, however exerted a delayed action on cytosolic calcium levels which may be due to some calcium mobilization from mitochondria or from endoplasmic reticulum. The release of calcium from isolated mitochondria by cAMP at comparable concentrations has been demonstrated (Borle, 1973) and more recently confirmed (Juzu & Holdsworth, 1980). Alternatively, cAMP may influence the plasma membrane calcium extrusion system. Indeed it has very recently been shown that if membrane protein kinase content is relatively high this cyclic nucleotide induces an inhibition of the Ca^{++}-pump in cardiac sarcolemma vesicles (Velema, Bolt & Zaagsma, 1983). Even after a 40 minute incubation with high extracellular cAMP (10^{-7} M) the intracellular Ca^{++} levels were clearly lower than after a 20 minute incubation with 10^{-11} M cGMP. This suggests that the delayed rise in intracellular calcium concentration most probably is not related to the initiation process but nevertheless may be required at a later time for some other event necessary for cell cycle progression. It is more likely that cAMP may initiate proliferation by raising intracellular Mg^{++} levels or even directly by stimulating some magnesium dependent event(s). There is evidence available to suggest that a transient cAMP increase will trigger DNA synthesis via its protein kinase and subsequent protein phosphorylation(s) although this remains a controversial area of investigation (section 2.2.4b). Since high concentrations of cGMP can inhibit thymocyte cAMP specific phosphodiesterase (Whitfield et al., 1971), it is possible that cGMP exerts its mitogenic effect by ultimately raising cAMP concentrations within the cell. This would certainly explain its magnesium dependence. The absence of a primary role for calcium in the proliferogenic actions of these high levels of cyclic nucleo-

tides is further substantiated by the failure of calmodulin inactivation to influence the mitotic response (figures 63 and 64). In contrast the low concentrations of cyclic-nucleotides were ineffective if trifluoperazine or M & B 13753 were added to the cultures.

Because of the many specific receptors it bears upon the plasma membrane, the thymic lymphocyte has proved to be an extremely useful model system for the general study of cell proliferation. In view of the evidence presented in this study it is clear that monovalent and divalent rearrangements are intimately associated with the induction of thymocyte mitosis. Isoprenaline is one of many magnesium-dependent mitogens (table ii). The receptors for these type 1 mitogens are probably of low affinity since rather high concentrations of hormone are required for activation. Receptor occupancy is probably linked to adenylate cyclase and cAMP metabolism (although conceivably compartmentalization could allow very high localised concentrations of cGMP to prevail). Certainly isoprenaline raises endogenous cAMP levels within thymic lymphocytes and 3T3 fibroblasts (Morgan, 1976; Rozengurt, 1982). The increase in intracellular cyclic nucleotide concentration may trigger a magnesium dependent reaction or alternatively promote magnesium influx which in turn leads to DNA synthesis and ultimately mitosis. In this way, a high extracellular magnesium concentration and exogenous cAMP (10^{-7} M) may be considered type 1 mitogens. Ca^{++}-calmodulin activation plays no part in this sequence of events. In other cultured cell lines such as 3T3 and lung fibroblasts where Mg^{++} may play a primary role in the coordinate control of cell proliferation, it is possible that some of the serum growth factors operate via a similar magnesium dependent axis (Rubin et al., 1977; Sanui & Rubin, 1982; McKeehan and McKeehan, 1980).

The receptors responding to type 2 mitogens (table ii) are probably few in number but of high affinity since only low concentrations of hormone are necessary to stimulate cell division via a calcium axis. Calcium influx is a direct or indirect consequence of membrane receptor occupation since calcium channel blockade invariably inhibited the mitogenic response to calcium-dependent mitogens. Parathyroid hormone is one such type 2 mitogen. Such substances are also thought to be associated with guanylate cyclase activation. Although increased cGMP concentrations have not been detected in response to mitogenic concentrations of thymocyte type 2 agents, PTH has been reported to stimulate guanylate cyclase in cultured osteoclasts (Davidovitch, Montgomery & Shanfield, 1977) and in isolated renal tubules (Wrenn, Currie & Biddulph, 1978). Significantly, the addition of Thymosin V to rat thymocytes also provokes cGMP production in a calcium dependent manner suggesting that this intrathymic 'hormone' is a type 2 candidate. (Naylor, Thurman & Goldstein, 1980). It is also conceivable that small localised alterations in concentration of cGMP serve to open transmembrane calcium channels. Since calcium ions will stimulate the guanylate cyclase (Levine, et al., 1979) a positive feed forward phenomenon may operate to raise intracellular calcium to threshold levels pertinent for cell activation. In support of cGMP-induced Ca^{++} influx in the rat thymic lymphocyte verapamil blocked the mitogenic actions of low cAMP and cGMP concentrations in this study. Furthermore, the observed elevations of intracellular calcium concentrations in response to cGMP were dependent on extracellular calcium ions. Calcium influx it appears, is followed by calmodulin activation and a subsequent protein phosphorylation(s) could commit a cell to DNA synthesis and mitosis. High extracellular

calcium concentrations may also initiate this same sequence of events. The inhibitory actions of oestradiol are thought to be exerted via potentiation of mitogen-induced calcium influx such that intracellular calcium levels exceed an upper limit and proliferation is severely compromised. These findings add to the growing body of evidence that calcium ions have a central role in stimulus-mitosis coupling in many normal cell types (see section 2.3.3a)

It is apparent from these studies however, that the distinction between the type 2 and type 1 mitogens is not as clear as formerly predicted. The results obtained appear to suggest complex overlap between the calcium- and magnesium-dependent mitogenic agents. One such anomalous grouping includes oestradiol and tamoxifen which at low concentrations stimulate mitosis. Their effects are magnesium dependent but involve the modulation of intracellular calcium homeostasis. Oestradiol for example, promoted the movement of calcium down its concentration gradient into the cell interior or across membranes of subcellular organelles. Such processes are facilitated (or possibly induced) by magnesium ions suggesting that oestradiol may also promote Mg^{++} uptake into the cell. It is however, ultimately the calcium ion which dictates the proliferative state of the cell since calmodulin inactivation prevents mitotic stimulation. This third axis may thus be considered one which is totally dependent upon the presence of both divalent cations. It may be relevant here to point out the duelism of oestrogen action upon cyclic nucleotide metabolism. In several cell types both cAMP and cGMP production can be promoted by oestradiol administration (Gunaga, Kawano & Menon, 1974; Tang, Martellock & Tang, 1982; Flandroy & Galand, 1978; 1980). The cyclic nucleotides in turn could influence the concentrations of the divalent cations.

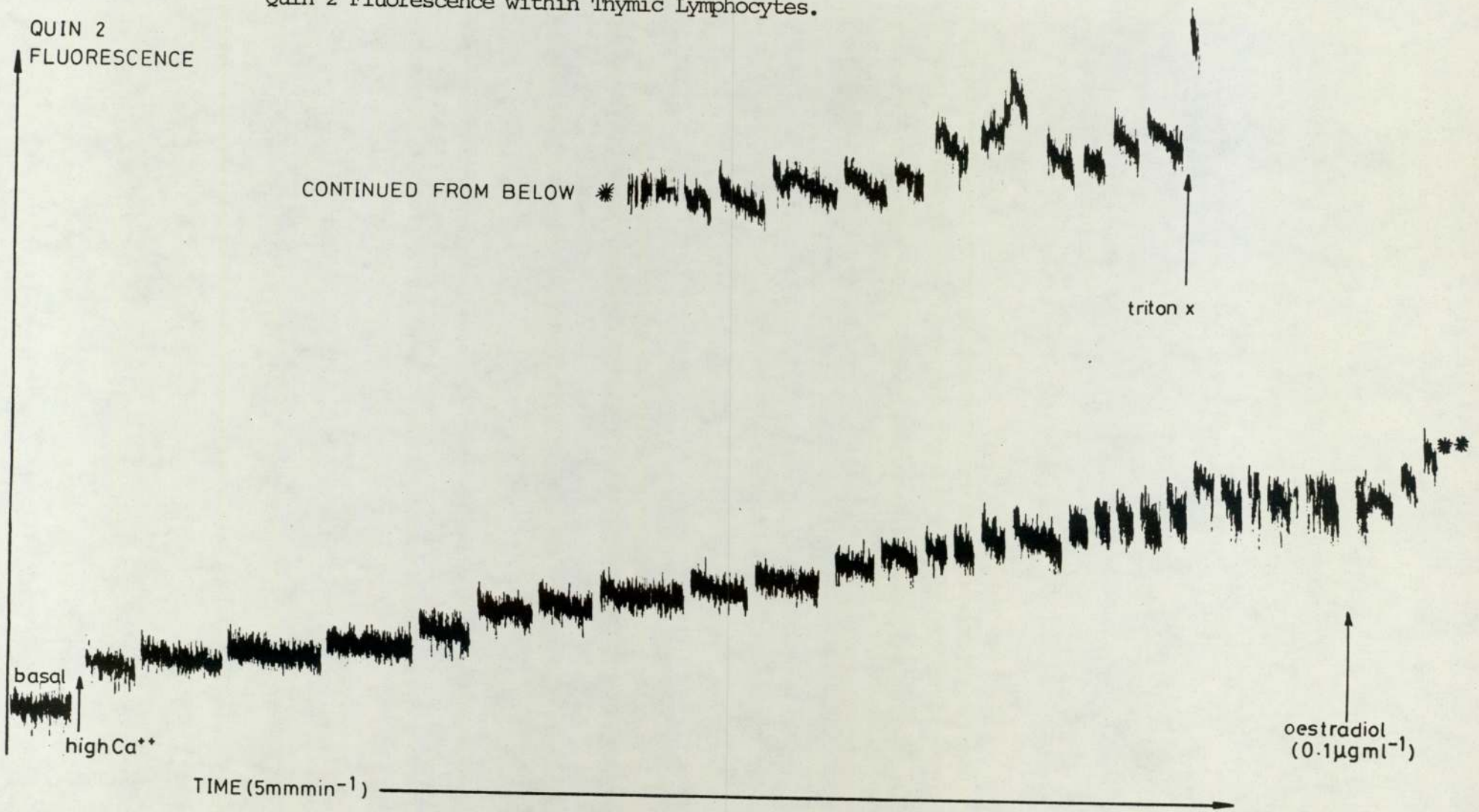
A fourth grouping might include agents which can indirectly influence the intracellular calcium status of a cell. Ouabain and sodium for example by a postulated increase in intracellular sodium ion concentration stimulated mitosis by inhibition of calcium exit. Calcium-dependence originated from the necessity to sustain basal rates of calcium influx in order to raise internal calcium content. Interestingly, Na^+ influx has also been shown to promote cGMP accumulation in cerebellar slices although this may be an indirect effect, the result of raised intracellular free Ca^{++} levels (Ahnert-Hilger & Habermann, 1981). Ouabain at a lower distinct concentration showed magnesium dependency (as did a greater Na^+ increment) but the mitogenicity was mediated via raised cytosolic calcium concentration and calmodulin activation. This may be the result of sodium-induced calcium release from mitochondrial stores (Nicholls, 1978) a process which in this system may be facilitated by magnesium ions.

This present report has clearly outlined that all four classes of mitogenic agents may operate via two major mitogenic axes regulated by calcium (and calmodulin activation) and magnesium respectively. In turn these axes can be influenced by many factors including monovalent cation metabolism, the cyclic nucleotides, and sex steroids. In such a complex and interrelated network of factors serving to control the intracellular divalent cationic climate the number of potential sites for modulation of the growth of normal cells and indeed for development of defects associated with neoplastic transformation will be huge and is likely to differ from cell to cell.

APPENDIX 1

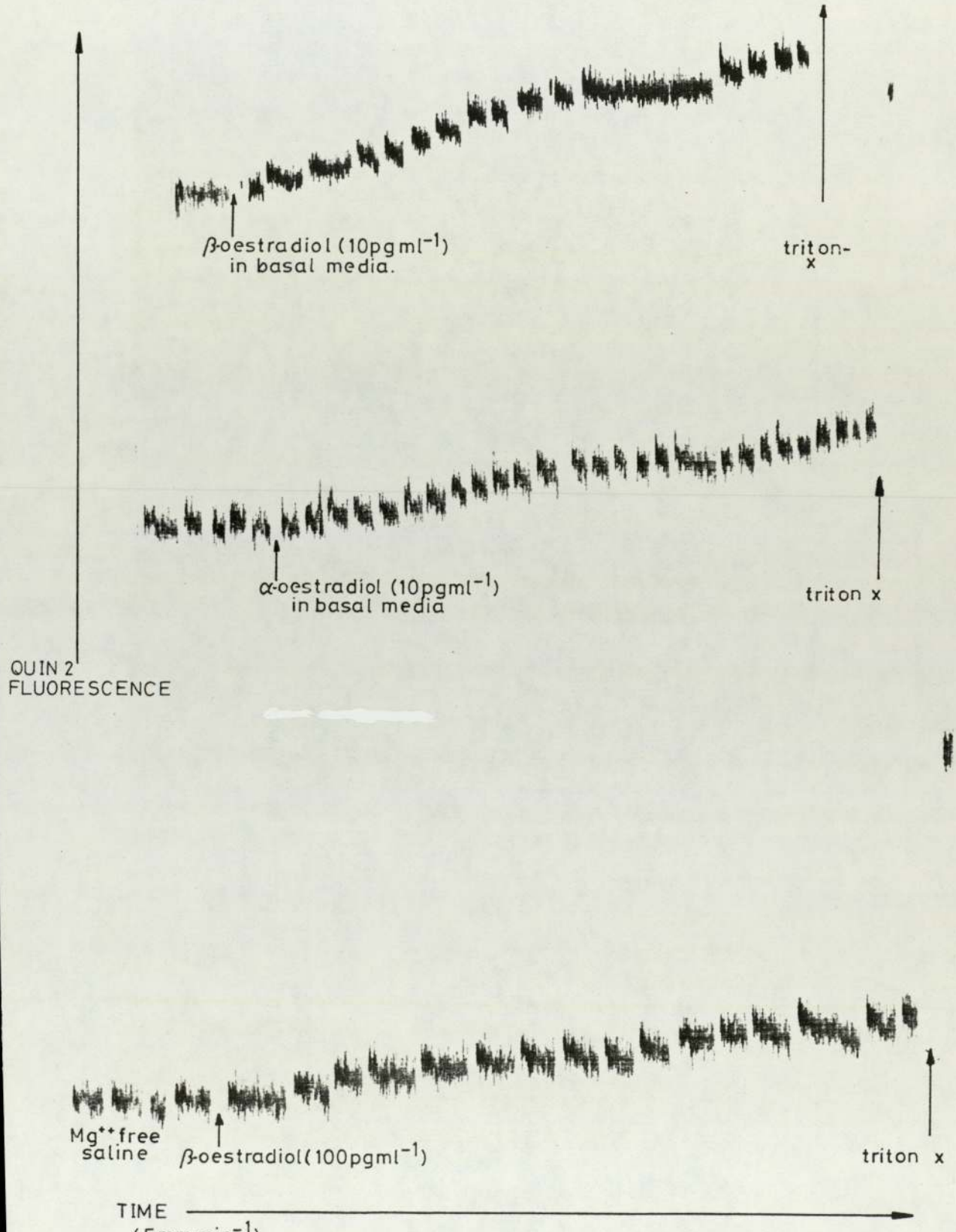
This includes records of intracellular calcium concentration as indicated by Quin 2 fluorescence. The traces were recorded on a flat bed recorder (section 3.3.1), and represent continuous monitoring of fluorescence (i.e. $[Ca^{++}]i$) during time periods shown. Gaps in the traces denote additions to or stirring of cell suspensions within the cuvette. Any additions made are clearly labelled on the traces included in this appendix. Traces are presented in the order of appearance in the results section (4.0). Basal saline, represents physiological saline containing 0.6 mM Ca^{++} and 1.0 mM Mg^{++}. (For futher details of methodology see section 3.3.1).

Records of Intracellular Calcium Concentration as Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.

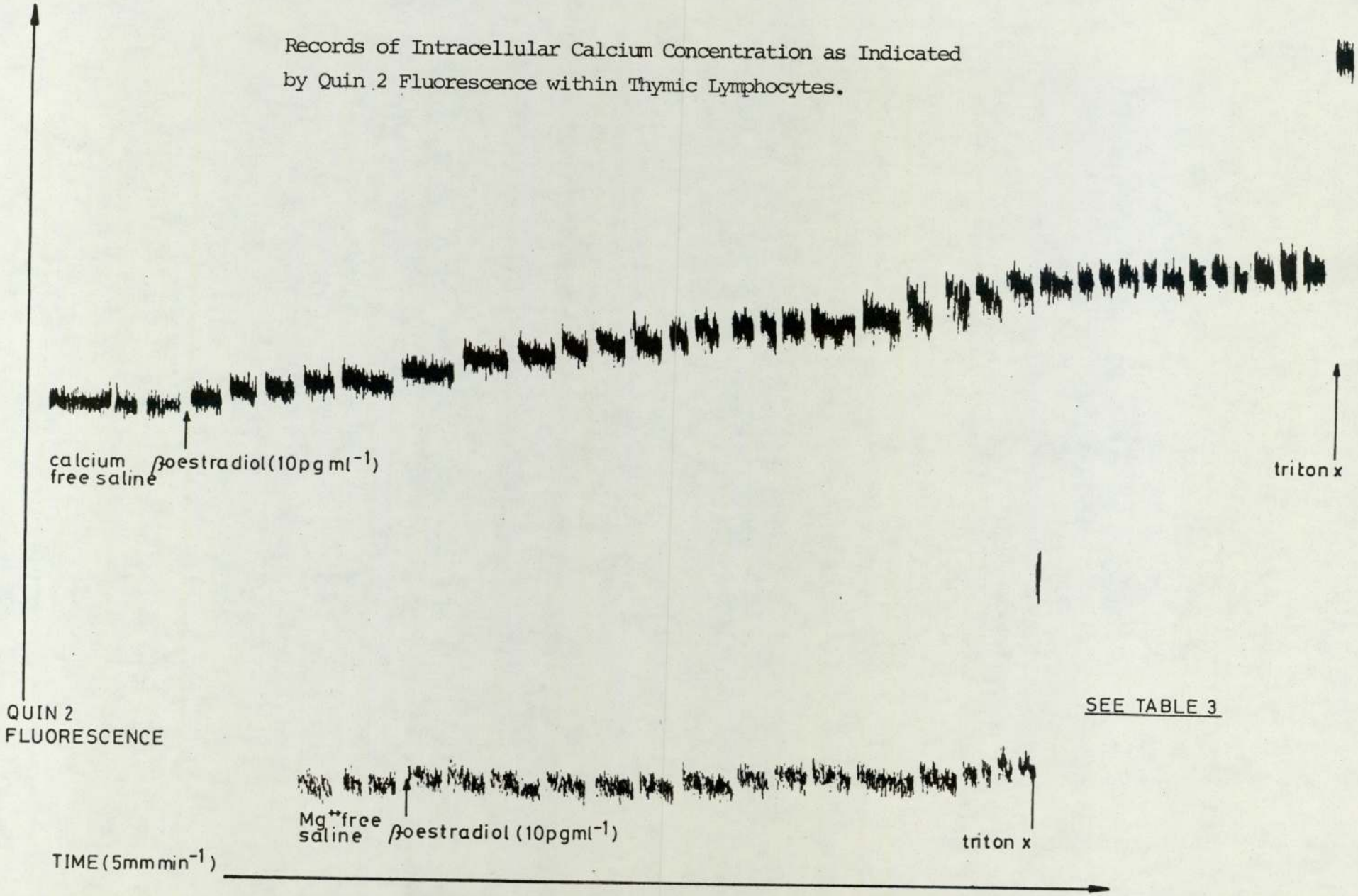


SEE TABLE 3

Records of Intracellular Calcium Concentration as Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.

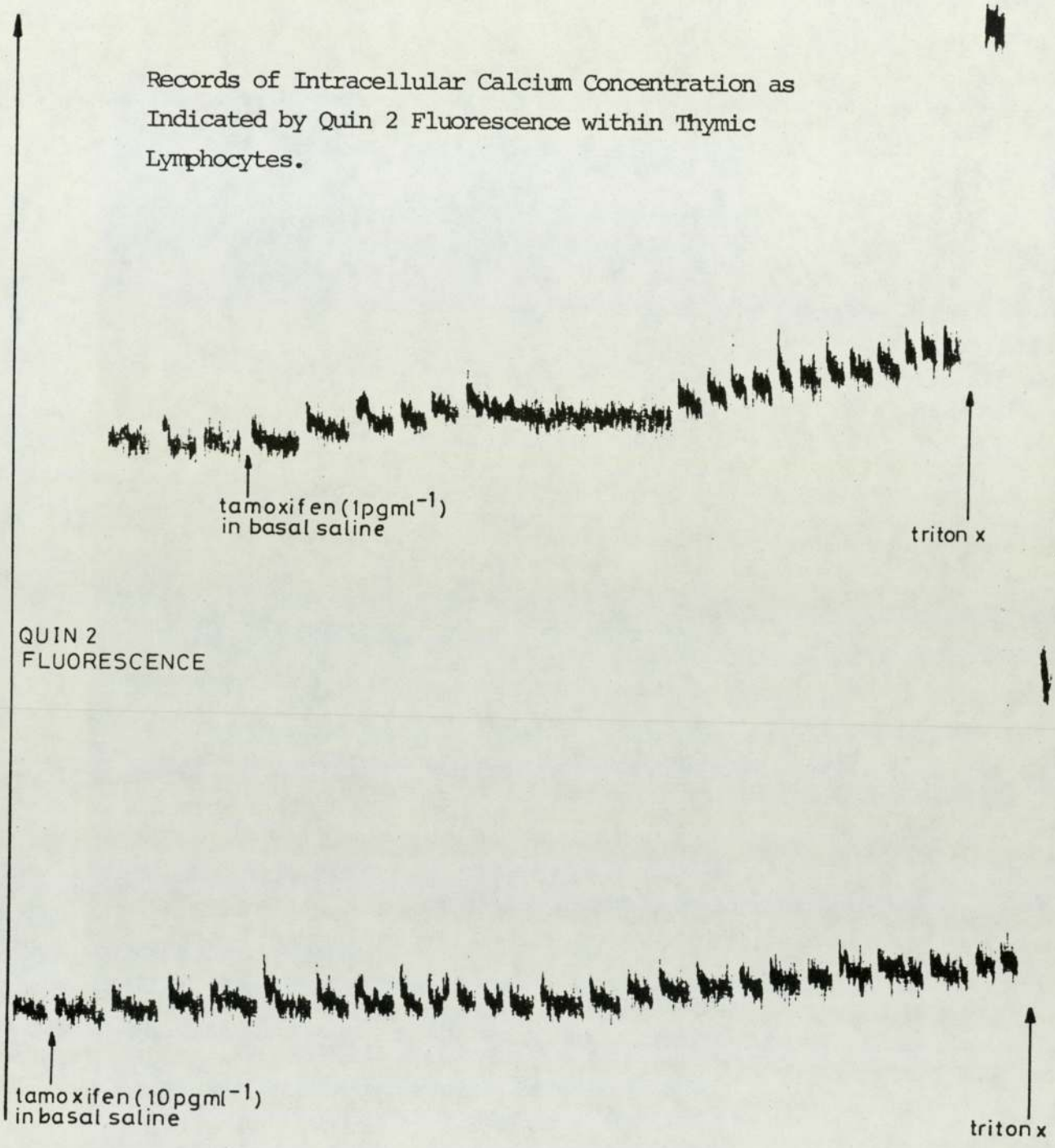


Records of Intracellular Calcium Concentration as Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.



SEE TABLE 3

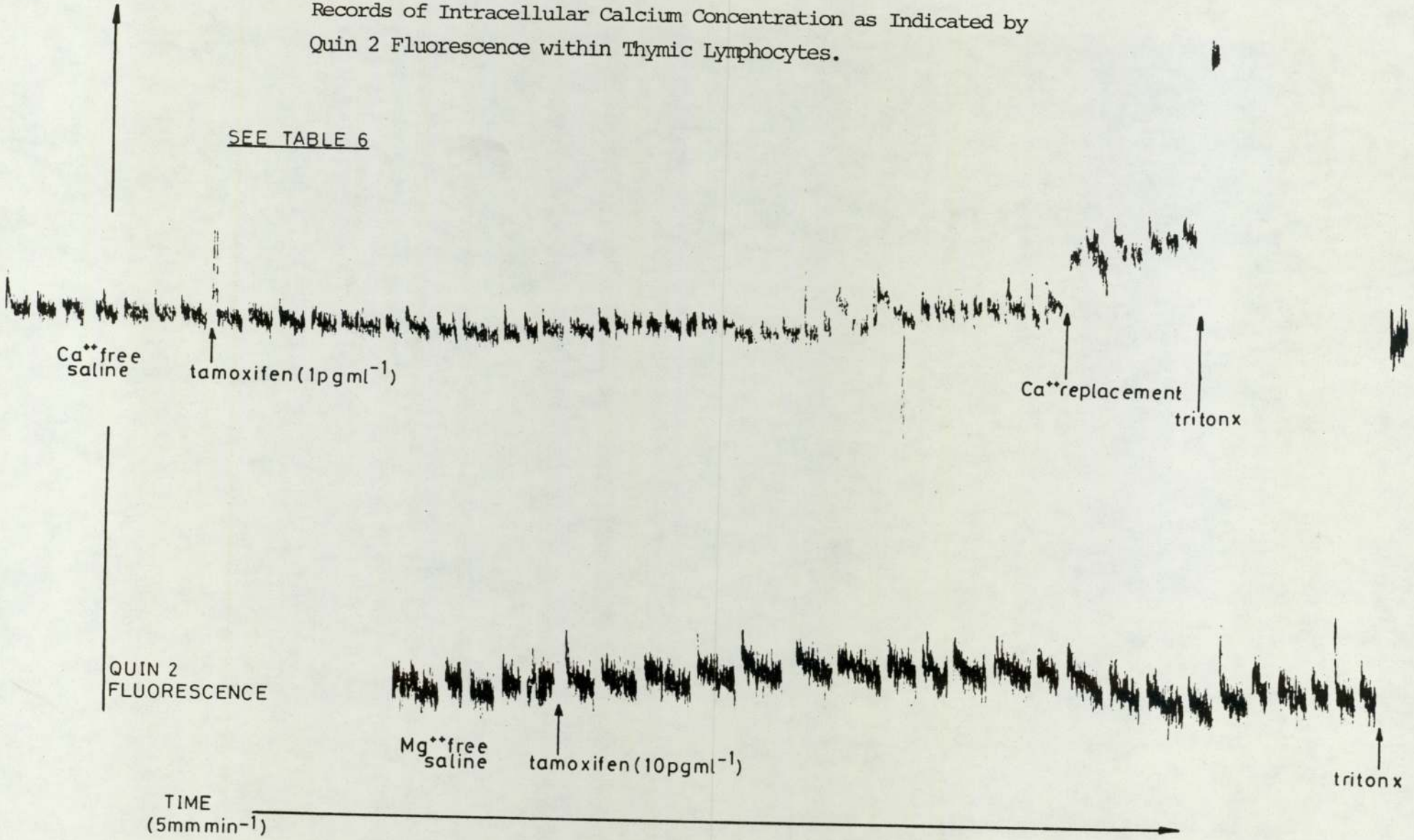
Records of Intracellular Calcium Concentration as Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.



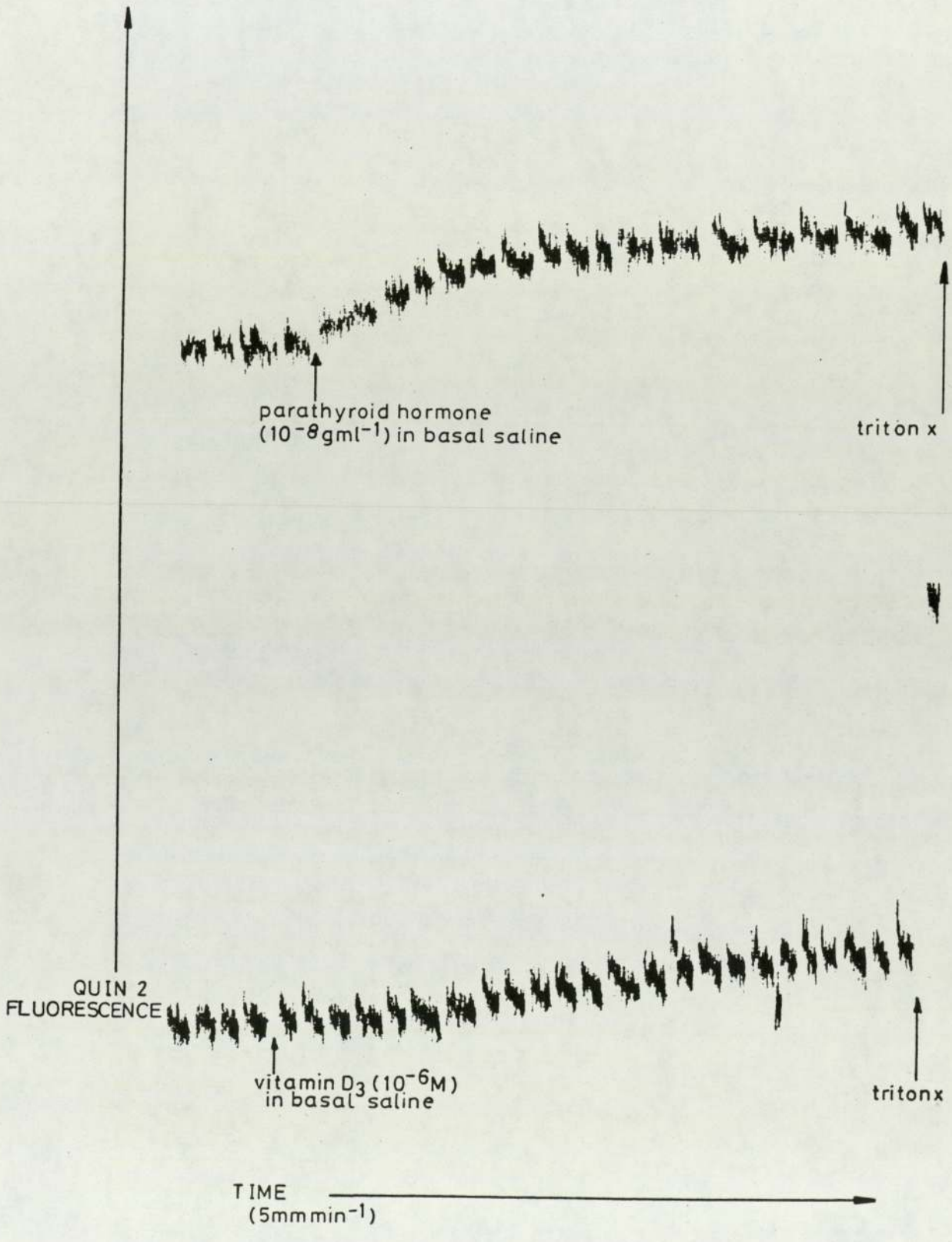
SEE TABLE 6

Records of Intracellular Calcium Concentration as Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.

SEE TABLE 6



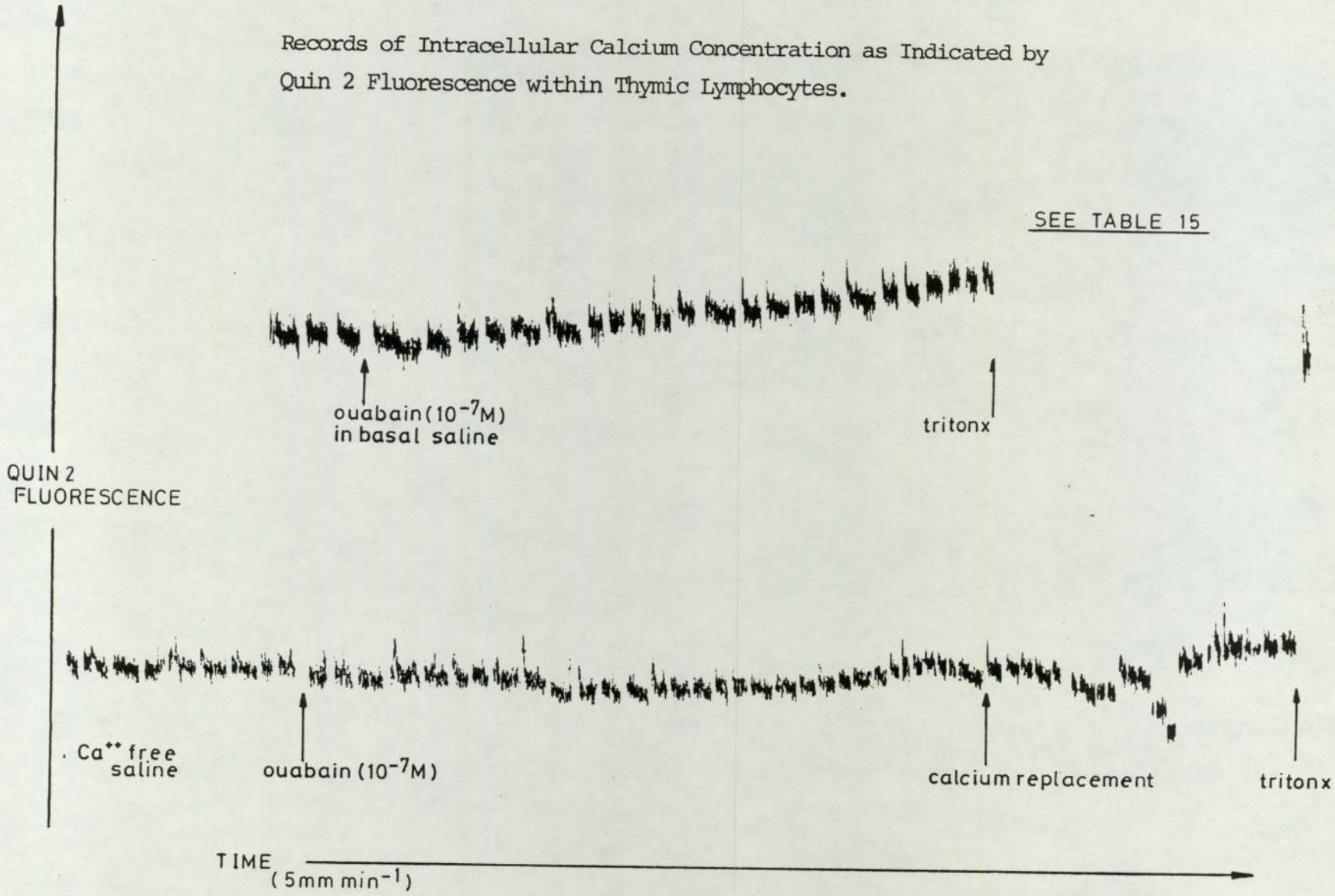
Records of Intracellular Calcium Concentration as Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.



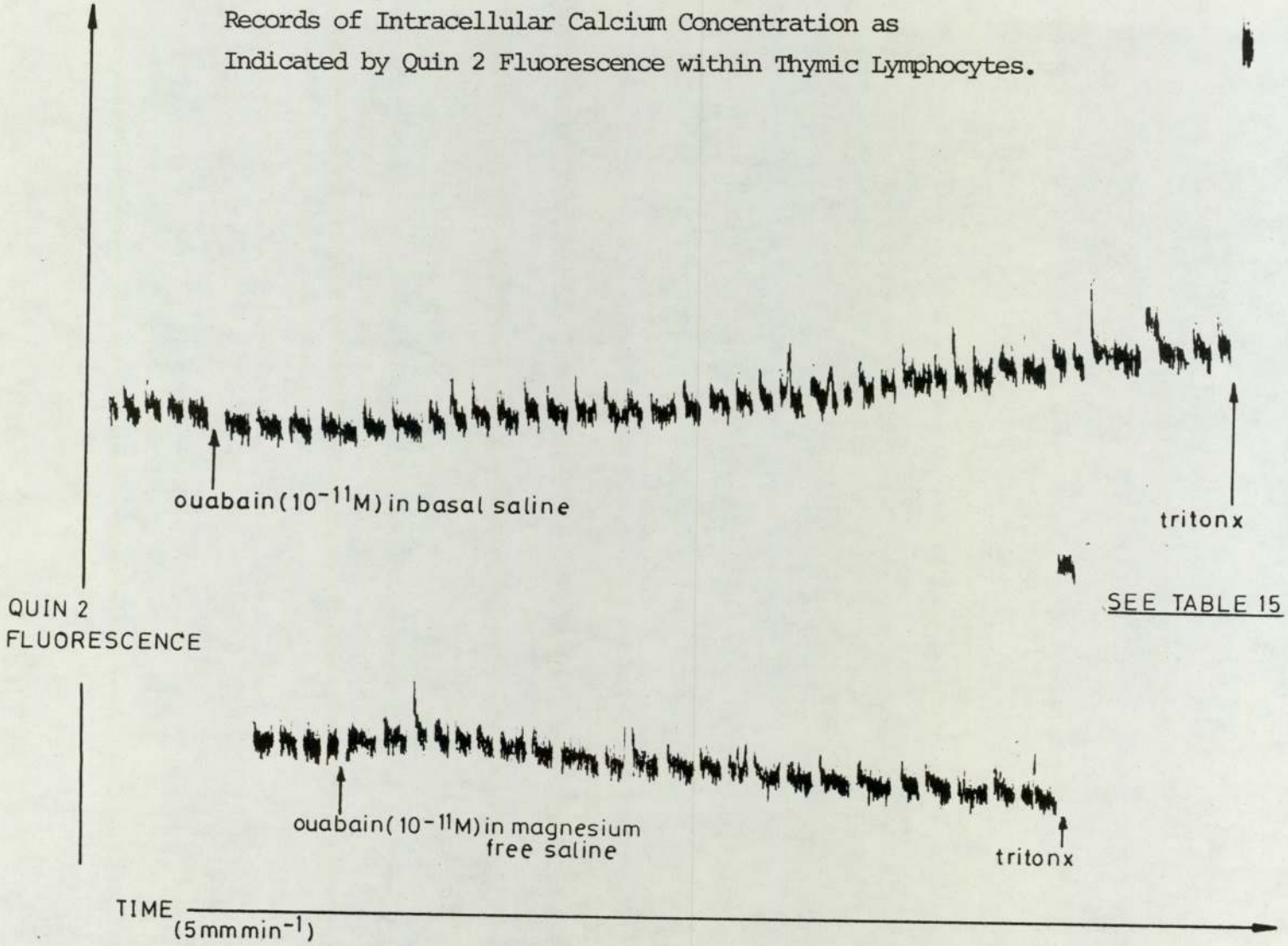
SEE TABLE 8

Records of Intracellular Calcium Concentration as Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.

SEE TABLE 15

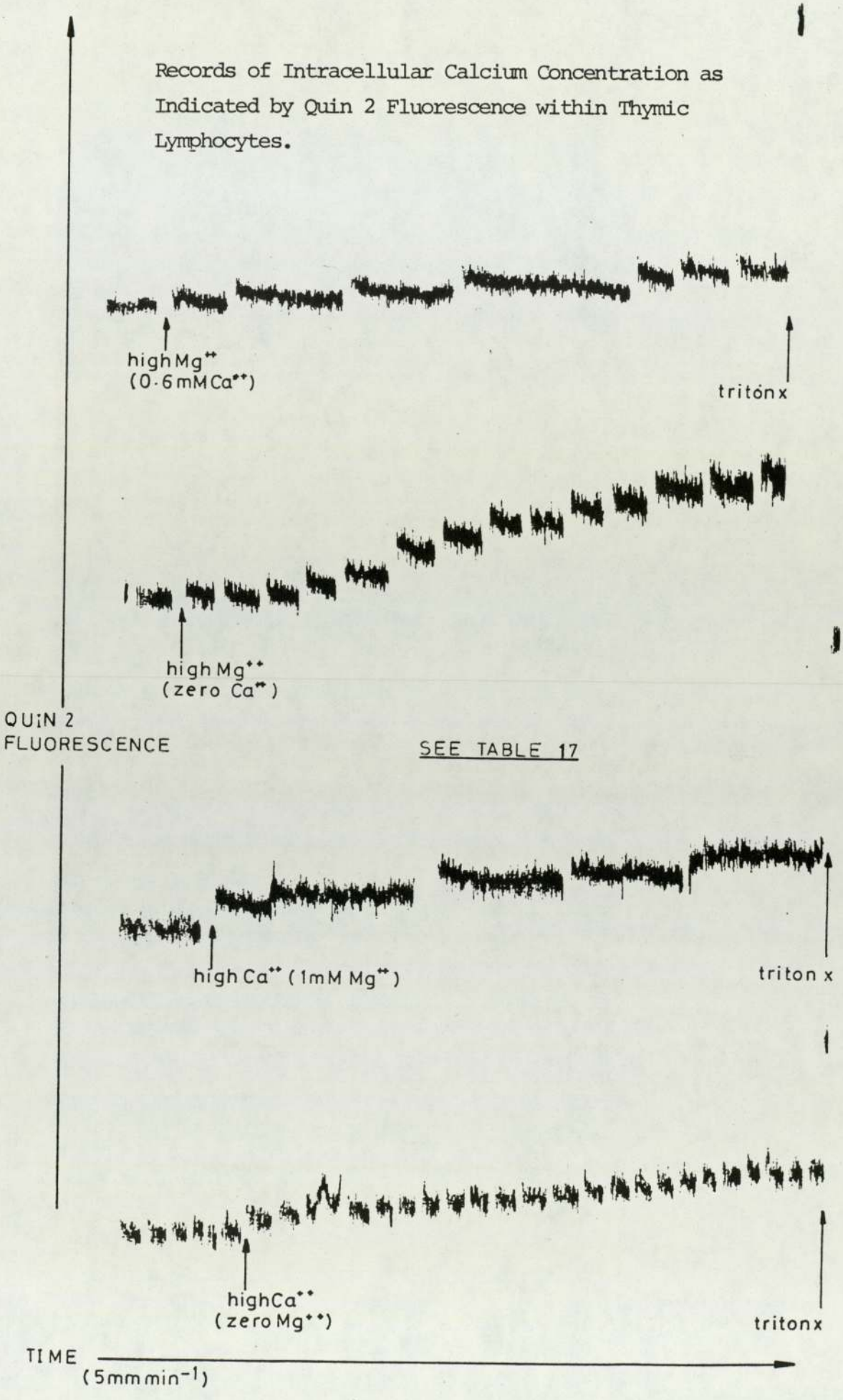


Records of Intracellular Calcium Concentration as
Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.



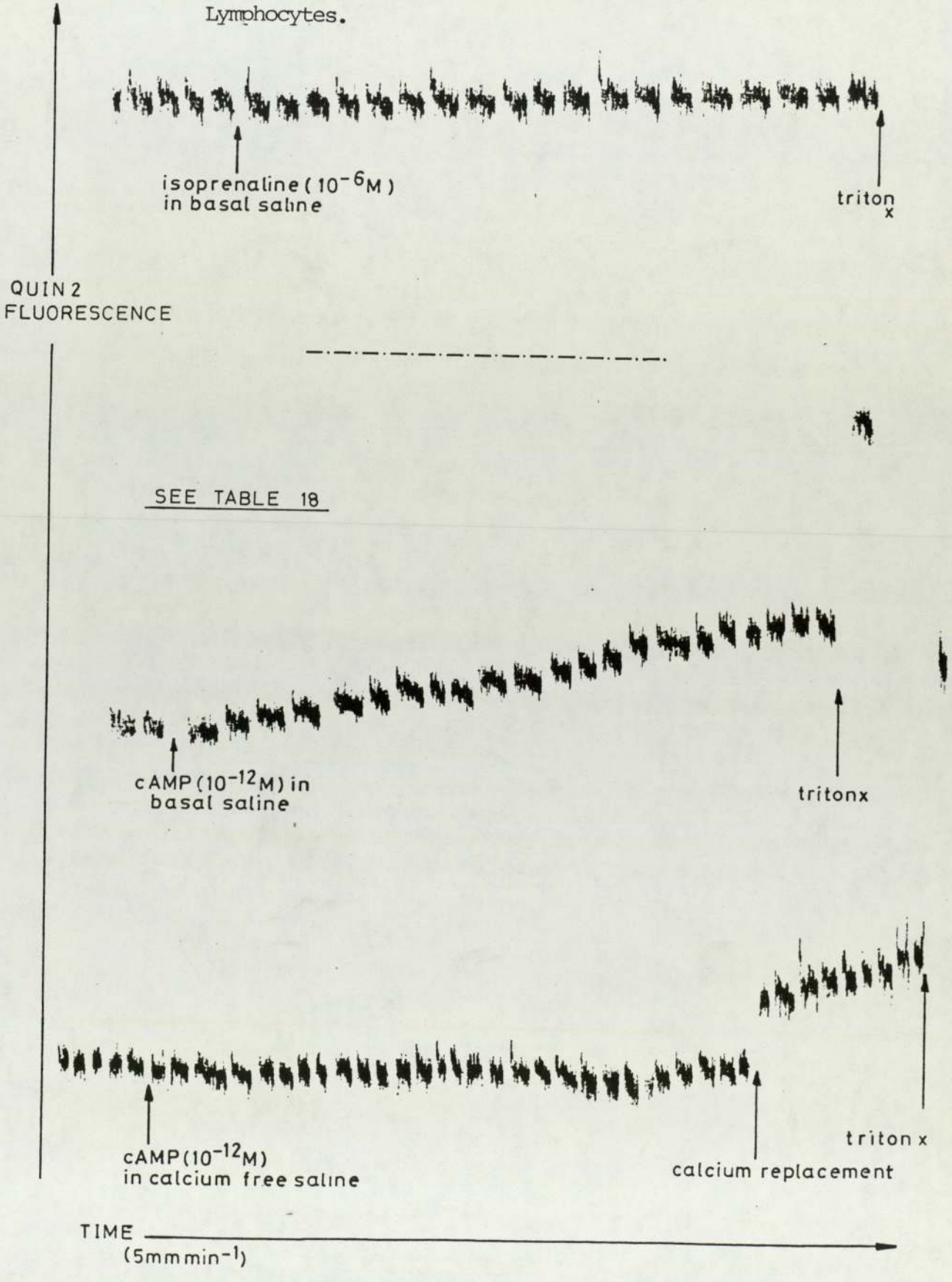
SEE TABLE 15

Records of Intracellular Calcium Concentration as Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.



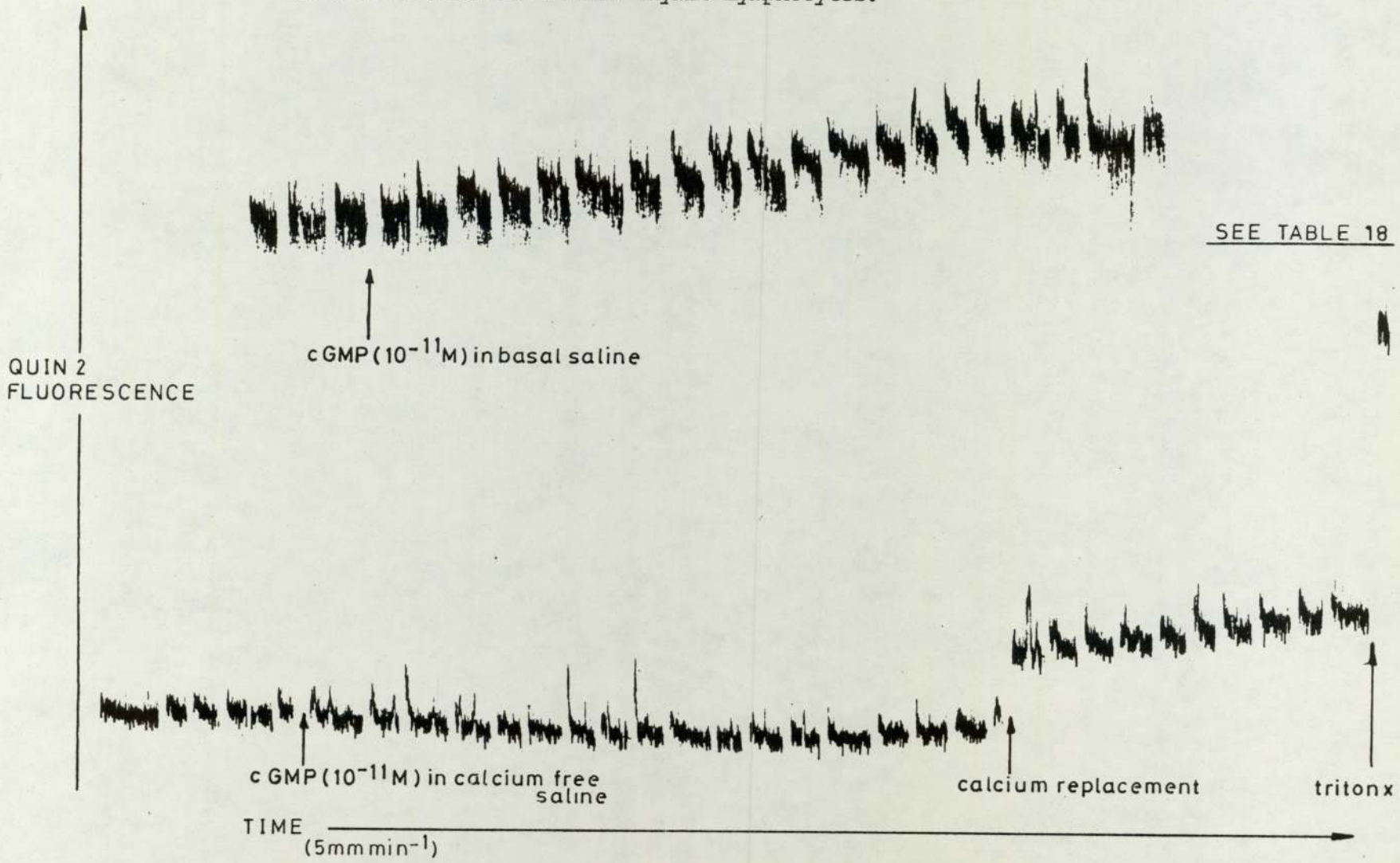
SEE TABLE 17

Records of Intracellular Calcium Concentration as Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.

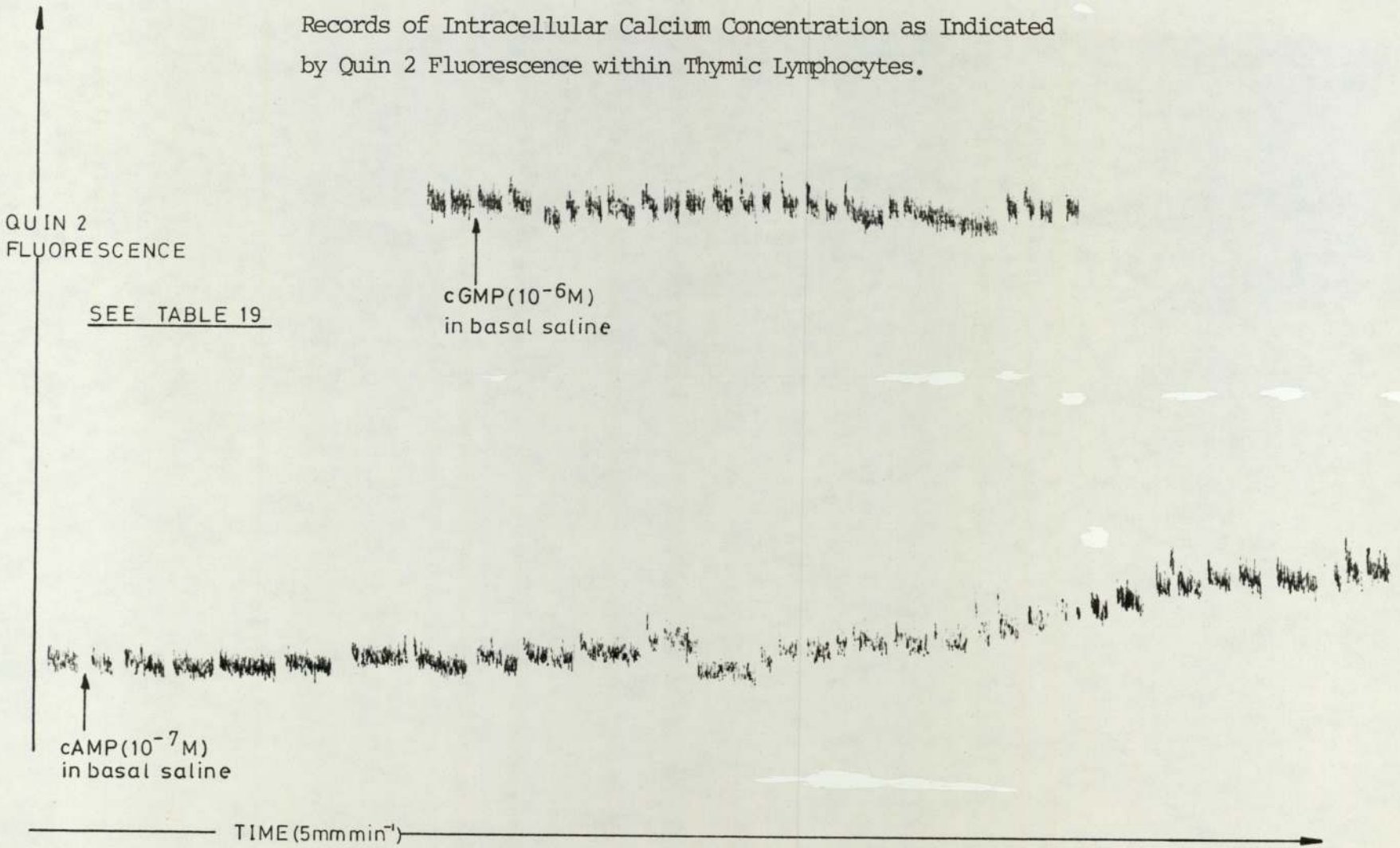


SEE TABLE 18

Records of Intracellular Calcium Concentration as Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.



Records of Intracellular Calcium Concentration as Indicated by Quin 2 Fluorescence within Thymic Lymphocytes.



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