'Signalling and control of locomotion in a T lymphocyte line'

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DEDICATION

This work is dedicated, with all my love, to my wife Ann, who supported, helped and encouraged me during the many months spent in the laboratory and subsequently at the keyboard of a computer. Her sense of organization and determination have been a valuable inspiration to me. I am very grateful for Ann's help with the proof-reading and with editing of the numerous drafts of this thesis.

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ABSTRACT

This study explored the intracellular signals that control the acquisition and maintenance of locomotory capacity in T lymphocyte line Jurkat. The majority of the actively dividing Jurkat cells show an immotile morphology. It was possible to induce the polarization and locomotion in the majority of these cells by culture in the presence of dibutyryl cyclic AMP for at least 3 days. Such culture resulted in a decrease in the Jurkat cell size, in a block in the first gap phase of the cell cycle and in an increased surface expression of CD3. These cells displayed a constitutively motile behaviour which was temperature and energy dependent. The polarization of the Jurkat cells was permanently reversed, in a short-term assay, by PHA and drugs which increase the phosphorylation of intracellular proteins (phorbol esters and phosphatase inhibitors) and which increase intracellular concentrations of cAMP. In contrast, the polarization was reversed transiently following a stimulation of the T cell receptor complex by an αCD3 monoclonal antibody or when the intracellular Ca^{2+} stores were emptied as a result of the inhibition of Ca^{2+} pump by thapsigargin. The effect involving PHA, αCD3 and thapsigargin is dependent on the presence of extracellular Ca^{2+}. The evidence suggests that PKC is the most important and common element in the inhibition of the polarization process in Jurkat cells. Indeed, the activity of most agents could be partially and dose-dependently inhibited using PKC inhibitors. The signal, which leads to the inhibition of polarization in dbcAMP-cultured Jurkat cells is coupled by a tyrosine kinase as well as by a pertussis toxin-sensitive G protein.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. GENERAL INTRODUCTION

1.1. Cells of the immune system

An organism is protected against invasion by foreign substances by three lines of defence: (1) the skin and other epithelial linings in contact with the environment; (2) the innate immunity comprising non-specific mechanisms interfering with the survival and growth of the pathogens; and (3) the immune system which defends the body by engaging humoral or cell-mediated immune response.

The immune system is mediated by leukocytes which comprise the 'white blood cells'. The leukocytes originate from primitive precursor stem cells residing principally in the bone marrow. Stem cells continuously divide and, under appropriate conditions, differentiate into cells of myeloid, mononuclear phagocyte, or lymphoid lineage.

Lymphocytes are small, free-living cells found in the blood and lymphatic circulatory systems and in all lymphoid organs and tissues. They may be also found in tissues underlying surface epithelium such as the lamina propria of the gut. The nucleus fills almost completely the perimeter of these cells. The remaining cytoplasm is relatively devoid of organelles. In an immune reaction, the lymphocytes are activated to undergo blast transformation and division. The cytoplasm of an activated lymphocyte resembles that of a metabolically active cell with abundant mitochondria and endoplasmic reticulum. After the infection has been eliminated, some of the cells revert to their small, apparently undifferentiated, form. However on restimulation, such lymphocytes, called memory lymphocytes, are capable of qualitatively different response compared to virgin lymphocytes.

Lymphocytes can be divided into three major groups based on function and cell surface characteristics. T cells undergo final maturation in the thymus. T helper cells interact with surface antigens present on the surface of B cells and assist them in the maturation process into antibody-

producing plasma cells. Cytotoxic T cells recognize pathogenic antigen structures presented by invaded tissues and destroy the infected cells.

1.2. Leukocyte adhesion

The exit of leukocytes from circulation and their migration to lymphoid tissues or inflammatory sites are prerequisites for eliciting an immune response. Leukocytes must adhere to endothelial cells to gain entry to tissues. Leukocyte adhesiveness is a precisely regulated process determined by presence of membrane receptors involved in cell-cell and cell-extracellular matrix recognition. Leukocytes express on their surface molecules which reflect, among others, their lineage, functional specificity and activation status. Similarly, endothelial cells express on their surface molecules that reveal the location and immunological state of the surrounding tissues. The regulation of differential migration of various leukocyte populations is thus mediated by altered expression of these surface molecules. Recently, the regulation of avidity of adhesion molecules has been implicated (Pardi *et al.*, 1995).

1.2.1. Selectins

The selectin or LEC-CAM family of adhesion molecules mediates the initial low-affinity reversible binding of leukocytes to the endothelium. Selectins are transmembrane glycoproteins which contain a single *N*-terminal lectin motif followed by a single epidermal growth factor repeat and a varying number of short consensus repeat homology units as found in complement binding proteins (Bevilacqua *et al.*, 1989; Lasky *et al.*, 1989; Camerini *et al.*, 1989; Tedder *et al.*, 1990; Siegelman *et al.*, 1989). All selectins recognize, via their lectin domain, ligands that are associated with fucosylated polylactosamines, such as sialyl-Lewis X tetrasaccharide (Rosen and Bertossi, 1994). However, additional tissue-specific ligands have been recently identified. These novel ligands are

mucin-like molecules, covered in dense patches of O-linked sugars (Hogg and Berlin, 1995). Standard nomenclature designates each family member according to the cell type on which it was originally identified: E-selectin (endothelium), P-selectin (platelets), and L-selectin (lymphocytes).

E-selectin (ELAM-1) expression is largely restricted to IL-1-, TNFα-, or LPS-activated endothelial cells (Bevilacqua *et al.*, 1987, 1989). Maximal surface expression is observed at 4 to 6 hrs followed by a decline toward basal levels by 24 to 48 hrs. IFN-γ alone does not induce the expression of E-selectin but it prolongs the expression in response to TNFα (Doukas and Pober, 1990; Leeuwenberg *et al.*, 1990). Although E-selectin can be expressed on inflamed endothelium in almost any tissue, it shows a biased expression on inflamed endothelium of skin (Picker *et al.*, 1991b). E-selectins were initially shown to mediate adhesion of neutrophils to cytokine-activated endothelium (Bevilacqua *et al.*, 1985; Gamble *et al.*, 1985) via their sialyl-Lewis X determinant. The scope of E-selectin binding activity was subsequently extended to monocytes and a highly restricted subpopulation of memory T lymphocytes (Graber *et al.*, 1990; Picker *et al.*, 1991a; Shimizu *et al.*, 1991; Carlos *et al.*, 1991; Hakkert *et al.*, 1991) marked by CLA.

P-selectin (PADGEM or GMP-140) is found in α and dense granules in resting platelets (Hsu-Lin *et al.*, 1984; Stenberg *et al.*, 1985; Israels *et al.*, 1992) and in Weibel-Palade bodies of endothelial cells (McEver *et al.*, 1989; Bonfanti *et al.*, 1989). A variety of mediators, including thrombin, histamine, and terminal complement components stimulate rapid surface expression of endothelial P-selectin (McEver *et al.*, 1989; Bonfanti *et al.*, 1989; Hattori *et al.*, 1989ab; Patel *et al.*, 1991). The expression of P-selectin at the cell surface is short lived, declining substantially within minutes. P-selectin expressed on activated platelets (Larsen *et al.*, 1989a; Hamburger and McEver, 1990; de Bruijne-Admiraal *et al.*, 1992) and on endothelium (Etingin *et al.*, 1991; Geng *et al.*, 1990) supports leukocyte adhesion, including neutrophils, monocytes and a subpopulation of T lymphocytes.

L-selectin (mLHR, Leu8, TQ-1, gp90^{MEL-14}, LAM-1, or LECAM-1) is constitutively expressed on lymphocytes, monocytes and neutrophils. The ligand for L-selectin has been identified as GlyCAM-1 and MAdCAM-1. GlyCAM-1 is a glycoprotein associated with peripheral lymph node HEVs (Lasky *et al.*, 1995) whereas MAdCAM-1 is expressed on mucosal lymphoid tissues (Hogg and Berlin, 1995).

Transient expression of selectins on activated leukocytes (Tedder *et al.*, 1990; Kishimoto *et al.*, 1989a) and endothelial cells (Bevilacqua *et al.*, 1989; Hattori *et al.*, 1989a) appears to be regulated by selective loss from the cell surface resulting from a proteolytic susceptibility built into selectin molecules.

1.2.2. Integrins

Bimolecular interactions between immunoglobulin-related cell adhesion molecules (IgCAMs) and integrins play key roles in firm adhesion and subsequent transmigration of blood lymphocytes. Integrins are heterodimers of noncovalently associated α and ß subunits categorized into subfamilies with members sharing a common ß subunit combined with a unique α subunit.

The members of IgCAM family, ICAM-1 (CD54), ICAM-2 (CD102) are transmembrane glycoproteins containing 5 and 2 extracellular Ig domains respectively (Simmons *et al.*, 1988; Staunton *et al.*, 1988, 1989). Recently, ICAM-3 has been identified (Defougerolles *et al.*, 1994). Resting lymphocytes lack ICAM-1 expression. However, this expression increases over days during T and B lymphocyte activation (Dustin *et al.*, 1986; Clark *et al.*, 1986; Wawryk *et al.*, 1989) to facilitate the cell-cell interactions. Activated lymphocytes in germinal centres and in sites of inflammation are strongly positive (Dustin *et al.*, 1986; Wantzin *et al.*, 1989). In contrast, monocytes contain an intracellular store of ICAM-1 which can be mobilized to the cell surface by adherence to fibronectin (Dougherty *et al.*, 1988). ICAM-1 is absent from most cells in normal,

nonlymphoid tissues, except for expression of low levels on endothelial cells. Local inflammation results in a rapid increase in ICAM-1 expression on endothelial cells and induction of ICAM-1 on epithelial and mesenchymal cells (Dustin *et al.*, 1986; Dustin and Springer, 1988; Wantzin *et al.*, 1989; Munro *et al.*, 1989). Like E-selectin, ICAM-1 is expressed on vascular endothelium after several hours of stimulation by IL-1 or TNFα (Dustin *et al.*, 1986; Pober *et al.*, 1986). However, there is a low level ICAM-1 expression on unstimulated endothelium and the cytokine-induced increase of expression is slower and prolonged in comparison to E-selectin. ICAM-2 expression on vascular endothelium does not appear to be regulated by cytokines (Dustin and Springer, 1988; Staunton *et al.*, 1989; Nortamo *et al.*, 1991). Neutrophils, monocytes, lymphocytes, and NK cells express LFA-1 ($\alpha_L\beta_2$ or CD11a/CD18) which binds both ICAM-1 and ICAM-2 (Stoolman, 1989; Carlos and Harlan, 1990; Springer, 1990; Arnaout, 1990). The ICAM-2/LFA-1 interaction accounts for two thirds of LFA-1-specific adhesion. In addition, neutrophils, monocytes, and NK cells (but not lymphocytes) express Mac-1 ($\alpha_M\beta_2$ or CD11b/CD18) and p150,95 ($\alpha_x\beta_2$ or CD11c/CD18) found at the cell surface as well as in storage granules. Activation of leukocytes results in increased adhesive capacity of β_2 integrins regulated by quantitative changes in the expression of Mac-1 and p150,95 (Stoolman, 1989; Carlos and Harlan, 1990; Springer, 1990; Arnaout, 1990) and by qualitative changes in LFA-1. Phorbol esters stimulate a rapid (<1 hr) increase in lymphocyte aggregation mediated by LFA-1/ICAM pathway (Patarroyo *et al.*, 1985; Rothlein and Springer, 1986; Rothlein *et al.*, 1986; Hamann *et al.*, 1986) which does not involve changes in LFA-1 or ICAM-1 surface density (Rothlein *et al.*, 1986). Indeed, phorbol esters, as well as cross-linking TcR on resting T lymphocytes, increase the avidity of cell surface LFA-1 for ICAM-1. Such an 'inside-out' signalling pathway offers a mechanism for antigen dependent adhesion (Dustin and Springer, 1989). In contrast to the phorbol ester-stimulated increase in LFA-1 avidity which persists for several hours, the increase triggered by cross-linking TcR is

transient, peaking at 10-20 min and decreasing to basal levels by 40 min.

A fourth memeber of IgCAM family has been identified as VCAM-1 (INCAM-110) containing 6 extracellular Ig domains (Osborn *et al.*, 1989). High levels of VCAM-1 are found on endothelial cells at sites of inflammation (Rice *et al.*, 1990) whereas expression on normal tissues is minimal (Rice *et al.*, 1991; Cybulski and Gimbrone, 1991; van Dinther-Janssen *et al.*, 1991). Indeed, VCAM-1 is expressed by non-specialized endothelial cells only after pretreatment with TNFα, IL-1ß, LPS (Rice and Bevilacqua, 1989; Osborn *et al.*, 1989) and IL-4 (Thornhill and Haskard, 1990; Schleimer *et al.*, 1992) but not IFN-γ (Carlos *et al.*, 1990). Cytokines upregulate the VCAM-1 expression with maximal activity at 6 to 12 hrs and like that of ICAM-1, the high level of expression is prolonged by comparison to E-selectin (Rice and Bevilacqua, 1989; Rice *et al.*, 1990; Schwartz *et al.*, 1990; Wellicome *et al.*, 1990). The binding of lymphocytes to peripheral lymph node HEV is mediated, in part, by VCAM-1 (May *et al.*, 1993). VCAM-1 is constitutively expressed by rat HEC *in vivo* and *in vitro* and is upregulated by TNFα, IL-1ß and IFN-γ (May *et al.*, 1993). So far, VCAM-1 has only been detected on HEV in inflamed lymph nodes of humans and sheep (Rice *et al.*, 1991; Mackay *et al.*, 1992a). VCAM-1 is also expressed on several non-vascular cell types, including dendritic cells found in lymph nodes and skin, bone marrow stromal cells, and synovial cells in inflamed joints (Rice *et al.*, 1991; Miyake *et al.*, 1991; Ryan *et al.*, 1991; Marlor *et al.*, 1992; Simmons *et al.*, 1992). Endothelial VCAM-1 was shown to support adhesion of lymphocytes and monocytes (but not neutrophils) through an interaction with the $ß_1$ integrin VLA-4 (LPAM-1, $\alpha_4ß_1$ or CD49d/CD29) (Graber *et al.*, 1990; Rice *et al.*, 1990; Schwartz *et al.*, 1990; Carlos *et al.*, 1990; Oppenheimer-Marks *et al.*, 1991; Elices *et al.*, 1990; Osborn *et al.*, 1989) which also binds fibronectin (Wayner *et al.*, 1989; Guan and Hynes, 1990). In contrast to VCAM-1, the fibronectin binding of VLA-4 is upregulated following treatment with IFN-γ (May and Ager, 1992). VLA-4 interacts with at least three HEC fibronectin sites, CS-1 and

REDV in the type III connecting segment, and H1 in the heparin-binding (HepII) region (Ager and Humphries, 1990; Szekanecz *et al.*, 1992). These regions are distinct from the RGD region used by another β_1 integrin VLA-5, the classical fibronectin receptor (Elices *et al.*, 1990; Guan and Hynes, 1990). In addition, other β_1 integrins mediate adhesion of different cell types to extracellular components such as collagens, fibronectin and laminin (Albelda and Bucks, 1990).

Integrin LPAM-2 ($\alpha_4\beta_7$ or $\alpha_4\beta_p$) is expressed on all peripheral blood lymphocytes and mediates their adhesion to VCAM-1 and fibronectin (Chan *et al.*, 1992a). In addition to L-selectin, a lymphocyte cell surface integrin LPAM-2 ($\alpha_4\beta_p$ or $\alpha_4\beta_7$) can support adhesion to mucosal HEV in Peyer's patches (Holzmann *et al.*, 1989; Holzmann and Weissman, 1989a). It is now clear that in the mucosa, the ligand MAdCAM-1 supports L-selectin binding via its mucin-like domain and LPAM-2 binding via its N-terminus ICAM/VCAM-like domain.

1.2.3. Mechanism of lymphocyte extravasation

The reversible binding mediated by the selectins causes the circulating leukocytes to decelerate and attach to the luminal surface of post-capillary venules. *In vivo*, leukocytes appear to roll along the inflammed endothelium (Arfors *et al.*, 1987; Fiebig *et al.*, 1991; Ley *et al.*, 1991; Tengelder and Arfors, 1991). The early phases of the response are dominated by P-selectin, followed by either L-selectin or E-selectin (Mayada *et al.*, 1993; Arbones *et al.*, 1994; Labow *et al.*, 1994; Ley *et al.*, 1995). In addition, lymphocytes use their α_4 integrins, especially LPAM-2, to mediate the rolling and firm adhesion under flow conditions (Berlin *et al.*, 1995).

The rolling phase occurs independently of leukocyte activation, while the phase of integrin-mediated arrest and firm adhesion require a leukocyte signalling event. The soluble mediators, released in the inflammatory sites, may be captured on endothelial glycosaminoglycans,

such as CD44 (Hermes-1) and syndecan (Hogg and Berlin, 1995). These surface molecules thus ensure localization of these mediators under blood flow conditions and act as scaffolding for optimal presentation to leukocytes.

The following sequence of events resulting in leukocyte migration was suggested by Dustin and Springer (1991). The initial transformation from a nonadherent cell in the blood to an adherent and polarized cell attached to the blood vessel wall results in activation of integrin cycling. Indeed, engagement of LFA-1 was shown to result in the production of intracellular secondary messengers known to be associated with lymphocyte activation (Wacholtz *et al.*, 1989). Once in the tissue, T lymphocytes remain polarized and locomote by forming transient bonds to the extracellular matrix and cell surfaces. The direction of migration may be dictated by chemotactic factors produced at a focus of inflammation (Larsen *et al.*, 1989b) or may be guided by extracellular matrix components or counter-receptors such as ICAM-1 induced in inflammatory sites (Dustin *et al.*, 1986). When lymphocytes encounter cells at their leading edge, weak adhesive interactions form which allow TcR time to encounter and bind appropriate foreign antigen/MHC complexes if present. TcR ligation leads to strengthening of adhesion and subsequent cell-cell interaction. Once this interaction is complete, as indicated by a change in the antigen-bearing cell, the avidity of integrins decreases to the basal migrating levels. The polarity of lymphocytes then becomes random resulting in their locomotion away from the target cell. The lymphocytes are active in this detachment process, using interactions with neighbouring extracellular matrix or cells to undermine the remaining adhesive interactions.

1.2.4. Role of extravasation in recruitment

T lymphocytes may leave the blood in peripheral vascular beds, particularly within inflammatory lesions. These cells drain to local lymph

nodes via afferent lymphatic vessels. The endothelium of the microvascular venules is activated by locally released cytokines inducing specific adhesion molecules on endothelial cells. Such adhesion molecules then bind and trigger transmigration of specific leukocyte subpopulations. P-selectin is translocated to the surface from preformed cytoplasmic stores within seconds of stimulation by acute inflammatory mediators (Hattori *et al.*, 1989ab; Bonfanti *et al.*, 1989). IL-1, TNFα and LPS induce expression of E-selectin (Bevilacqua *et al.*, 1987), VCAM-1 (Elices *et al.*, 1990), and ICAM-1 (Pober *et al.*, 1986). E-selectin peaks within hours after activation returning to baseline within 24 h (Bevilacqua *et al.*, 1987). In contrast, VCAM-1 peaks after 24 h and remains elevated for days *in vitro* (Elices *et al.*, 1990). These differences have lead to the speculation that P- and E-selectins function early in the inflammation with the contribution from VCAM-1 increasing as the lesion matures or goes to chronicity. Indeed, mAb to E-selectin inhibit neutrophil flux into the lung 6 h following deposition of immune complexes (Mulligan *et al.*, 1991) and mAb to VLA-4 partially inhibit lymphocyte migration into delayed-type hypersensitivity lesions in the skin and joints (Issekutz and Wykretowicz, 1991; Issekutz, 1991). The sequence observed with monocytes and skin-homing memory T lymphocytes indicates that cellular arrest occurring via α_4 integrin is followed by firm adhesion and transmigration by means of the LFA-1/ICAM-1 interaction (Luscinskas *et al.*, 1994; Babi *et al.*, 1995). This proposal is supported by the following observations: (1) adhesion of T lymphocytes to cultured EC is mostly inhibited by mAb to LFA-1 (Haskard *et al.*, 1986); and (2) the kinetics of lymphocyte entry into sites of inflammation are similar to kinetics of ICAM-1 upregulation.

1.2.5. Role of extravasation in recirculation

Lymphocyte recirculation differs from recruitment in that trafficking through tissues occurs constantly (Yednock and Rosen, 1989; Stoolman, 1989). The vast majority of T cells recirculates by crossing the walls of

specialized lymph node post capillary venules lined with high endothelial cells (HEV). Following binding to the luminal surface of HEV, lymphocytes reach the basal lamina within 5 to 10 min (Fossum *et al.*, 1983). After 18 to 20 hr residency in the node, T cells leave through the efferent lymphatic duct. HEV of lymphoid organs are the most efficient sites of lymphocyte extravasation. HEV also occur at sites of chronic inflammation although postcapillary venules can become adhesive for lymphocytes in acute inflammation without acquiring the high phenotype (Duijvestijn and Hamann, 1989; Smith *et al.*, 1970; Iguchi and Ziff, 1986). Other abundant leukocytes, such as neutrophils, do not normally migrate across these vessels (Gowans and Knight, 1964). The majority of unactivated lymphocytes recirculate through the spleen and lymph nodes for efficient antigen encounter (Duijvestijn and Hamann, 1989). The terms 'homing receptor' and 'vascular addressin' are used to describe cell surface adhesion molecules on lymphocytes and endothelium (usually in HEV) respectively. These molecules contribute to tissue-specific localization of lymphocytes (Picker and Butcher, 1992; Holzmann and Weissman, 1989b). L-selectin appears to function as a homing receptor for peripheral lymph nodes where L-selectin binds the lymph node addressin originally identified by antibody MECA 79 (Streeter *et al.*, 1988). L-selectin mediates trafficking or homing of lymphocytes to peripheral and mesenteric lymph nodes due to selective expression of a unique counterreceptor for L-selectin on the HEV in these organs (Lasky *et al.*, 1992). An organ specific mechanism for lymphocyte adhesion to peripheral lymph node HEV is inhibited by mAb MEL-14 to the L-selectin (Gallatin *et al.*, 1983). In contrast, the β_1 integrin LPAM-1 appears to mediate homing to Peyer's patches and mesenteric lymph nodes (Issekutz, 1991; Holzmann *et al.*, 1989; Holzmann and Weissman, 1989ab). Adhesion to Peyer's patch HEV is inhibited by mAb to LPAM-1, VLA-4 and CD44 (Holzmann and Weissman, 1989b; Jalkanen *et al.*, 1987). Lymphocyte adhesion to peripheral and mucosal lymph node and to synovial HEV is inhibited 50-60% by mAb to LFA-1 (Duijvestijn and Hamann, 1989; Hamann *et al.*,

1988; Pals *et al.*, 1988). Consistent with expression of ICAM-2 on endothelial cells *in vitro*, mAb to ICAM-1 does not inhibit lymphocyte binding to HEV (Duijvestijn and Hamann, 1989).

1.2.6. Memory versus naive T lymphocytes

Memory and naive lymphocytes recirculate differently, based on differences in recirculation patterns in neonatal versus adult animals. Although naive and memory T lymphocytes are morphologically indistinguishable, stable changes in the expression of a variety of surface molecules reflect the prior activation. E-selectins bind only memory-type T cells (Picker *et al.*, 1991a) consistent with observations reporting the accumulation of memory T cells in inflammatory lesions containing antigens or pathogens which the host has encountered previously. Memory T cells are also the only T cells which recirculate through uninflamed tissues (Mackay *et al.*, 1990b), providing the necessary surveillance. In contrast, new antigens drain to local lymph nodes which are frequented by lymphocytes of diverse specificities, particularly naive T cells (Mackay *et al.*, 1990b).

Together with other surface molecules, a number of adhesion receptors are also increased on memory T lymphocytes. LFA-3 is negative on naive and positive on memory T lymphocytes while the expression of others, including LFA-1, CD2, β_1 integrin (CD29) and CD44, is two- to four-fold higher on the memory T lymphocytes. In general, the differences in naive and memory T cell subpopulations are independent of CD4/CD8 phenotype although all CD8 cells seem to have higher LFA-1 levels (Pardi *et al.*, 1989; Mackay *et al.*, 1990b). It appears that only naive cells enter lymph nodes through HEV, while memory lymphocytes leave the blood at other sites and enter lymph nodes through lymphatic drainage from these tissues (Mackay *et al.*, 1990b). This situation may arise by a more efficient binding of memory lymphocytes to other postcapillary venules or less efficient binding to lymph node HEV. Memory T lymphocytes show

stronger adhesion to monokine-stimulated EC cultures than do naive cells (Pitzalis *et al.*, 1988). Activated T lymphocytes adherent to purified ICAM-1 were enriched for cells expressing higher LFA-1 suggesting that, in lymphocytes in similar activation state, differences in LFA-1 surface expression on the order of 2.5-fold are functionally significant (Dustin *et al.*, 1988).

1.3. Lymphocyte motility

1.3.1. Morphology of motile lymphocytes

The earliest studies that yielded observations of motile lymphocytes were performed by Lewis and Webster (1921) using explants of rat lymph nodes. In contrast, McCutcheon (1924) used lymphocytes from blood cultured over a period of hours. In his subsequent studies, Lewis (1931) used time-lapse cinematography to analyze the locomotion of rat lymph node lymphocytes in detail:

> "During a period of rest the lymphocyte is spherical but pseudopodia are sometimes thrust out and retracted without producing more than a slight wobbling motion of the whole cell. When, however, the rest period is over and the lymphocyte begins to migrate, a single pseudopodium flows out from a relatively small area. As the pseudopodium enlarges, a groove develops at its base where it joins the body of the cell. This groove or constriction ring corresponds to the rim about the softened area which gives rise to the pseudopod. As the pseudopod increases in length and breadth the groove usually becomes more marked. Thus, when the groove first appears, in front of it is the expanding pseudopod and behind it the body of the lymphocyte with its large nucleus."

The constriction ring remained stationary while the lymphocyte moved towards or away from it. The locomotory lymphoblasts from rabbit lymph nodes observed by Rich *et al.* (1939) were characterized as having "...the general outlines of a handmirror." Similar observations were made by de Bruin (1944) who recognized that:

> "... a leukocyte moving on flat surface can be divided into three portions: (1) an anterior portion, which is the most active in locomotion; this portion gives rise to pseudopodia, (2) a relatively quiet body, which contains the nucleus, (3) a posterior part, the tail, which appears to be completely passive."

He suggested that such functional partitioning of a motile lymphocyte may be expressed as polarity and that:

> "It is this polarisation, or rather the degree of polarisation, which is characteristic for the different leukocytes in motion."

1.3.2. Acquisition of locomotory capability by lymphocytes

Soluble chemical substances in the lymphocyte environment may determine the speed at which the cells move (chemokinesis), as well as the direction of their locomotion (chemotaxis). The early studies reported that lymphocytes moved into filters placed over a compartment with culture supernatants of immunized guinea pig lymph node cells restimulated with the antigen *in vitro* (Ward *et al.*, 1971) or with proteolytically modified immunoglobulin (Higuchi *et al.*, 1975). The methods used were unable to distinguish between chemokinesis and chemotaxis. The first report of chemotactic response was observed in B lymphoblasts examined in checkerboard assays (Russell *et al.*, 1975).

1.3.3. Locomotion in lymphoblasts

The lymphocytes which most clearly localize in inflammatory lesions *in vivo* are members of the blast-transformed population (Asherson *et al.*, 1973; Moore and Hall, 1973; McGregor and Logie, 1974). Indeed, human B lymphoblasts from continuous cell-line cultures showed an increase in locomotion in the presence of a chemoattractant (*i.e.* endotoxin-activated plasma or casein) although their motility was low in an unstimulated state (Russell *et al.*, 1975). Several B cell lines showed a

response consistent with chemotactic reaction. On the other hand, oxazolone-sensitized mouse T lymphoblasts showed high unstimulated motility and only a chemokinetic response to the chemoattractants (Russell *et al.*, 1975).

1.3.4. The acquisition of motility during cell culture

The morphological examination of lymphocytes direct from blood revealed that these cells are mostly immotile (Wilkinson, 1986). When cultured for 24 hrs with only FCS, such cells responded by increased polarization to FCS, endotoxin-activated serum and alkali-denatured HSA; however, the majority of these cells remained round and unresponsive. In addition, human peripheral blood lymphocytes, cultured for 3 days with FCS in absence of mitogens, migrated only very short distances into filters and were not stimulated by chemoattractants (Wilkinson *et al.*, 1976).

On the other hand, lymphocytes stimulated by T cell mitogens PHA and Con A responded by chemokinesis and chemotaxis to casein, alkali-denatured HSA, endotoxin-activated plasma and even PHA (Wilkinson *et al.*, 1976). Many of the responding cells were blasts but cells of all sizes entered the filters. The need for the presence of mitogen during the culture was questioned by O'Neill and Parrott (1977) who determined that the presence of Con A was not essential during culture of human peripheral blood lymphocytes to induce locomotor responses to endotoxin-activated serum or casein.

The cultures without the mitogen contained virtually no blasts yet showed equivalent chemokinesis towards the chemoattractants (O'Neill and Parrott, 1977). Wilkinson *et al.* (1977) showed that even small post-blast cells obtained from lymph nodes of immunized mice, 10 days after challenge, migrated actively when compared to non-immunized small lymphocytes. Time-course studies with PHA revealed that although 80-90% of lymphocytes polarized after a 48-hr culture, 50-70% of lymphocytes changed shape within 24 hrs which is before the time that

lymphoblasts appear (Wilkinson, 1986).

1.3.5. Locomotory properties of lymphocyte subpopulations

The examination of various lymphocyte subsets revealed complexity reflecting the differences in activation requirements and the ability to respond to chemoattractants. Schreiner and Unanue (1975) observed spontaneous motility in mouse lymph node T lymphocytes while B lymphocytes showed chemokinesis when stimulated with high concentrations of αIg. In a later study they showed that T-enriched lymphocytes from rat spleens were able to respond to supernatants from MLC and phagocytosing macrophages while B-enriched cells responded by chemotaxis only to low concentrations of αIg (Ward *et al.*, 1977). In contrast, separated T and non-T human peripheral blood lymphocytes both responded to casein and endotoxin-activated serum; however, T lymphocytes had to be cultured to acquire the ability for locomotor response while non-T cell population was often motile when isolated from blood (O'Neill and Parrott, 1977). Experiments exploring the locomotion of human T lymphocyte subsets revealed that the suppressor Tγ lymphocytes showed little ability to respond to casein while helper Tμ lymphocytes moved very well into filters placed over the chemoattractant (Parrott *et al.*, 1978).

1.3.6. Physiological induction of motility in lymphocytes

The proposed physiological activator of lymphocyte locomotion is antigen. Thus, mouse lymphocytes from the lymph nodes draining a site of contact sensitization (Shields *et al.*, 1984) or draining a site of challenge with a protein antigen (Wilkinson *et al.*, 1977) are more motile than lymphocytes from control nodes. Here, the antigenic sensitization could be detected *in vitro* as these lymphocytes responded to the same antigens by increased locomotion in a micropore filter assay. The analogue of

TcR/CD3 in B lymphocytes is the immunoglobulin molecule expressed on its surface. Indeed, as stated above, cross-linking of this activation molecule with αIg induced motility in B cells. Similarly, direct activation through TcR/CD3 stimulated in MLC or by culture with PPD or αCD3 induced an increase in number of polarized cells within 24 hrs and reached maximum after 48 hrs (Wilkinson, 1986).

The mitogenic activity of αCD3 was demonstrated in many systems as a wide range of activation-associated events. Further inquiry into the mechanism of αCD3 action showed that little polarization occurred before 8 hrs of culture (Wilkinson and Higgins, 1987a). In addition, the polarization of lymphocytes exposed to the monoclonal antibody only for the first 30 min of the culture was comparable to that in cultures which remained in the presence of αCD3 for the duration of the incubation (Wilkinson and Higgins, 1987a). Some original observations suggested that mitogen-activated cultures release factors which induce locomotor capacity in lymphocytes; these related to the presence of cell density-dependent polarization in FCS cultures (Wanger *et al.*, 1985). Active factors were identified in Con A stimulated mononuclear cell cultures (Van Epps *et al.*, 1983), in mitogen-stimulated lymphocyte cultures (Center and Cruikshank, 1982) and in mixed lymphocyte cultures (El-Naggar *et al.*, 1982). In contrast, Wilkinson and Higgins (1987a) determined that the effect of αCD3 and Con A, although cell density-dependent, was not mediated by a soluble factor but required direct contact with the AC. Indeed, the effect of αCD3 was abrogated by an excess of intact human IgG molecule or once the Fc portion was removed by pepsin digestion (Wilkinson and Higgins, 1987a). Similarly, the polarization in response to αCD3, but not PHA, was inhibited in cultures depleted of Fc receptor-bearing cells. Thus, it was determined that αCD3 cross-links Fc receptor-bearing cells such as monocytes or B cells resulting in activation of locomotory capability (Wilkinson and Higgins, 1987a) and of other events such as proliferation.

1.3.7. Induction of lymphocyte motility by phorbol esters

Phorbol esters were shown to induce lymphocyte growth following a long-term exposure (Touraine *et al.*, 1977; Abb *et al.*, 1979; Kabelitz *et al.*, 1982). When used with lymphocytes fresh from blood, PMA polarized about 40% compared to 43% with colchicine (Wilkinson and Higgins, 1987b). The observation of time-course of PMA-induced shape change showed that the cells started to deviate from a round morphology within 1-2 min and reached maximum effect in 5-15 min (Wilkinson *et al.*, 1988). However, although the short-term PMA treatment resulted in antero-posterior polarity, this was not as well defined as the 'classical' polarized morphology and constriction rings were rare. Many cells showed rapidly extended and retracted veils at one or more sites on the cell surface. Due to this difficulty, the term 'non-spherical' was used to describe these morphologies and they constituted 40-50% of all cells treated with PMA for 30 min (Wilkinson *et al.*, 1988). In addition, these cells were determined to be non-motile as they did not enter collagen gels or nitrocellulose filters in comparable numbers. The shape change resulting from 30 min exposure to PMA was not affected by the absence of divalent cations in the medium or by the presence of PKC inhibitors H-7 or W-7 (Wilkinson *et al.*, 1988).

When PMA, at a concentration unable to produce a shape change in a short-term assay, was added to the culture of lymphocytes for 24 hrs, 70-80% of these cells displayed a polarized morphology (Wilkinson and Higgins, 1987b). Although the morphology induced by PMA was not identical to that seen with other activators, these cells vigorously invaded collagen gels and nitrocellulose filters (Wilkinson and Higgins, 1987b; Wilkinson *et al.*, 1988). The morphology of these cells was more elongated than that seen following a 30 min treatment. Also, the protrusions and veils were more persistent and the constriction rings were more common. The PMA-induced lymphocyte shape change was not affected by culture in divalent cation-free medium (Wilkinson *et al.*, 1988).

1.3.8. Induction of lymphocyte motility by 'cell growth'

The common feature of observations made with the various mitogens was the relative length of time required by lymphocytes to acquire the locomotor capacity. The reason for this became apparent when the motile lymphocytes obtained following a 24-hr culture with PHA were found generally larger and more active in RNA and protein synthesis (Wilkinson, 1986). Similar observations were made with B cell-enriched lymphocytes cultured with growth factors such as IL-4 or IFN-γ, where the increase in size was associated with motility as measured by polarization and collagen gel invasion (Wilkinson and Islam, 1989). Under such conditions, the increase in lymphocyte motility preceded the DNA synthesis and was inhibited by protein synthesis inhibitor cycloheximide but not by DNA synthesis inhibitor mitomycin *c* (Wilkinson, 1986). Cycloheximide had no effect when added to PHA-activated lymphocytes indicating that once the motility is acquired, protein synthesis is not necessary for its maintenance (Wilkinson, 1986). This sequence of events suggests that the locomotor capacity increases as the cells progress through the G_1 phase towards the S phase of the cell cycle. Cyclosporin A and FK506 are immunosuppressants that inhibit the expression of mRNA for activation lymphokines and thereby inhibit activation of lymphocytes at some point in G_1 (Klaus and Chisholm, 1986; Klaus and Hawrylowicz, 1984). As predicted, both of these drugs inhibited dose-dependently the acquisition of the locomotory capability as seen in 24-hr cultures of: (1) T lymphocytes with αCD3, PHA, or B lymphocytes with *Staphylococcus aureus* Cowan strain in the presence of cyclosporin A (Wilkinson and Higgins, 1987b); (2) B-enriched mononuclear lymphocytes with IL-4 in the presence of cyclosporin A (Wilkinson and Islam, 1989); (3) T lymphocytes with αCD3 and (4) B lymphocytes with IL-4 in the presence of FK506 (Wilkinson and Watson, 1990). However, motile lymphocytes fresh from blood (*i.e.* stimulated with FCS or PMA) or activated in a culture (*i.e.* with PMA or HSA) were not affected by either cyclosporin A (Wilkinson

and Higgins, 1987b) or FK506 (Wilkinson and Watson, 1990). Ratner *et al.* (1988) examined, using flow cytometry, the cell cycle profiles of mouse lymphocytes stimulated with Con A and subsequently allowed to invade collagen gels. They also concluded that the Con A-induced motility commences before S phase and diminishes shortly before or during G_2/M. Therefore, does the cellular synthetic activity generate some component of locomotory machinery or some element of signal transduction pathway linking this machinery to the extracellular signal?

1.3.9. Evidence for constitutively functioning locomotory machinery

The ability of colchicine to polarize about 46% of lymphocytes fresh from blood over the period of 30 min (Wilkinson, 1986) showed that simple microtubule disassembly allows for the expression of motile behaviour. Indeed, lymphocytes treated with colchicine or vinblastine are actively motile and rapidly invade collagen gels (Haston *et al.*, 1982; Haston and Shields, 1984). Similarly, 80% of fresh neutrophils may be polarized by colchicine (Keller *et al.*, 1984). On the other hand, D_2O which stabilizes microtubule polymers, completely but reversibly inhibited polarization of neutrophils (Zimmermann and Keller, 1984; Zimmermann *et al.*, 1988) and lymphocytes cultured in the presence of FCS (Wilkinson, 1986). In addition, the existence of functioning locomotory machinery in fresh lymphocytes was suggested by the ability of chemoattractants, such as FCS and endotoxin-treated human serum, to induce a shape change in many such cells within 30 min (Wilkinson, 1986). Thus, the responsiveness to chemoattractants seems to stem from activation of a signal transduction pathway which links surface receptors for such factors with the intracellular locomotory machinery.

1.3.10. Importance of chemoattractants in induction and maintenance of lymphocyte motility

The chemoattractants may be generated at the same time as the signal pathways are activated. This point was illustrated in experiments where B-enriched cells, activated by culture with IL-4 and washed, were able to repolarize only in 24-hr culture supernatant but not in fresh medium or in pure IL-4 (Wilkinson and Islam, 1989). Although the existence of such chemoattractant was not ascertained by Wilkinson and Higgins (1987a) their experiments monitored an effect of 24-hr culture supernatants on lymphocytes cultured without αCD3 or Con A. Consequently, such cells would not express an activated transduction pathway capable of transmitting the stimulatory signal for locomotion.

IL-8, also known as NAP-1 has been recently identified as a lymphocyte chemoattractant (Larsen *et al.*, 1989b; Leonard *et al.*, 1990). In human mononuclear cell cultures containing αCD3 or PPD, IL-8 was released into the culture medium reaching maximal levels after 48 hrs (Wilkinson and Newman, 1992). These supernatants and human recombinant IL-8 induced increase in polarization and collagen gel invasion in lymphocytes cultured with FCS; this activity could be inhibited by αIL-8. In orientation assays it was shown that IL-8 is a true chemotactic factor for lymphocytes (Wilkinson and Newman, 1992). Interestingly, although the levels of IL-8 detected in the presence of PPD were superior to those observed with αCD3, PPD-activated cultures yielded a smaller proportion of polarized cells. In agreement with the suggestion that cells first have to be activated through TcR/CD3 before lymphocytes can respond to IL-8, it is clear that PPD-specific lymphocytes would constitute a minority of the general population while αCD3 is a polyclonal activator. This may be supported by the superior ability of memory or effector $CD45RO^+$ cells to polarize in response to hrIL-8 and the supernatants compared to naive or resting $CD45RA^+$ cells (Wilkinson and Newman, 1992).

2. AIMS OF THE STUDY

2.1. BACKGROUND

2.1.1. Requirements for physiological T cell activation

Proliferation of T lymphocytes *in vivo* is driven by response to antigens. In general, antigens are recognized by T lymphocytes via the TcR during cell-cell interactions with AC. The antigens must be first 'processed' by an AC before they are displayed on its surface bound to a protein product of the MHC genes. The TcR interacts with the processed antigen and simultaneously with a class I or class II MHC glycoprotein. Activation of the T cell induced by interaction of TcR with antigen presented on the surface of AC results in transmembrane signals, the generation of intracellular second messengers and ultimately in the cell proliferation.

2.1.2. Events defining T lymphocyte activation

The G_0, or resting, T lymphocytes have a relatively low RNA content. These lymphocytes, when activated *in vivo*, respond by mitogenesis. The process of activation can be divided in terms of different stages of cell cycle as a progress from G_0 into G_1 and finally into the $S/G_2/M$ phase.

At 4 to 16 hrs after appropriate stimulation, the cells enter G_1 phase which is accompanied by the 'early' events of lymphocyte activation. These 'early' events comprise the increase in RNA content, *de novo* expression of activation-associated surface proteins such as IL-2r (Cotner *et al.*, 1983; Cantrell and Smith, 1983; Bruszewski *et al.*, 1984; Williams *et al.*, 1985) and insulin receptor (Helderman and Strom, 1979). The expression of activation-associated antigens correlates highly with T cell entry and transit of the S phase (Cotner *et al.*, 1983; Cantrell and

Smith, 1983) and thus may constitute an obligatory step in the induction of T lymphocyte proliferation (Cantrell and Smith, 1984). The synthesis of IL-2 is dependent upon the availability of AC-derived IL-1 (Larsson *et al.*, 1980) and is followed by proliferation of IL-2r-positive cells triggered by the interaction of IL-2 with its receptor (Smith, 1980; Cantrell and Smith, 1983). The cells in the $S/G_2/M$ phase are characterized by the 'late' events of T cell activation namely detectable DNA synthesis and cell proliferation.

2.1.3. Polyclonal lymphocyte activation with chemical agents

The AC-dependent molecular events of primary T cell activation cannot be examined conveniently *in vitro* due to the small size of the antigen-reactive clone population (Williams *et al.*, 1984b; Ythier *et al.*, 1985). A possible exception is the use of PPD to induce blast transformation and mitogenesis in a relatively large population of human lymphocytes from tuberculin-sensitive donors (Schrek, 1963).

The polyclonal proliferation of peripheral T lymphocytes can be activated by a variety of chemical agents including lectins (Nowell, 1960), sodium periodate (Novogrodsky and Katchalski, 1972), PMA (Touraine *et al.*, 1977), calcium ionophore A23187 (Luckasen *et al.*, 1974), galactose oxidase (Novogrodsky and Katchalski, 1973), and a range of polyspecific antisera which recognize multiple lymphocyte determinants (Brochier *et al.*, 1976; Owen and Fanger, 1974; Woody *et al.*, 1975; Woodruff *et al.*, 1967).

Lectins are widely used to induce a polyclonal T cell response. These agents mediate their effect via activation signals delivered by binding to specific sites on the lymphocyte membrane (Toyoshima *et al.*, 1972) which include a variety of lectin-binding glycoproteins (Henkart and Fisher, 1975). The ambiguity of lectin interaction with lymphocytes makes these agents unsuitable for *in vitro* enquiries into specific biochemical mechanisms involved in T lymphocyte activation. Furthermore, lectin

activation is not fully supported by exogenous IL-1 and requires the presence of AC (Williams *et al.*, 1984a; Hünig *et al.*, 1983).

2.1.4. The use of mitogenic monoclonal antibodies

The use of monoclonal antibodies to various components of TcR/CD3 complex has facilitated the study of its involvement in signalling leading to primary activation and proliferation of T cells. Although the activation, induced by the antibodies, occurs in a large polyclonal population of normal T cells, the mechanisms seem to be analogous to activation by specific antigen (Meuer *et al.*, 1983c, 1984; Kammer *et al.*, 1984). In addition, these antibodies bind to a specific cell membrane structure. Indeed, most epitopes recognized by αTcR/CD3 are on the 20-kDa CD3ε subunit (Tunnacliffe *et al.*, 1989).

Four subgroups depending on αCD3 specificity were suggested (Tunnacliffe *et al.*, 1989). Antibodies in group I (*i.e.* UCHT1 and αLeu-4) recognize the native CD3ε. Antibodies in group II (*i.e.* OKT3, WT32 and BMA030) recognize conformational determinants of CD3ε which rely on its association with other subunits in the complex. Antibodies in group III (*i.e.* WT31) recognize a nonpolymorphic determinant on the TcRα or TcRß (Spits *et al.*, 1985).

The use of αCD3 or αTcR introduced new complexity, such as epitope specificity and affinity, and their effect on the resulting mitogenicity (Geppert and Lipsky, 1988; Pichler *et al.*, 1987) or even on the activation mechanisms triggered. For example, although UCHT1 and OKT3 interact with the same antigenic structure on the T cell surface (Burns *et al.*, 1982) and have comparable affinity and mitogenicity (Tax *et al.*, 1983; Burns *et al.*, 1982), UCHT1 exerts its effect through an IL-2-independent pathway while OKT3 activity is IL-2-dependent (Van Wauwe *et al.*, 1984).

2.1.5. Effect of αTcR/CD3 on unseparated PBMC cultures

Monoclonal αCD3, such as OKT3 (Van Wauwe *et al.*, 1980), αLeu-4 (Kaneoka *et al.*, 1983; Van Wauwe and Goossens, 1983), and 64.1 (Williams *et al.*, 1985) and αTcR (Kappler *et al.*, 1983; Meuer *et al.*, 1983b; Tax *et al.*, 1983; Kayc *et al.*, 1984; Weiss and Stobo, 1984) antibodies have been used extensively to induce unseparated PBMC to express IL-2r (Meuer *et al.*, 1984; Tsoukas *et al.*, 1985; Ledbetter *et al.*, 1986), to secrete IL-2 or IFN-γ (Von Wussow *et al.*, 1981; Chang *et al.*, 1981; Venuta *et al.*, 1983; Van Wauwe *et al.*, 1984), and finally to proliferate (Van Wauwe *et al.*, 1980; Chang *et al.*, 1981).

The mitogenic effect of soluble αCD3 is markedly diminished in AC-depleted PBMC cultures indicating that mere binding of the αCD3 to T lymphocytes is not sufficient for their activation (Van Wauwe and Goossens, 1981; Tax *et al.*, 1983; Williams *et al.*, 1985; Kaneoka *et al.*, 1983). In the human PBMC, the AC are contained within the adherent cell population (Tax *et al.*, 1983).

2.1.6. Role of AC in αCD-induced lymphocyte activation

The first signal *in vivo* is induced by redistribution of TcR/CD3 via interaction with antigen presented on the AC surface in the association with class II MHC. This signal can be mimicked by αCD3 bound to Fc receptors on AC, or to various artificial substrata.

The second function of the AC is demonstrated by the failure of immobilized αCD3 or lectins to induce proliferation of AC-depleted T cells (Williams *et al.*, 1985; Ledbetter *et al.*, 1986; Weiss *et al.*, 1986). These additional signals are thought to be supplied by soluble materials liberated by AC, at least one of which is IL-1 (Mizel, 1982). The IL-1 receptors are expressed, after antigenic stimulus is perceived (Williams *et al.*, 1985).

2.1.7. Signals required for TcR/CD3-mediated lymphocyte activation

In order to distinguish between the involvement of cross-linking and IL-1 production by AC, AC-depleted cultures were stimulated with αCD3 bound to Sepharose beads (Tax *et al.*, 1984; Manger *et al.*, 1985; Williams *et al.*, 1985; Palacios, 1985; Meuer and Meyer zum Buschenfelde, 1986), to polystyrene beads (Halvorsen *et al.*, 1987) or onto the surface of culture dishes (Ceuppens *et al.*, 1985). The requirements for activation of T cells by αCD3 or αTcR presented in this way were dependent on the event of T cell activation examined (Tsoukas *et al.*, 1985; Ledbetter *et al.*, 1986), on the αCD3 or αTcR clone and the mode of antibody immobilization (Verwilghen *et al.*, 1991), as well as on the basal activation state of the T cells.

There is a fundamental difference in the AC requirement for the re-activation of antigen-primed T cell clones as compared with the primary activation of resting T cells by αCD3. Thus, Seph-αTcR (Meuer *et al.*, 1983c; O'Flynn *et al.*, 1985) or Seph-αCD3 (Meuer *et al.*, 1984, 1983c; Meuer and Meyer zum Buschenfelde, 1986; Manger *et al.*, 1985) was sufficient to induce IL-2 production and subsequently proliferation in T cell clones. Similar observations were made in leukaemic cell lines HUT-78 and Jurkat[1] where the former has a phenotype of an activated T cell while the latter resembles a resting T cell. Indeed, full IL-2 production was induced with Seph-αCD3 in HUT-78 while Jurkat required the costimulatory signal of exogenous IL-1 (Manger *et al.*, 1985).

2.1.8. Phorbol esters mimic second signal

Exogenous IL-1 substituted for AC and induced IL-2 production and

[1]The cell line Jurkat was derived from a patient with T lymphoblastic lymphoma, whereas HUT 78 cultured from peripheral blood of a patient with Sezary syndrome (Kaplan *et al.*, 1976; Gazdar *et al.*, 1980).

DNA synthesis in AC-depleted PBMC cultures activated with Seph-αCD3 (Manger *et al.*, 1985; Williams *et al.*, 1985; Palacios, 1985) while others could not demonstrate such properties of IL-1 (Davis *et al.*, 1986; Halvorsen *et al.*, 1987). The exogenous IL-1 enhanced IL-2r expression, IL-2 production, and proliferation in the presence of stimulating concentrations of αCD3; however, the main role for IL-1 may rest in a synergism with suboptimal αCD3 concentrations to induce T cell activation (Verwilghen *et al.*, 1991).

PMA was shown to be able to replace the second signal delivered by IL-1 in several studies (Rosenstreich and Mizel, 1979; Abraham *et al.*, 1987). Indeed, IL-1 can initiate the production of DAG independent of phosphoinositide metabolism by the hydrolysis of phosphatidyl choline (Rosoff *et al.*, 1988).

Jurkat cells stimulated with PHA are activated to produce IL-2 and this is further increased by the addition of PMA (Gillis and Watson, 1980; Weiss *et al.*, 1984c). Although PMA alone was shown to increase IL-2 production in peripheral blood cell cultures (Sando *et al.*, 1981) it did not induce detectable IL-2 production in Jurkat (Weiss *et al.*, 1984c).

In view of the established mitogenicity of αCD3 in PBMC cultures the effect of αCD3 (Weiss *et al.*, 1984c) and αTcR (Imboden *et al.*, 1985) on Jurkat was examined. In the presence of PMA, αCD3 or αTcR activate Jurkat to produce IL-2 (Weiss *et al.*, 1984bc; Imboden *et al.*, 1985). Neither PMA nor the antibodies alone induced IL-2 production. This synergism is not limited to Jurkat but can be observed in peripheral T cells (O'Flynn *et al.*, 1985) and HUT-78 (Manger *et al.*, 1985). The same two stimuli were necessary for the appearance of IL-2 mRNA (Weiss *et al.*, 1984c) or IFN-γ mRNA (Weiss *et al.*, 1984a) indicating that the signals act on pretranslational events.

2.1.9. Involvement of Ca^{2+} in T cell activation

Early studies measuring cell-associated $^{45}Ca^{2+}$ uptake suggested

increased $[Ca^{2+}]_i$ in mitogen-stimulated T cells (Allwood *et al.*, 1971; Whitney and Sutherland, 1972). Indeed, when the extracellular Ca^{2+} was removed with citrate, EDTA or EGTA, the lectin-induced lymphocyte mitogenesis was inhibited (Alford, 1970; Whitney and Sutherland, 1972). The role of Ca^{2+} as a mitogenic signal was supported by observations of a blastogenic response (Maino *et al.*, 1974) or of DNA synthesis (Hesketh *et al.*, 1977) in lymphocytes exposed to Ca^{2+} ionophore.

A more recent technique (Tsien, 1981) for loading intact small cells with fluorescent derivatives of the Ca^{2+} chelator EGTA (Tsien, 1980) allowed for direct and sensitive determination of $[Ca^{2+}]_i$ in mouse thymocytes and pig lymphocytes (Tsien *et al.*, 1982). The intracellular $[Ca^{2+}]$ of resting cells was about 100 nM; within minutes of stimulation with Con A the $[Ca^{2+}]_i$ increased to 200-250 nM (Tsien *et al.*, 1982).

The Ca^{2+} ionophores together with PMA were used instead of αCD3 in an successful attempt to activate $CD3^-$ mutants of Jurkat cells (Weiss *et al.*, 1984b). These mutants fail to respond to PHA (Weiss and Stobo, 1984) and αCD3 (Weiss *et al.*, 1984b) even in the presence of PMA. The combination of Ca^{2+} ionophores and phorbol ester was used to activate peripheral blood T lymphocytes (Truneh *et al.*, 1985ab; Kaibuchi *et al.*, 1985; Granelli-Piperno *et al.*, 1986), HUT-78 (Manger *et al.*, 1985), immature $CD4^-$ $CD8^-$ thymocytes unresponsive to surface-acting ligands (Lugo *et al.*, 1986) and replaced the antigen-specific signal in T cell lines and clones (Albert *et al.*, 1985; Isakov and Altman, 1985; Koyasu *et al.*, 1987; Goronzy *et al.*, 1987).

The combination of Ca^{2+} ionophore and PMA mimics the effect of activation by antigen but cannot replace IL-2-mediated proliferative signal in IL-2-dependent cell lines which are dependent on exogenous IL-2 (Albert *et al.*, 1985; Isakov *et al.*, 1985) indicating that IL-2 triggers a pathway distinct from TcR/CD3.

2.2. EXPERIMENTAL

2.2.1. Intracellular signals involved in the acquisition and maintenance of the locomotory capacity in lymphocytes

T lymphocyte proliferation and differentiation into competent cells are mediated by a complex series of intracellular signalling events (Isakov *et al.*, 1987; Altman *et al.*, 1990; Klausner and Samelson, 1991). Signals through TcR/CD3 lead to expression of early activation genes such as those encoding c-*fos*, c-*myb*, c-*myc*, IL-2 and IL-2r (Crabtree, 1989) and eventually result in cell proliferation. The wide scope of data reviewed above, that defines signalling pathways and mechanisms involved in the initiation of many activation-associated events, contrasts with the lack of information on specific biochemical events resulting in lymphocyte locomotion.

It has been established that 'cell growth', including the expression of new genes, correlates with the acquisition of the locomotory capacity (Wilkinson, 1986). Indeed, a mitogenic monoclonal αCD3 antibody has the ability to induce polarized morphology in PBL cultures (Wilkinson and Higgins, 1987a). In line with the evidence presented above, the acquisition of locomotory capacity by PBL exposed to mitogenic monoclonal antibodies is dependent on the presence of Fc receptor-positive AC (Wilkinson and Higgins, 1987a). In addition, the increase in polarization of lymphocytes exposed to PMA was demonstrated by Wilkinson *et al.* (1988). However, the mechanism by which this phorbol ester induces the shape change was not examined. The coupling elements comprising the signalling pathway involved in the locomotor response to chemoattractants seem to include a pertussis toxin-sensitive G protein (Wilkinson and Watson, 1990). However, its relative position within the activation pathway and the effector enzymes regulated by this G protein are not known.

There is an obvious need for specific information that would clarify

the intracellular mechanisms and relationships that regulate the acquisition and maintenance of locomotory capability in T lympohocytes. For this purpose, a study, described in this document, was conducted in an attempt to identify the key biochemical components involved in the regulation of this important T lymphocyte function.

3. MATERIALS AND METHODS

3.1. SUPPLIERS

Amersham

Amersham International plc, Amersham Place, Littlechalfont, Buckinghamshire, U.K.

Applied Biosystems

Applied Biosystems, Warrington, Cheshire, U.K.

BDH

BDH Ltd., Poole, Dorset, U.K.

Beckman

Beckman Instruments Inc., 2500 Harbor Boulvard, Fullerton CA, U.S.A.

Becton Dickinson

Becton Dickinson, Cowley, Oxon., U.K.

Biological Industries

Biological Industries Ltd., Cumbernauld, Strathclyde, U.K.

Bio-Rad

Bio-Rad Laboratories, 3300 Regatta Boulvard, Richmond CA, U.S.A.

Calbiochem

Calbiochem, Nottingham, U.K.

Canberra Packard

Canberra Packard, Pangbourne, Berks, U.K.

Ciba-Geigy

Ciba-Geigy, Basel, Switzerland

Coulter

Coulter Electronics, Luton, Beds, U.K.

CP Pharmaceuticals

CP Pharmaceuticals Ltd., Wrexham, U.K.

Dako

DAKO A/S, Produktionsvej 42, Postbox 1359, DK-2600 Glostrup, Denmark.

Dynal

Dynal Laboratories Ltd., Billingshurst, West Sussex, U.K.

Fisons

Fisons Chemicals, Loughborough, Leics., U.K.

Flow

ICN Flow Ltd., High Wycombe, Bucks, U.K.

Gibco

Life Technologics Ltd., Paisley, Strathclyde, U.K.

Graticules Ltd.

Graticules Ltd., Tonbridge, Kent, U.K.

Hoechst-Behring

Hoechst U.K. Ltd., Twickenham, Middlesex, U.K.

LKB Wallac

Wallac Oy, Turku, Finland

Luckham

Luckham Ltd., Burgess Hill, Sussex, U.K.

McQuilkin

McQuilkin & Co., Glasgow, U.K.

Molecular Probes

Molecular Probes, P.O. Box 22010, Eugene, Oregon, U.S.A.

Nikon

Nikon U.K. Ltd., Telford, Shropshire, U.K.

Olympus

Olympus Optical Co. (U.K.), London, U.K.

Ortho

Ortho Diagnostic Systems Inc., Raritan, NJ 08869, U.S.A.

Packard

Packard Instrument Company Inc., One State Street, Meriden CT, U.S.A.

Perkin-Elmer

Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, U.K.

Pharmacia

Pharmacia LKB, Biotechnology AB, Uppsala, Sweden

Roche Products

Roche Products, Welwyn Garden City, U.K.

SAPU

Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, U.K.

Scotlab

Scotlab, Coatbridge, Strathclyde, U.K.

Sigma

Sigma Chemical Company Ltd., Poole, Dorset, U.K.

Sterilin

Sterilin Ltd., Teddington, Middlesex, U.K.

Transduction Labs

Transduction Laboratories, Affinity, Mamhead Castle, Mamhead, Exeter, EX6 8HD, U.K.

Weber

Weber Scientific International Ltd., Lancing, Sussex, U.K.

Whatman

Whatman International Ltd., Maidstone, U.K.

3.2. MATERIALS

3.2.1. Reagents

A23187 (10^{-2}M)

5.00 mg of calcium ionophore A23187 (Sigma C7522; $C_{29}H_{37}N_3O_6$; FW 523.6) was dissolved in 955 μl DMSO, aliquoted at 200 μl/tube and stored at 4°C.

Acetic acid (5%)

5 ml acetic acid (CH_3.COOH; MW 60.05; d 1.048-1.050 g/ml; glacial 17.5 M) was made up to 100 ml with dH_2O.

Acrylamide solution (30%)

29.2 g acrylamide (BDH 44299; CH_2:CH.CO.NH_2; MW 71.08) and 0.8 g N,N'-methylenebisacrylamide (Fisons H/P210/46) dissolved in 100 ml ddH_2O.

αCD3 (2 μg/ml)

25 μg of OKT3 (Ortho) was diluted in 12.5 ml HBSS/MOPS/HSA and dialysed against three changes of 1 l HBSS/2.5 mM MOPS at 4°C. The solution was then filter-sterilized, aliquoted at 100 μl/tube and stored at -20°C.

Azide (1%)

1.00 g sodium azide (BDH 30111; NaN_3; MW 65.0) was dissolved in dH_2O at room temperature. The reagent was stored at 4°C.

$CaCl_2$ (100x)

185 mg calcium chloride dihydrate (Sigma C-3881; $CaCl_2.2H_2O$; FW 147.0) was dissolved to final concentration of 126 mM in 10 ml dH_2O. The solution was then filter-sterilized and stored at 4°C.

CGP41251 (10^{-2}M)

The reagent (dissolved in DMSO) was a gift to Prof. P.C. Wilkinson from Ciba-Geigy.

Cholera toxin (200 μg/ml)

1.0 mg from *Vibrio cholerae* with Tris buffer salts, sodium azide and Na_2EDTA was diluted in 5 ml HBSS/MOPS and dialysed against three changes of 500 ml HBSS/2.5 mM MOPS at 4°C. The solution was then filter-sterilized, aliquoted at 1 ml/tube and store at 4°C.

Collagen (2x)

A stock solution of type I collagen was prepared from rat tail tendons using a method described by Elsdale and Bard (1972). Briefly, the tails were washed in ethanol and the skin was stripped. The four longitudinal bundles of tendons were removed from each tail and were placed in 200 ml of 5% acetic acid. The collagen was allowed to dissolve at 4°C for 24 to 48 hrs giving a tropocollagen in solution. The debris was removed from the solution by coarse-filtering through Nitex gauze and then by centrifugation at 3,000 g for 45 min at 4°C. The collagen was precipitated from the supernatants overnight at 4°C by adding NaCl to final concentration of 10% (w/v). The precipitate was collected by centrifugation at 3,000 g for 45 min at 4°C. The precipitate was removed from the top of the centrifuge tubes and redissolved in minimal volume of 5% acetic acid overnight at 4°C. The collagen was re-precipitated as above and then dialyzed against three changes of water at 4°C. A small sample of collagen was freeze-dried and then redissolved in 5% acetic acid to give a 1 mg/ml solution. A standard curve was obtained by reading the absorbance of several dilutions on spectrophotometer set at 243 nm. The concentration of the stock was determined to be 4.5 mg/ml. The purity of this preparation was verified under denaturing conditions by polyacrylamide gel electrophoresis (Ian Newman, personal communication). The collagen solution was stored in 20 ml aliquots at -20°C.

The minimum concentration of collagen giving a satisfactory

gel matrix is about 1.0 mg/ml (Dr. I. Newman, personal communication). In order to assure a reproducible firm collagen gel the final concentration of 1.5 mg/ml was used in all the experiments. The collagen was prepared as 2x working solution by mixing the following for each 1.0 ml: 667 μl of 4.5 mg/ml stock collagen, 100 μl of RPMI 1640 medium (10x) without L-glutamine & $NaHCO_3$ (Gibco 042-02511H), 10 μl HEPES buffer solution (1M; Gibco 043-05630H), 15 μl 1 N NaOH, and 208 μl of dH_2O. The RPMI 1640 and HEPES was sometimes replaced by 10x Hanks' balanced salt solution without sodium bicarbonate (Flow 19-101-54) and 1 M MOPS respectively. The 2x collagen solution obtained was then kept in an ice bath to prevent a premature gelling.

dbcAMP (10 mM)

50.6 mg N^6-2'-O-dibutyryl adenosine-3':5'-cyclic monophosphate sodium salt (Sigma D-0627; $C_{18}H_{23}N_5O_8PNa$; anhydrous MW 491.4; 97% pure) was dissolved in 10 ml with RPMI/HEPES or HBSS/MOPS, filter-sterilized, aliquoted at 1 ml per sterile vial and stored at -20°C. Each vial was used with 9 ml of suspension (final concentration of 1 mM dbcAMP) containing Jurkat cells in an exponential phase of growth.

2-deoxy-glucose/PBS (5.5 mM)

90.3 mg 2-deoxy-D-glucose (Sigma D-8375; $C_6H_{12}O_5$; FW 164.2) was dissolved in 100 ml PBS.

DMSO

Dimethyl sulphoxide (Sigma; $(CH_3)_2SO$; MW 78.1)

EDTA (100 mM)

4.162 g ethylenediaminetetra-acetic acid tetrasodium salt (BDH 28026; $[CH_2.N(CH_2.COONa)_2]_2.2H_2O$; FW 416.2) was dissolved in 100 ml of dH_2O, filter-sterilized and stored at 4°C.

EGTA (100 mM)

3.804 mg Ethylene glycol-bis(ß-aminoethylether) N,N,N',N',

tetraacetic acid (Sigma E-4378; FW 380.4) was partially dissolved in 80 ml dH_2O. The pH of the solution was increased using NaOH pellets until EGTA is was completely dissolved. The solution was made up to 100 ml with dH_2O, filter-sterilized and stored at 4°C. When added to buffers, final pH was adjusted with HCl.

Forskolin (10^{-1}M)

10 mg forskolin (Sigma F-6886; 7ß-acetoxy-1α,6ß,9α-trihydroxy-8,13-epoxy-labd-14-en-11-one; from *Coleus forskohlii*; $C_{22}H_{34}O_7$; FW 410.5) was dissolved in 244 μl of DMSO and stored at -20°C.

Freezing medium

For every 10 ml of the freezing medium, 1 ml of DMSO was mixed with 9 ml of neat foetal calf serum (Biological Industries 04-001-1B). The medium was made fresh before each freezing.

Fura 2-AM (2 mg/ml)

0.5 mg of Fura 2-AM (Sigma F-0888; FW 1001.9) was dissolved in 250 μl of DMSO and stored at -20°C.

GF109203X (10^{-3}M)

The reagent purchased from Calbiochem (dissolved in DMSO) was a gift to Prof. P.C. Wilkinson from Dr. N. Matthews (Yamanouchi Research Institute, Littlemore Hospital, Oxford, U.K.

Glucose/PBS (5.5 mM)

100 mg D-glucose (BDH 10117; $O^{\cdot}.(CH.OH)_4C^{\cdot}H.CH_2OH$; FW 180.16) was dissolved in 100 ml PBS.

Glutaraldehyde (2.5%)

10 ml glutaraldehyde (Sigma G-6257; grade II; 25% aqueous solution; $C_5H_8O_2$; FW 100.1) was made up to 100 ml with sterile saline and stored at 4°C.

H-7 (10^{-1}M)

10 mg (1-(5-Isoquinolinylsulphonyl)-2-methylpiperazine dichloride (Calbiochem I-4756; $C_{14}H_{17}N_3O_2S_2.HCl$; FW 364.29) was dissolved in 274 μl DMSO and stored at -20°C.

HCl (1 M)

8.59 ml glacial hydrochloric acid (36%; 11.6 M) was made up to 100 ml with dH_2O, filter-sterilized and stored at 4°C.

Herbimycin A (1 mg/ml)

100 μg Herbimycin A (Sigma H-6649) was dissolved in 100 μl of DMSO and stored at -20°C. Working solution of 5 μg/ml was obtained by dilution in HBSS/HEPES prior to each experiment.

IBMX (10^{-1}M)

22.22 mg IBMX (Sigma I-5879; 3-isobutyl-1-methyl-xanthine; $C_{10}H_{14}N_4O_2$; FW 222.2) was dissolved in 1.00 ml of DMSO. A fresh solution was made before each experiment.

Iso H-7 (10^{-1}M)

5 mg 1-(5-Isoquinolinylsulphonyl)-2-methyl piperazine (Sigma I-6891; $C_{14}H_{17}N_3O_2S$; FW 291.4) was dissolved in 172 μl DMSO and stored at -20°C.

$MgCl_2$ (100x)

100 mg magnesium chloride (BDH 10149; $MgCl_2.6H_2O$; FW 203.30) was dissolved in 10 ml of dH_2O to final concentration of 49 mM. The solution was then filter-sterilized and stored at 4°C.

MOPS (1 mM)

20.93 g 3-[N-morpholino]propanesulphonic acid (Sigma M-1254; MW 209.3; pH optimum 6.5-7.9) and 17 sodium hydroxide pellets (BDH 10252) were dissolved in 50 ml dH_2O. The pH was then adjusted with 2M NaOH to 7.4. Finally, the solution was made up to 100 ml with dH_2O, filter-sterilized and stored at 4°C.

NaOH (2 M)

8.00 g sodium hydroxide (BDH 10252; NaOH; MW 40.0) was dissolved in 100 ml dH_2O, aliquoted and stored at 4°C.

Paraformaldehyde (1%)

1.00 g paraformaldehyde (BDH 29447 4L; $[H.CHO]_n$) was placed in 100 ml of PBS and was dissolved by heating to 60°C for 60 min. The reagent was stored at 4°C.

PDB (10^{-2}M)

5.00 mg porbol 12,13-dibutyrate (Sigma P1269; $C_{28}H_{40}O_8$; FW 504.6) was dissolved in 91 μl DMSO, aliquoted at 100 μl per tube and store at 4°C.

Pertussis toxin (200 μg/ml)

Pertussis toxin solution in sterile distilled water (Sigma P6561) was kept at -20°C.

PGE_2 (10^{-2}M)

1 mg prostaglandin E_2 (Sigma P-5640; [5Z,11α,13E,15S]-11,15-dihydroxy-9-oxoprosta-5,13-dienoic acid; $C_{20}H_{32}O_5$; FW 352.5) was dissolved in 284 μl of DMSO and stored at -20°C.

Phosphorylation lysis buffer

100 μl 2M Tris (BDH 27119) pH 7.4, 240 μl 5M NaCl (BDH 10241), 80 μl 100% Nonidet P40 (BDH 56009), 400 μl 20 mM sodium orthovanadate (Sigma S-6508), 80 μl 100 mM EDTA, 160 μl 100 mM EGTA, 80 μl 1M NaF, 80 μl 100 mM DTT (Sigma D-9163; DL-dithiothreitol; $C_4H_{10}O_2S_2$; MW 154.2), 80 μl 5 mg/ml leupeptin (Sigma L-2884; $C_{20}H_{38}N_6O_4.1/2H_2SO_4$; MW 475.6), 80 μl 1.4 mg/ml aprotinin (Sigma A-1153), 40 μl 10 mg/ml PMSF (Sigma P-7626; phenyl methylsulphonyl fluoride; $C_7H_7FO_2S$; MW 174.2) in total volume of 8 ml of ddH_2O.

Pluronic F-127 (20%)

20 mg pluronic F-127 (Molecular Probes P-1572) was dissolved in 100 μl of DMSO and stored at -20°C.

PMA (10^{-2}M)

5.00 mg phorbol 12-myristate 13-acetate (Sigma P8139; 4ß,9α,12ß,13α,20-pentahydroxytiglia-1,6-dien-3-one12ß-myristate 13-acetate; PMA; 12-O-tetradecanoylphorbol 13-acetate; TPA; $C_{36}H_{56}O_8$; FW 616.8) was dissolved in 811 μl DMSO, aliquoted at 100 μl per tube and stored at 4°C.

Propidium iodide

10 mg propidium iodide (Sigma P-4170; 95-98%) was dissolved

in 2 ml dH_2O and stored at 4°C. The final concentration used was 10 μg/ml. Working solution of 10 μg/ml propidium iodide was prepared fresh before each experiment by diluting 10 μl of stock solution in 5 ml of dH_2O.

RNase (500 U/ml)

5,000 units ribonuclease (Sigma R-7003; 0.65 mg solid; 9,900 units/mg solid; 126,000 units/mg protein [Lowry]) were dissolved in 10 ml phosphate buffer pH 7.4, aliquoted at 1 ml per tube and stored at -20°C.

Ro31-8220 (10^{-2}M)

The reagent (dissolved in DMSO) was a gift to Dr. A. Severn and Prof. F.Y. Liew from Roche Products.

Separating gel (7.5%)

Mini gel: 1.55 ml 1.5 M Tris pH 8.9, 7.7 ml ddH_2O, 3.125 ml acrylamide solution (30%), 125 μl 10% SDS (BDH 10032; sodium dodecyl sulphate; $C_{12}H_{25}OSO_3.Na$; MW 288.38), 20 μl TEMED (Pharmacia 80-1128-13; NNN'N'-tetramethylenediamine; $(CH_3)_2.N.CH_2.CH_2.N(CH_3)_2$) and 25 μl 10% ammonium persulphate (BDH 10032; $(NH_4)_2S_2O_8$; MW 228.19).

Separating gel (10%)

Large gel: 4 ml 1.5 M Tris pH 8.9, 19.7 ml ddH_2O, 8 ml acrylamide solution (30%), 320 μl 10% SDS, 80 μl TEMED and 25 μl 10% ammonium persulphate.

Stacking gel (3%)

Large gel: 3.75 ml 0.5 Tris pH 6.8, 8.88 ml ddH_2O, 2.37 ml 30% acrylamide solution, 150 μl 10% SDS, 15 μl TEMED and 40 μl 10% ammonium persulphate

Mini gel: 1.38 ml 0.5 Tris pH 6.8, 3.0 ml ddH_2O, 0.5 ml acrylamide solution (30%), 50 μl 10% SDS, 20 μl TEMED and 20 μl 10% ammonium persulphate.

Staurosporine (10^{-3}M)

0.1 mg staurosporine (Sigma S-4400; from *Streptomyces* sp.;

$C_{28}H_{26}N_4O_3$; FW466.5) was dissolved in 214 μl DMSO and stored at -20°C.

Thapsigargin (10^{-3}M)

0.5 mg thapsigargin (Sigma T-9033; $C_{34}H_{50}O_{16}$; FW 650.8) was dissolved in 768 μl DMSO and stored at -20°C.

W-7 (10^{-1}M)

25mg W-7 (N-[6-aminohexyl]-5-chloro-1-napthalenesulphonamide; Sigma A3281; FW 377.3) was dissolved in 663 μl of DMSO and stored at -20°C.

3.2.2. Buffers

HBSS/MOPS

10 ml 10x Hanks' balanced salt solution without sodium bicarbonate (Flow 19-101-54) and 1.0 ml of 1 M MOPS were made up to 100 ml with ddH_2O. The solution was occasionally stored at 4°C for maximum of a week.

HBSS/MOPS/EDTA

10 ml 10x Hanks' balanced salt solution without calcium and magnesium (Gibco 042-04180M), 1.0 ml 1 M MOPS and 10 ml 100 mM EDTA were mixed. The pH was adjusted to 7.4 with HCl. Finally, the buffer was made up to 100 ml with dH_2O.

HBSS/MOPS/$NaHCO_3$

10 ml 10x Hanks' balanced salt solution without sodium bicarbonate (Flow 19-101-54), 1.0 ml 1M MOPS and 3.0 ml Sodium bicarbonate (7.5%; Gibco 043-05080H) were made up to 100 ml with dH_2O. The pH was adjusted to 7.4 with NaOH.

HBSS/MOPS/HSA

100 mg purified lyophilized human albumin (Hoechst-Behring; ORHA 20/21) was dissolved in 10 ml HBSS/MOPS.

Phosphate buffer pH 7.4

35.3 mg sodium dihydrogen orthophosphate (BDH 10245; $NaH_2PO_4.2H_2O$; FW 156.01) and 168.9 mg *di*-sodium hydrogen orthophosphate (Fisons S/4520/53; Na_2HPO_4; FW 141.96) were dissolved in 10 ml dH_2O.

PBS pH 7.4 (25x Phosphate Buffered Saline)

200.0 g NaCl (FW 58.44; 137 mM), 28.75 g Na_2HPO_4 (FW 141.96; 8 mM), 5.00 g KCl (FW 74.55; 2.7 mM), and 5.00 g KH_2PO_4 (FW 136.09; 1.5 mM) were dissolved in 1 l of dH_2O. The buffer was autoclaved before use.

PBS

4 ml 25x PBS pH 7.4 was made up to 100 ml with dH_2O.

PBS/azide

4 ml 25x PBS pH 7.4 and 1 ml of 1% azide were made up to 100 ml with dH_2O. The buffer was then stored at 4°C.

Protease inhibitor solution (100x)

10 mg TLCK (Sigma T-4376; N-tosyl-L-phenylalanine chloromethyl ketone; $C_{17}H_{18}ClNO_3S$; MW 351.8), 75 mg PMSF (Sigma P-7626; phenyl methylsulphonyl fluoride; $C_7H_7FO_2S$; MW 174.2), 2.5 mg TPCK (Sigma T-7254; Nα-p-tosyl-L-lysine chloromethyl ketone; $C_{14}H_{21}ClN_2O_3S.HCl$; MW 369.3) in 5 ml of ethanol.

Protease inhibitor solution

0.5 ml 2M Tris pH 7.6, 0.5 ml 100 mM EDTA, 1.0 ml 100 mM EGTA, and 100 μl protease inhibitor solution (100x) in 10 ml of ddH_2O.

RPMI (10%FCS)

87 ml RPMI 1640 medium (1x) without L-glutamine and $NaHCO_3$ (Gibco 042-02511H) was supplemented with 1.0 ml HEPES buffer solution (1M; Gibco 043-05630H), 1.0 ml L-glutamine 200mM (100x; 29.2 mg/ml; Gibco 043-05030H), 1.0 ml Gentamycin (10 mg/ml; Gibco 043-05710D) or Penicillin-Streptomycin solution (10,000 IU/ml - 10,000 μg/ml; Gibco 043-05140D), and 10 ml foetal calf serum (Biological Industries 04-001-1B).

RPMI (D_2O)

1.0 ml RPMI 1640 medium (10x) without L-glutamine & $NaHCO_3$ (Gibco 042-02511H) was mixed with 0.3 ml sodium bicarbonate (7.5%; Gibco 043-05080H), 0.1 ml HEPES buffer solution (1M; Gibco 043-05630H) and the pH was adjusted to 7.4 with NaOH. Finally the volume was make up to 10 ml with deuterium oxide (Sigma D-4501).

Running buffer (10x)

30.3 g Tris, 144.1 g glycine (BDH 101196; $C_2H_5NO_2$; MW 75.07), 10 g SDS in total volume of 1 L ddH_2O

Sample buffer (2x)

0.76 g Tris, 10 ml glycerol (BDH 10118; $CH_2OH.CHOH.CH_2OH$; MW 92.09), 0.78 g DTT (Sigma D-9163; DL-dithiothreitol; $C_4H_{10}O_2S_2$; MW 154.2) and 2.3 g SDS in total volume of 50 ml of ddH_2O pH 6.8

TBS/vanadate

1.25 ml 2 M Tris pH 7.6, 3 ml 5M NaCl, 5 ml 20 mM sodium orthovanadate (Sigma S-6508) and ddH_2O to 100 ml final volume.

Transfer buffer (10x)

30.3 g Tris, 144.1 g glycine, 20 g SDS in total volume of 1 L of ddH_2O. Final pH should be 8.3 to 8.4.

Transfer buffer

100 ml transfer buffer (10x), 200 ml methanol (BDH 10158) and 700 ml ddH_2O.

Tris (1 M)

12.1 g of Tris(hydroxymethyl)methylamine (BDH 27119); $NH_2.C(CH_2OH)_3$; MW 121.14) was disolved in ddH_2O to the final volume of 100 ml and was stored at 4°C.

T-TBS

5 ml 2M Tris pH 7.6, 20 ml 5M NaCl, 1 ml Tween-20 (polyoxyethylene (20) sorbitan monolaurate; BDH 66368) and ddH_2O to 1 L final volume.

T-TBS (high salt)

5 ml 2M Tris pH 7.6, 50 ml 5M NaCl, 1 ml Tween-20 and ddH_2O to 1 L final volume.

3.3. METHODS

3.3.1. Preparation of peripheral blood mononuclear cells for experiments

3.3.1.1. Separation of whole blood

Blood was collected from healthy volunteers into universals (Sterilin) each with two drops of heparin (CP Pharmaceuticals). The blood was then carefully layered onto lymphocyte separation medium (Flow 16-922-49) using a pasteur pipette. For each 5 ml of blood, a conical sterile tube (McQuilkin) with 2.5 ml of separation medium was used. The tubes were centrifuged at 275 g for 30 min. Using a pasteur pipette, the platelet-rich plasma was aspirated and discarded, leaving the cloudy interface just above the clear separation medium. This layer containing mononuclear cells was aspirated and collected in a tube with 5 ml of HBSS/MOPS. The volume in the tube was then made up to 10 ml and the cells were pelleted by centrifugation at 275 g for 10 min. The wash was repeated, collecting all of the mononuclear cells in one tube. Finally, the cells were resuspended at a desired concentration in HBSS/MOPS/HSA.

3.3.2.2. Activation by culture with αCD3

The peripheral blood monocytes were cultured at a cell density of $2\text{-}3\times10^6$/ml. Aliquots of 1 ml were placed in wells of 24-well plates (Flow 76-063-04) and 5 μl of αCD3 were added to wells to give a final concentration of 10 ng/ml. The cells were then cultured in a moist box at 37°C for 24 to 48 hrs.

3.3.2. Maintenance of Jurkat cell cultures

3.3.2.1. Cell culture conditions

Jurkat cells used in this project were a kind gift of Dr. Cushley of the Department of Biochemistry, University of Glasgow. The cells were maintained in 50 ml flasks (Flow 3013; 25 cm^2 growth area) containing 10 ml RPMI (10% FCS) and incubated at 37°C in humid atmosphere containing 5% CO_2. The cultures were regularly (*i.e.* every 2 to 3 days) split by removing an aliquot of the 'old' culture into a new 25 cm^2 tissue culture flask and adding RPMI (10% FCS) to the final volume of 10 ml. On the other hand, prior to the freezing or the dibutyryl cAMP treatment, 10 ml of RPMI (10% FCS) were added to the culture one day before the procedure. Thus, a sufficient number of cells at an exponential phase of growth was obtained.

3.3.2.2. Freezing of cells

Jurkat cells were harvested at an exponential phase of growth by centrifugation at 275 g for 10 min. The cells were tapped loose from the bottom of the centrifuge tube and the freezing medium was added to give a suspension with a density of $5x10^6$ cells per ml. The cells were distributed in 1 ml aliquots into sterile 1.8-ml cryovials (Gibco 3-68632A) and were placed in a styrofoam box. The cells were left for at least two days at -70°C and then were transferred into a liquid nitrogen storage.

3.3.3. Counting of cells

Cells were placed on the bridge of a Neubauer Improved haemocytometer (Weber) and the number of cells within the 1 mm^2 area was counted under the inverted microscope with phase contrast optics (Nikon Diaphot). To assure a consistent count, cells lying on the upper

and left border were excluded from the count. All the cells lying within the area and on the right and bottom border were included in the final cell count. Knowing that the distance between the surface of the bridge and the bottom surface of the coverslip is 0.1 mm, the count could be expressed as cells/ml by multiplying the count by the factor of 10^4. In the counts where the number of viable cells was evaluated, the trypan blue exclusion test was performed. Just prior to the count, 50 μl of the cell suspension was mixed with 50 μl of 0.4% trypan blue (Flow 16-910-49). The cells were then counted as above, keeping in mind that viable cells actively exclude the stain. Such cells appear, under phase contrast, as phase-bright images. On the other hand, dead cells take up the stain resulting in a dark blue appearance.

3.3.4. Methods for evaluation of lymphocyte locomotion

3.3.4.1. Collagen gel invasion of chromium^{51} labelled cells

The cells were collected by centrifugation at 275 g for 10 min, saving the supernatant if necessary. The cells were then washed twice with a buffer (*i.e.* RPMI/HEPES or HBSS/MOPS). The cells were placed in a glass tube with four drops of neat FCS. $NaCr^{51}$ (1 MBq/ml) was added to final activity of 0.5 MBq per $1x10^6$ cells and the tube was placed at 37°C in 5%CO_2 atmosphere for 1 hr. The cells were then collected by centrifugation as above and were washed three times with the buffer. A 200 μl sample was taken after each wash and was kept as a 'wash control'.

Equal volumes of 2x collagen solution and test media (*i.e.* supernatants or a buffer with or without chemotactic agents) were mixed and the pH was adjusted if necessary. An aliquot of 0.5 ml was placed in a plastic luminometer tube (Luckham LP3; 10.5 x 63.5 mm) and was allowed to gel for 30 min at room temperature. After this time, 50 μl of a buffer was added to each tube to prevent the gel surface from drying.

A 200 μl aliquot of the cell suspension at $2x10^6$ cells/ml was added

to each tube making certain that the surface of the gel was not disturbed by the pressure of the liquid. The tubes were then incubated for 2 hrs at 37°C in 5% CO_2 atmosphere or at 4°C for the negative controls. After this time, the cells on the surface of the gel were gently agitated by pipetting with a 200 μl pipette and were finally removed into a small test tube. A 200 μl aliquot of a fresh buffer was added to the surface of the gel and was removed into a tube as above. This washing procedure, aimed to recover any residual cells, was repeated three times. The tubes with collected loose cells from the gel surface and the corresponding gels were finally counted on a Compugamma Universal Gamma Counter (LKB Wallac 1282). In addition the 'wash controls' were counted to asses the efficiency of the washing procedure.

To determine the contribution of cell adhesion to the surface of the gel as a source of radioactivity retention by the gels, a short term collagenase treatment was used to strip a thin layer of collagen gel. Thus, it was possible to count the radioactivity and therefore relative number of cells contained on or near the surface of the gel. For this modified gel invasion procedure, cells were washed from the surface and collected as described above. Then, collagenase (BDH 39058 2E) was added to each tube to a final activity of 12.5 U/ml and the gels were incubated at 37°C for various time intervals. The washing procedure described above was then repeated for each tube and the collagen and the cells contained in the collagenase digest were collected as a third fraction. The remaining gels, the digests and the cells washed off the gel surface were then counted on a gamma counter.

3.3.4.2. Collagen invasion of lymphocytes determined by visual method

The cells were collected by centrifugation at 275 g for 10 min, saving the supernatant if necessary. The cells were then washed twice with a buffer (*i.e.* RPMI/HEPES or HBSS/MOPS).

Gels were prepared as described above. However, they were

poured into a well of a 24-well tissue culture plate (Flow 76-063-04). An aliquot of 400 μl of a buffer was used to cover the surface of the gel to prevent it from drying out.

An aliquot of 600 μl containing $2x10^6$ cells was carefully added to each of the gels and the plate was incubated at 37°C for 2 hrs. After this time the gels were examined using x400 magnification on an inverted microscope (Nikon Diaphot) equipped with phase contrast optics and a graticule (Graticules Ltd.) with a 1 mm^2 area ruled into 10 x 10 grid. First, the number of cells, lying on the surface of the gel within the grid area, was counted. Then, by focusing into the gel interior the number of cells, which entered the gel within the grid area, was determined. Four independent fields within each well were calculated and the mean percentage of cells entering the gel was calculated for each well.

3.3.4.3. Polarization assay

Previous section described in detail the early observations which identified the 'cell polarization' as an initial and crucial phase of a locomotory response of lymphocytes. The polarization assay, described by Haston and Wilkinson (1988), takes advantage of this correlation between lymphocyte morphology and the ability of the cell to locomote. Essentially, a 1 ml test sample containing at least $5x10^5$ lymphocytes was isolated. The cells were fixed with an equal volume of 2.5% glutaraldehyde and were incubated for 10 min at 37°C. The cells were then collected by centrifugation at 500 g for 10 min and were washed twice with saline and finally resuspended in the remaining drop (*i.e.* 50 to 100 μl) of saline. Cells fixed in such a manner could be stored at 4°C for several days if necessary.

To analyze the polarized cells, slide-and-coverslip preparations were viewed under a x40 phase contrast objective (Olympus BH-2). When necessary, microphotographs were taken using the attached camera system (Olympus PM-10AK-1). To prepare a slide for enumeration, the sample

contents were mixed by tapping and a 10 μl drop was placed on the coverslip. The slide was then lowered at an angle over the coverslip until the contents of the drop spread over the entire area of the coverslip. Six fields of 50 cells each were counted representing a random sample from different areas of the slide. Every such count, where cell morphology is evaluated and a category is assigned, is associated with standard error which is defined by the binomial distribution:

$$S.E. = \sqrt{p(1-p)} / \sqrt{(n-1)},$$

where p is the proportion of cells falling into a particular category and n is the total number of cells counted. All the polarization assay data presented here were calculated based on the total of 300 cells counted. Standard error was calculated and expressed as a function of number of cells counted for the full range of 0-100% (figure 1). It can be seen that the function has a maximum corresponding to a standard error of 3.3% for counts of 50%. Thus, when examining the polarization assay data, the reader may conservatively assume standard error to be 4-5%.

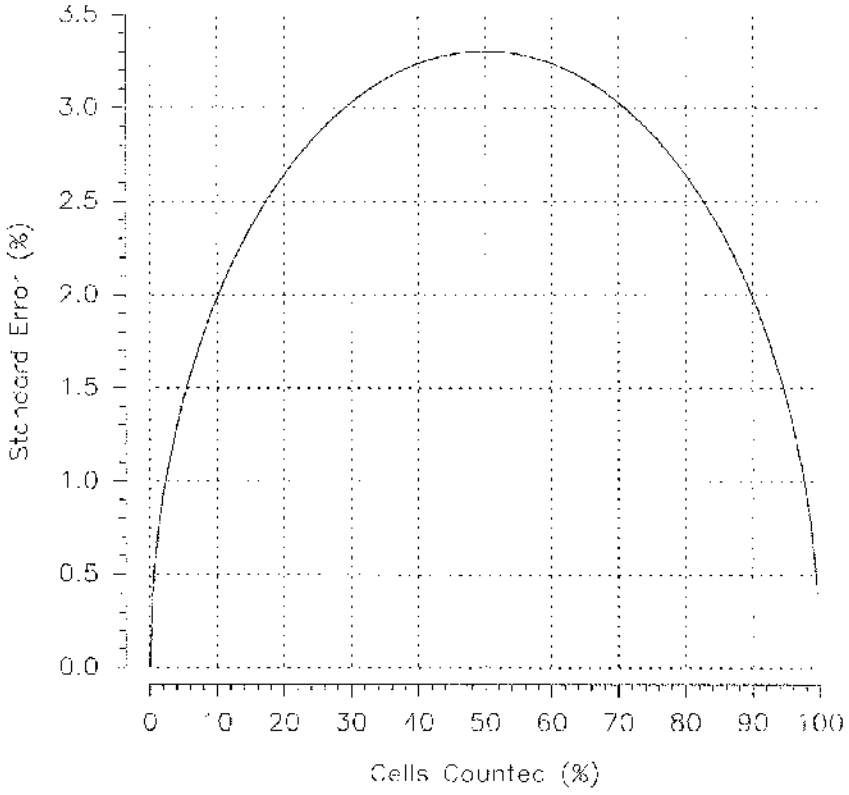
For the purpose of reproducible enumeration, the following categories of cell morphology were defined. Polarized cells included any cell with a defined anterior leading edge, tapered body and uropod, as well as any cell with a well defined constriction ring. The cells were considered round if they projected a circular contour with the occasional 'veil' or spike on its surface. Any other cell morphologies were considered to be irregular.

3.3.5. FACS analysis of surface markers

3.3.5.1. Monoclonal antibodies used

The primary monoclonal antibodies used were as follows: mouse anti-human CD3 (UCHT1 from Dako M 835 or SAPU S048-201; OKT3 from Ortho OKT3), mouse anti-human CD25 (ACT-1 from Dako M 731), mouse anti-human CD4 (fluorescein isothiocyanate-conjugated MT310 from

Figure 1: Standard error for counts of cells determined by the polarization assay. The standard error function $\sqrt{p(1-p)} / \sqrt{(n-1)}$ is based on binomial distribution, where p is the proportion of cells in a particular category and n is the total number of cells counted (set to 300).



Dako F 766), mouse anti-human CD8 (R-phycoerythrin-conjugated DK25 from Dako R 806), mouse anti-human CD2 (RFT11 from SAPU S051-201), anti-CD45RO (UCHL1, a gift of Prof. P.C.L. Beverley, Imperial Cancer Relief Fund, London, U.K.), and anti-CD45RA (2H4LDH11LDB9 from Coulter). The secondary antibody used was rabbit anti-mouse immunoglobulin (fluorescein isothiocyanate-conjugated $F(ab')_2$ fragment from Dako F 313). The working dilution of the secondary antibody (1:100 in PBS/azide) was prepared prior to each staining with an non-conjugated mouse monoclonal antibody.

3.3.5.2. Staining procedure

A staining procedure published on a Dako 'Specification sheet' supplied with the monoclonal antibodies was used with some modifications. Briefly, an aliquot of cell suspension containing about $5x10^5$ cells was washed in PBS/azide and the pellet was resuspended in the remaining drop of about 100 μl. A 10 μl aliquot of the primary antibody was added to the resuspended cells and the tubes were incubated on ice at 4°C for at least 30 min. The antibody was diluted in a single wash with 10 ml PBS/azide and the cells were pelleted by centrifugation at 275 g for 10 min. If required, 50 μl of secondary antibody dilution was added to 50 μl of resuspended cells and the tubes were again incubated on ice at 4°C for at least 30 min. The antibody was removed as above. Finally, the cells were fixed by adding 50 μl 1% paraformaldehyde to 50 μl resuspended cells. After a minimum of 10 min on ice, additional 250 μl 0.5% paraformaldehyde was added and the contents were transferred into a labelled tube. The data acquisition and analysis was performed on FACScan (Becton Dickinson).

3.3.6. Coating polystyrene beads

Dynabeads^R M-450 are uniform, magnetisable, polystyrene beads with the following physical characteristics: diameter 4.5 μm (CV_{max} 5%),

specific gravity 1.5 g/cm^3, surface area 3-5 m^2/g, per g dry weight $1.4x10^{10}$/g, supplied as suspension $4x10^8$ beads/ml (30 mg/ml). The beads were coated with αCD3 as follows. Fifty μl of sheep anti-mouse IgG (Dynal 110.01) was mixed with 50 μl αCD3 (OKT3) and placed at 4°C for 2 hrs with occasional mixing. The beads were washed twice in HBSS/MOPS, resuspended in 2.0 ml, counted and the suspension was adjusted to $8x10^6$ beads/ml. In experiments, 0.5 ml of the suspension was added to wells containing 10^6 cells in a 0.5 ml volume.

3.3.7. Measurement of intracellular cAMP concentration

For the intracellular cAMP determination, a commercial 'Cyclic AMP [^{3}H] assay system' from Amersham (TRK 432) was used. All the reagents used were those supplied with the kit.

3.3.7.1. Cyclic AMP extraction

The method outlined in 'Simple buffer extraction' section of the literature supplied was used with some modifications. Briefly, the cells were centrifuged at 10,000 rpm in microcentrifuge (MSE micro centaur from Scotlab) for 20 sec and the supernatant was pipetted off using a pasteur pipette. The tube was placed in a boiling waterbath and 250 μl of hot 'Assay buffer' (Tris/EDTA) were added and the pelleted cells were resuspended by pipetting. After 10 min of incubation in boiling water the cells were sonicated for 30 min. Finally, the cell debris was collected by centrifugation for 10 min at 13,000 rpm in microcentrifuge and the supernatant was collected for subsequent cAMP determination.

3.3.7.2. Cyclic AMP assay

The 'Assay protocol' was used as published in the literature supplied. The charcoal blank consisted of 150 μl of 'Assay buffer' and 50

μl of 'Tracer' (18 nM [8-^{3}H]adenosine 3',5'-cyclic phosphate). The negative control consisted of 50 μl of 'Assay buffer', 50 μl of 'Tracer' and 100 μl of 'Binding protein' (bovine muscle cAMP-binding protein). Each of the tests consisted of 50 μl of the unknown sample or of a 'Standard' (adenosine 3',5'-cyclic phosphate standard) dilution, 50 μl of 'Tracer' and 100 μl of 'Binding protein'. Each of the tubes was mixed by vortexing for 5 sec and placed in a ice bath. All of the tubes were then incubated at 4°C for at least 2 hrs. Working with 12 tubes at a time, 100 μl of ice cold 'Charcoal' (charcoal adsorbent resuspended in 'Assay buffer') was added to all the tubes and 2 min after addition to the last tube they were centrifuged for 10 min at 13,000 rpm in the microcentrifuge. A 200 μl aliquot of the clear supernatant was removed from each tube and added to a scintillation vial with 10 ml of liquid scintillation cocktail (Betaplate Scint from Fisons). All the samples were finally counted on a beta scintillation counter (Minaxi Tricarb 4000 Series from Canberra Packard).

Each of the counts was corrected by subtraction of the charcoal blank and the ratio of C_0/C_x was calculated for each sample. Here C_0 is the corrected count for the negative control and C_x is a corrected count for each of the unknowns. A standard curve was used to convert the ratio into the absolute cAMP concentration for each of the unknowns.

3.3.8. Measurement of changes in intracellular Ca^{2+} concentration

Jurkat cells were cultured for 3 days in the presence 1 Mm dbcAMP. The cells were pelleted by centrifugation at 275 g and washed twice with HBSS without phenol red (Gibco 14065-049).

Cells were resuspended at $2x10^6$ cells/ml in total volume of 10 ml of HBSS without phenol red supplemented with 1% HSA. Aliquots of 20 μl 2 mg/ml Fura 2-AM and 20 μl 20% pluronic F-127 were mixed and vortexed. An aliquot containing 20 μl of the mixture were then added to the cell suspension in a container wrapped in aluminum foil. The cells were incubated for 20 min at room temperature. Finally, the cells were

washed twice as above, were resuspended at $2x10^6$ cells/ml in HBSS without phenol red and were left to equilibrate for 15 min at room temperature.

An aliquot containing 1 ml of cell suspension was placed in a disposable methacrylate cuvette (Whatman 6602 4008). The cuvette was then inserted into a temperature controlled chamber of Perkin-Elmer LS-50B spectrofluorimeter equipped with a magnet stirrer. After initial baseline was established, 10 μl of activator (5 μg/ml OKT3, 0.1 mg/ml PHA, or 100 μM thapsigargin) were added and the fluorescence was recorded at 510 nm using a dual excitation source at 340 nm and 380 nm. The data were collected for 5 min and saved in a computer data file. At the end of the experiment, the instrument was calibrated for R_{max} by adding 10 μl of calcium ionophore 100 μM A23187 and for R_{min} by adding 50 μl of 100 mM EGTA buffered to pH 9.4 with 1 M Tris. The data were imported into Microsoft Excel and intracellular Ca^{2+} levels were calculated according to the formula:

$$[Ca^{2+}]\ nM = K_d \times (R-R_{min})/(R_{max}-R) \times (S_{f2}/S_{b2}),$$

where R is the ratio of fluorescence intensities I_{340}/I_{380}, K_d is the effective dissociation constant of Fura 2-AM for calcium binding (225 at pH 7.15, 37°C), and S_{f2}/S_{b2} is the ratio of the fluorescence intensity at blue wavelengths of the calcium-free and bound dye (an instrument constant). The data for the three activators were then plotted and a polynomial regression curve was drawn through the data points.

3.3.9. Cell cycle analysis

A modification of method for cell cycle analysis by flow cytometry by Gray and Coffino (1979) was used. The cells were pelleted by centrifugation at 275 g for 10 min, the culture medium was decanted and the pellet was tapped loose. The cells were fixed by adding 70% ethanol and incubated at 4°C for at least 30 min. The cells were centrifuged again, the ethanol was decanted and replaced with 1 ml of 500 U/ml RNase. The

cells were mixed by vortexing and incubated at 37°C for 15 min. The cells were pelleted again by centrifugation, the RNase solution was removed and replaced with 1 ml of 10 μg/ml propidium iodide. The cells were mixed again by vortexing, incubated at room temperature for 30 min and finally analyzed on FACScan (Becton Dickinson).

3.3.10. Western blot analysis of protein tyrosine-phosphorylation

3.3.10.1. Extraction of total cell protein

Jurkat cells were cultured for 3 days in the presence of 1 mM dbcAMP. Prior to the experiment, the cells were washed twice with HBSS/HEPES and resuspended at 6.25×10^6 cells/ml and 1 ml of cell suspension each was placed in four test tubes.

Two aliquots were preincubated at 37°C for 30 min with 50 ng/ml Herbimycin A and two in the buffer only. Then, 20 μl of 5 μg/ml OKT3 were added, one aliquot to the Herbimycin A preincubated cells and one to the buffer control. The incubation was continued for another 15 min.

The test samples were mixed with an equal volume of ice-cold TBS/vanadate and the cells were collected by centrifugation at 400 g for 5 min. The cells were washed once with 10 ml of ice-cold TBS/vanadate. Finally, the pellet was tapped loose and 200 μl of PTK lysis buffer were added. The cells were lysed at 4°C for 15 min. The particulate matter was sedimented by centrifugation in microfuge at 13,000 rpm for 15 min at 4°C. The concentration of protein (440 μg/ml) in the supernatants was determined using Micro BCA Protein Assay Kit (Pierce 23235).

3.3.10.2. Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE)

Mixture for 10% polyacrylamide separating gel was prepared and poured between two clean glass plates (16 x 19 cm) fitted with 0.75 mm

spacers. After the separating gel polymerized, 3% stacking gel mixture was prepared, poured and comb for 8 mm wells was inserted. The gel was stored at 4°C overnight. Prior to the use the wells were washed with three changes of running buffer. A 180 μl aliquot of each of the supernatants was mixed with 30 μl of sample buffer and boiled for 5 min. A 70 μl aliquot of each sample (26.4 μg of protein) was applied to the wells in duplicate. The apparatus was assembled, filled with the running buffer and the current was applied at 100 V/30 mA for about 2 hrs.

3.3.10.3. Western blot

Following the electrophoretic separation, the apparatus was disassembled and the gels were submerged in transfer buffer, while the transfer casette (Scotchbrite pad, Whatman filter paper, gel, Trans-Blot nitrocellulose membrane [Bio-Rad 162-0115], filter paper, pad) was assembled and inserted in the chamber of the transfer apparatus. The chamber was filled with transfer buffer and the current was applied at 30 V/200 mA overnight at 4°C.

3.3.10.4. Immunodetection

The transfer casettes were disassembled and the nitrocellulose membranes were blocked in T-TBS with 2% BSA for 1 hr at room temperature with gentle shaking. Subsequently the HRPO-conjugated anti-phosphotyrosine monoclonal antibody RC-20 was diluted in blocking buffer and was applied to the membranes for 1 hr as above. The membranes were then washed for 10 min at room temperature with low-salt T-TBS, then 10 min with high-salt T-TBS (to decrease non-specific binding) and finally for 10 min with low-salt T-TBS again. The specific bands were visualized using the ECL immunodetection kit (Amersham RPN 2105) and Kodak X-OMAT AR film (Sigma F-5513).

3.3.11. Western blot analysis of PKC isoform expression

3.3.11.1. Extraction of total cell protein

Jurkat cells were cultured for 3 days in the presence or absence of 1 mM dbcAMP. One batch of Jurkat with 1 mM dbcAMP was treated with 10^{-7} M PMA throughout the culture. The cells were washed twice with HBSS/HEPES.

The pellet was tapped loose and 750 μl of lysis buffer were added. The cells were lysed at 4°C for 15 min. The particulate matter was sedimented by centrifugation in microfuge at 13,000 rpm for 15 min at 4°C. The concentration of protein in the supernatants (2.3, 2.2, and 2.2 mg/ml for control, dbcAMP, and dbcAMP/PMA cells respectively) was determined using Micro BCA Protein Assay Kit (Pierce 23235). Several aliquots of 14 μl each of the samples were mixed with 6 μl of sample buffer and stored at -20°C for future use.

3.3.11.2. Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE)

Mixture of 7.5% polyacrylamide separating gel was prepared and poured between two clean glass plates (8 x 10 cm) fitted with 0.75 mm spacers. After the separating gel polymerized, 3% stacking gel was prepared, poured and comb for 5 mm wells was inserted. The Mini-Gel apparatus was assembled, the tank was filled with running buffer and 20 μl of each sample were applied to the wells. The current was applied at 100 V/30 mA for about 45 min.

3.3.11.3. Western blot

Following the electrophoretic separation, the apparatus was disassembled and the gels were submerged in transfer buffer, while the

transfer casette (Scotchbrite pad, Whatman filter paper, gel, Trans-Blot nitrocellulose membrane [Bio-Rad 162-0115], filter paper, pad) was assembled and inserted in the chamber of the transfer apparatus. An icepack was inserted into the tank and the chamber was filled with transfer buffer. Current was applied at 100 V/400 mA for 2 hrs at 4°C.

3.3.11.4. Immunodetection

The transfer casettes were disassembled and the nitrocellulose membranes were blocked in T-TBS with 5% non-fat dry milk for 1 hr at room temperature with gentle shaking. Subsequently, monoclonal antibodies specific for the PKC isoforms (Transduction Labs S85080) were diluted (1:1000 for α, ß, γ, δ; 1:500 for ϵ, ι, λ, μ, ζ; and 1:250 for Θ) in blocking buffer and were applied to the membranes as 5 ml aliquots inside a sealed plastic bag. The membranes were incubated for 1 hr as above. The membranes were then washed in three 5 min changes of T-TBS. HRPO-conjugated sheep anti-mouse IgG monoclonal antibody (SAPU S081-201) was diluted 1:1000 in blocking buffer and was applied to the membranes for 1 hr as above. Finally, the membranes were washed in six 5 min changes of T-TBS and the specific bands were visualized using the ECL immunodetection kit (Amersham RPN 2105) and Kodak X-OMAT AR film (Sigma F-5513).

3.3.12. Measurements of PKC activity

Jurkat cells were cultured for 3 days in the presence 1 mM dbcAMP. The cells were washed twice with HBSS/HEPES and resuspended at 12-16x10^6 cells/ml.

Cells were preincubated at 37°C for 30 min. Then, 10 μl of the activators (5 μg/ml OKT3, 0.1 mg/ml PHA, 100 μM thapsigargin, or 10^{-6}M PMA) were added. The incubation was then continued for various time

periods. At the end of each time period an aliquot of 250 μl was withdrawn into a tube with 250 μl of ice-cold protease inhibitor solution. At the end of the experiment, the cells were collected by centrifugation at 300 g for 15 min at 4°C. The cells were washed once with the ice-cold protease inhibitor solution and finally resuspended in 200 μl of the solution. The cells were then disrupted by 3 series of 6 bursts using sonicator set at 20, cooling the sample on ice after each set. The probe was cooled in ice after each sample was processed. The particular fraction was collected by centrifugation at 23,000 g for 1 hr at 4°C. The resulting supernatants was transferred into a second set of tubes and the pellets were resuspended in 50 μl of the protease inhibitor solution by rapid pipetting. The PKC activity in each of the fractions was determined using a kit (Amersham RPN77). The incorporation oflabelled phosphorus supplied by [^{32}P]ATP (Amersham PB168) was measured using a scintillation counter (Beckman LS 6500) and scintillation fluid Ultima-FLO AF (Packard 6013589). Finally, the counts were interpreted as percent increase in the membrane-associated activity compared to the unstimulated control.

4. THE GROWTH AND MORPHOLOGY OF JURKAT CELLS FROM ROUTINE CELL CULTURE

4.1. BACKGROUND

The acquisition of locomotor capacity is a physiologically essential event which determines the ability of a lymphocyte to seek the sites of inflammation. The locomotory capacity is acquired during the process of antigen-stimulated lymphocyte activation (Wilkinson, 1986). The investigations into signalling pathways, utilizing blood lymphocytes, present difficulties with the interpretation of the data mostly due to the heterogeneity of the lymphocyte populations. In addition, the presence of various other cell types adds to the complexity of the signalling systems. Indeed, in an attempt to manipulate the lymphocyte population, these contaminating cell types may themselves be affected resulting in the secretion of various factors and in the initiation of cell-to-cell interactions whose impact would further obscure the investigations.

In an attempt to simplify the experimental system, a leukaemic T cell line Jurkat was selected. The additional advantage of this system is the vast information on signal transduction derived from experiments with this leukaemic T cell line.

4.2. EXPERIMENTAL

4.2.1. Growth characteristics of normal Jurkat cells

The Jurkat cells were maintained at 37°C in a 5% CO_2 atmosphere. The growth medium consisted of RPMI-1640 medium containing sodium bicarbonate, supplemented with 100 mM HEPES buffer, 2 mM L-glutamine, 100 U penicillin, 100 μg streptomycin, and 10% FCS. The cells displayed characteristic growth kinetics typical of actively dividing cell culture populations. Three cycles of two cultures grown in parallel are

shown in figure 2.

In general, a two-day 'lag' phase was followed by the 'log' phase with doubling time of about 16 hours and finally the 'stationary' phase. In normal culture, the cells were never allowed to reach this phase. To ensure a well growing population, the cells were maintained at a cell density between 5 and $15x10^5$ cells per ml. Cells sub-cultured at the log phase resumed growth without any noticeable decrease in growth rate.

4.2.2. Morphology of normal Jurkat cells

The cultured Jurkat cells in culture, observed under phase contrast, appeared mostly as clusters of round cells. When filmed under phase contrast using a time-lapse video camera, several polarized cells could be observed as they performed shape changes usually associated with locomotion. However, the inability of Jurkat cells to adhere to the plastic culture dish made an actual translocation of the cell difficult. At times, the cells were able to push off the surrounding cells and cell clusters.

Figure 3 shows cells removed from routine culture, washed, incubated at 37°C for 30 min and analyzed by the polarization assay. Usually, several shapes resembling typical locomotory cells were observed in glutaraldehyde-fixed cell samples from routine Jurkat cell cultures. However, in all the tests performed, about 70% of the cells were round.

4.3. SUMMARY

In conclusion, normal Jurkat cells were shown to have the capability to locomote, or at least to become polarized. However, only a small proportion of the population displays this morphology at any given time. What are the conditions which would result in the acquisition of the locomotory morphology in the majority of constitutively dividing Jurkat cells?

Figure 2: Growth curves of Jurkat cell from routine culture. The cells were maintained in RPMI 1640 medium with sodium bicarbonate supplemented with 100 mM HEPES buffer, 2 mM L-glutamine, 100 U penicillin, 100 μg streptomycin, and 10% FCS. The cultures were kept at 37°C in an incubator with a moist atmosphere containing 5% CO_2. The cells were subcultured on day 1, 7, and 13. The viable cells capable of exclusion of 0.4% trypan blue stain were counted using a haemocytometer. The symbols represent two independent cultures examined in parallel.

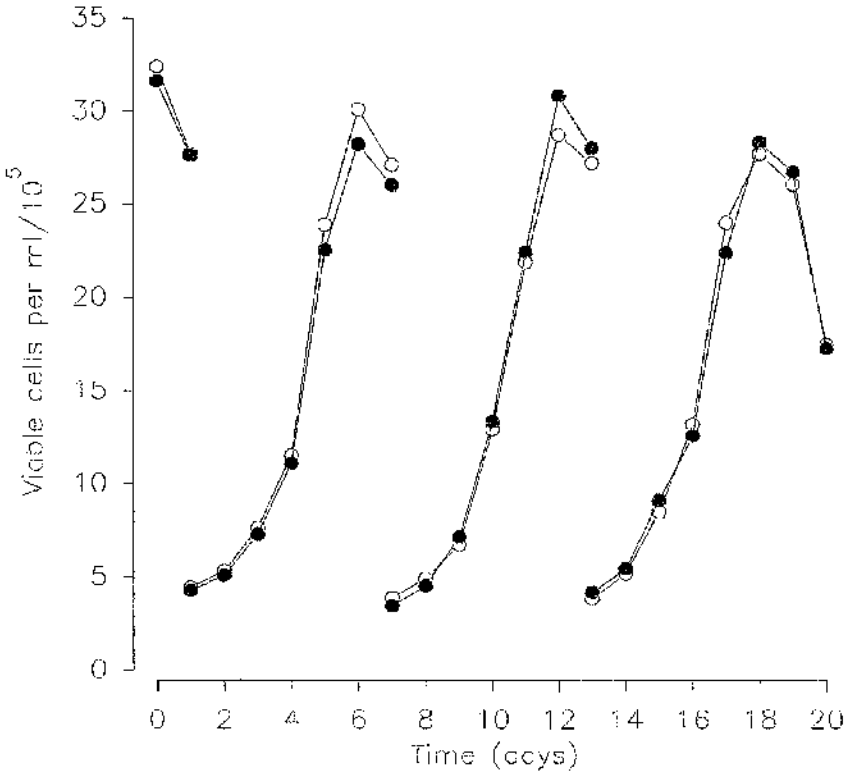
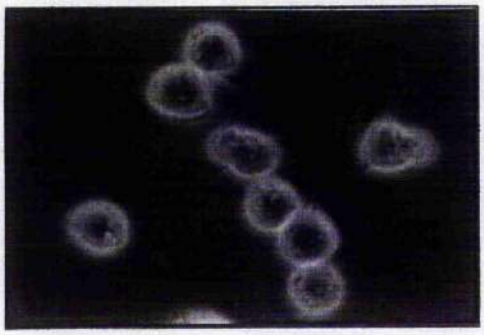


Figure 3: Morphology of Jurkat cells from routine culture. The cells were maintained in RPMI 1640 medium with sodium bicarbonate supplemented with 100 mM HEPES buffer, 2 mM L-glutamine, 100 U penicillin, 100 μg streptomycin, and 10% FCS. The cultures were kept at 37°C in an incubator with a moist atmosphere containing 5% CO_2. A sample was withdrawn from the culture, was washed at room temperature with HBSS/MOPS, and then incubated in 1 ml of HBSS/HSA at 37°C for 30 min. The cells were finally fixed with glutaraldehyde and photographed under phase contrast (x400).



5. EFFECT OF cAMP ON GROWTH AND LOCOMOTOR ACTIVITY OF JURKAT CELLS IN LONG-TERM CULTURE

5.1. BACKGROUND

Only very few Jurkat cells in routine culture resemble motile lymphocytes. It was suggested that only cells at G_o/G_1 phase of the reproductive cycle have the ability to polarize and to become motile (Wilkinson, 1986). It is conceivable that only a minority of actively dividing Jurkat cells would be passing through G_o/G_1. The ability to slow down or to arrest the dividing cells at this phase of the cycle should 'synchronize' the cell cultures and subsequently result in an increased proportion of polarized cells.

The control of mammalian cell proliferation is a very complex process affected by numerous environmental signals such as hormones, growth and differentiation factors and extracellular matrix. The development of cell lines which are able to grow, to become quiescent, or to differentiate in response to pure hormones and growth factors in chemically defined media has facilitated the studies. An actively growing cell line, such as Jurkat, maintained in the absence of growth factors therefore lacks certain intracellular signals responsible for the cell growth inhibition. There is much evidence that cAMP and agents which cause an increase in intracellular cAMP levels are potent growth inhibitors for lymphocytes, smooth muscle cells and many types of fibroblasts (Pouysségur *et al.*, 1990). The effect of dbcAMP resulting in a block at the G_1 phase of cell cycle has been reported by Coffino and Gray (1978).

In a subsequent study the effectiveness of the physiological second messenger, cAMP, in arresting Jurkat T lymphocytes in G_1 and inducing the polarization, was investigated.

5.1.1. PKA is the intracellular target of cAMP

The second messenger, cAMP, exerts nearly all its effects by activating PKA which is ubiquitous and phosphorylates many intracellular proteins (Cohen, 1992). The resulting intracellular physiologic effects of cAMP are mediated by the phosphorylation of protein hydroxy-amino acid residues by the PKA catalytic subunits while the cAMP-regulatory subunit complex was shown to enter the nucleus, bind to DNA and alter gene transcription (Krebs and Beavo, 1979). The phosphorylation patterns induced by cAMP are distinct from those induced by mitogen, antireceptor antibodies, PMA or Ca^{2+} ionophore (Dasch and Stavitski, 1985). Several PKA-specific protein substrates have been identified in human T cells (Kammer *et al.*, 1988), however the physiological substrates of PKA in lymphocytes remain mostly unknown (Perlmutter *et al.*, 1993).

5.1.2. PKA structure

The intracellular receptors for cAMP are two isozymes of cAMP-dependent protein kinase that have identical 40-kDa catalytic subunits but differ in their regulatory subunits (Krebs and Beavo, 1979; Flockhart and Corbin, 1982). These isozymes, designated type I and type II PKA, comprise 48-kDa R_I and 54-kDa R_{II} regulatory subunits. Both PKA isozymes are activated by binding of cAMP to the regulatory subunits resulting in dissociation of the holoenzyme into a dimer of cAMP-bound regulatory subunits and a couple of active catalytic subunits. The two PKA isozymes are distinguished on the basis of the different properties of R_I and R_{II}. In resting lymphocytes, type I PKA is localized in the cytoplasm; type II associates with the plasma membrane (Kammer, 1988) and its activity did not seem to be translocated to the membrane upon lymphocyte activation (Farrar and Anderson, 1985).

5.1.3. Inhibition of lymphocyte activation

The pretreatment of lymphocytes with PGE_1, a cAMP-inducing ligand, inhibited Con A-induced phosphorylation (Chaplin *et al.*, 1980), contradicting some reports which suggested cAMP to have a role in mitogen-induced T cell activation (Byus *et al.*, 1977). Indeed, the increase of intracellular cAMP through physiological or pharmacological interference results in general T cell activation inhibition (Bourne *et al.*, 1975).

The activity of cAMP was shown to interfere with several events associated with TcR/CD3-mediated T cell activation such as phosphatidylinositol hydrolysis (Klausner *et al.*, 1987; Patel *et al.*, 1987; Muthusamy *et al.*, 1991; Bismuth *et al.*, 1988; Lerner *et al.*, 1988), CD3γ serine and CD3ζ tyrosine phosphorylation (Klausner *et al.*, 1987; Patel *et al.*, 1987; O'Shea *et al.*, 1987), lymphokine production and secretion (Rappaport and Dodge, 1982; Baker *et al.*, 1981; Chouaib and Fradelizi, 1982) and proliferation (Isakov and Altman, 1985; Kim *et al.*, 1988; Goodwyn *et al.*, 1977). The phosphatidylinositol metabolism was suggested to be uncoupled by phosphorylation and inhibition of $PLC\gamma_1$ (Park *et al.*, 1992; Kim *et al.*, 1989). In addition, the inhibition of $[Ca^{2+}]_i$ increase (Gray *et al.*, 1988) may result from PKA-mediated phosphorylation of Ca^{2+} channels (Curtis and Catterall, 1985) or IP_3 receptor (Supattapone *et al.*, 1988a). However, increased cAMP was found to inhibit also activation induced by Ca^{2+} ionophore and phorbol esters (Chouaib *et al.*, 1987; Isakov and Altman, 1985; Takayama *et al.*, 1988) which bypass the generation of phosphatidylinositol-derived second messengers. Indeed, evidence suggests that cAMP interferes in various ways with 'downstream' activation events. The IL-2-induced transcription resulting in activation-associated mRNA such as IL-2, and c-*myc* is inhibited while the accumulation of IL-2r, c-*fos* and c-*myb* mRNA is enhanced; however, the translation of these activation products is invariably reduced (Kammer, 1988). Thus, an effect of cAMP on cell

cycle control mechanisms may be expected.

5.1.4. Inhibition of cell cycle progression

Activation of B lymphocytes leads to proliferation, differentiation and finally to antibody production. In order to proceed from G_1 to S phase of the cell cycle a decrease in intracellular cAMP is necessary in the late G_1 (Jegasothy *et al.*, 1978; Wang *et al.*, 1978). Resting B cells activated in the presence of forskolin, an activator of adenylate cyclase, increased in size and RNA content (G_0 to G_1) but did not synthesize DNA (G_1 to S) in response to B cell growth factor (Muraguchi *et al.*, 1984). Similarly, forskolin and a cAMP analogue blocked cell cycle progression from G_1 to S phase in normal B lymphocytes and in a neoplastic B cell line (Blomhoff *et al.*, 1987). In T cells, cAMP interfered with IL-2-induced proliferation at the early G_1 phase (Isakov and Altman, 1985; Beckner and Farrar, 1986; Johnson *et al.*, 1988).

Studies done with PKA mutants (Coffino *et al.*, 1975a; Bourne *et al.*, 1975; Insel *et al.*, 1975) and 'deathless' mutants (Lemaire and Coffino, 1977) of S49 mouse T cell lymphoma revealed that one consequence of PKA activation by cAMP is inhibition of cell growth (Coffino *et al.*, 1975b). The DNA content and size of 'deathless' S49 treated with dbcAMP was examined by flow cytometry (Coffino and Gray, 1978). The data revealed that only about 30% of the S49 exponentially growing population is in G_1 and comprises the smallest cells. The treatment with dbcAMP induced progressive depletion first of the S phase cells and subsequently of the G_2/M cells. However, even under the most efficient conditions, the G_1 population reached maximum of about 90%. Thus, it was suggested that the cAMP block is not absolute but rather results in a reduced rate of progression through G_1.

5.2. EXPERIMENTAL

5.2.1. Growth and morphology of Jurkat cells cultured in the presence of dbcAMP

5.2.1.1. Growth of dbcAMP-cultured Jurkat cells

The typical growth curve of a cell culture of Jurkat supplemented with 1 mM dbcAMP is shown in figure 4. The cells were sub-cultured from a log-phase population on day 1. The rate of growth of the cells decreased slightly just one day after the introduction of dbcAMP as compared to a normal logarithmic growth seen between day 0 and 1. The growth was almost completely abrogated after 3 days of the dbcAMP treatment. The viability of cells maintained at such a culture usually started to deteriorate after 7 to 8 days probably due to the exhaustion of nutrients in the culture medium.

5.2.1.2. Size of the dbcAMP-treated Jurkat cells

The Jurkat cells cultured with 1 mM dbcAMP for 3 days, washed at room temperature, glutaraldehyde fixed and observed under phase contrast (x400) appeared to be smaller and more phase-bright than those obtained from routine cultures (figure 5). The appearance of the dbcAMP-cultured cells was found to resemble the PBL observed under similar conditions.

This apparent decrease in size was confirmed by a decrease in forward scatter as detected by FACScan and presented as a dot plot (figure 6a) and a histogram (figure 6b). The FACScan results show that the FSC of the normal population shifted toward the range consistent with a smaller size following the 3 day culture with 1 mM dbcAMP. In addition to this shift, the size of the cells was more uniform as indicated by the narrower peak. The granularity of the dbcAMP cultured cells remained constant as

Figure 4: Growth curve of Jurkat cells in a regular culture medium supplemented with dbcAMP. The Jurkat cells from a routine culture, in log phase of growth, were subcultured on day 1 and dbcAMP was added to final concentration of 1mM. The viable cells capable of exclusion of 0.4% trypan blue stain were counted daily using a haemocytometer.

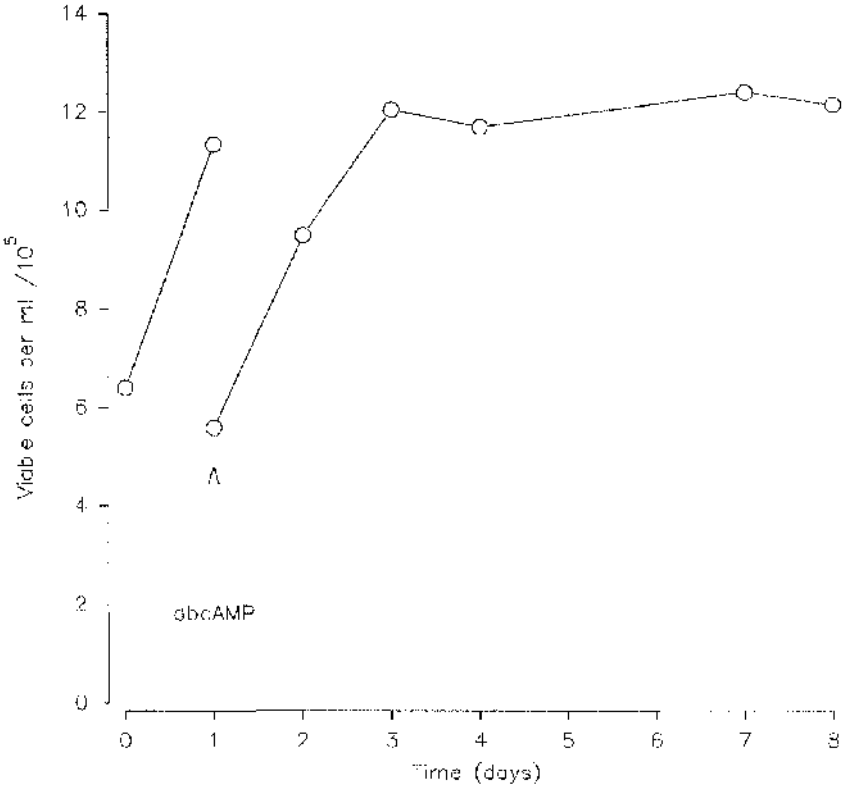
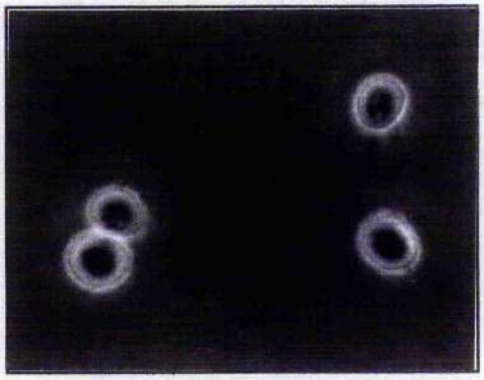


Figure 5: Size of Jurkat cells from routine culture (A) or following a culture in the presence of dbcAMP (B). Samples were removed from a routine culture and from a 3-day culture containing 1 mM dbcAMP and each sample was washed at room temperature with HBSS/MOPS. The cells were finally fixed with glutaraldehyde and photographed under phase contrast (x400).

A.



B.

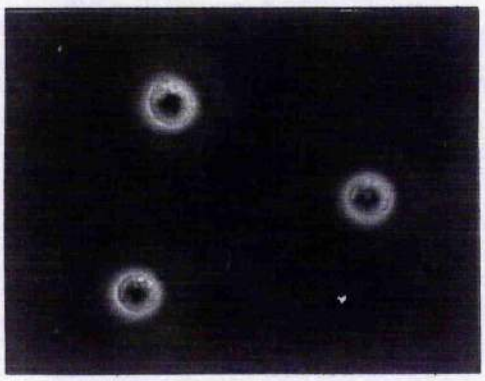


Figure 6a: Size of Jurkat cells from a routine culture (right panel) or following a culture in the presence of dbcAMP (left panel). Samples were removed from a routine culture and from a 3-day culture containing 1 mM dbcAMP and each sample was washed at room temperature with PBS. The cells were finally fixed with paraformaldehyde and examined using FACScan. The cell size is associated with the magnitude of the FSC while the SSC reflects the granularity of the cells.

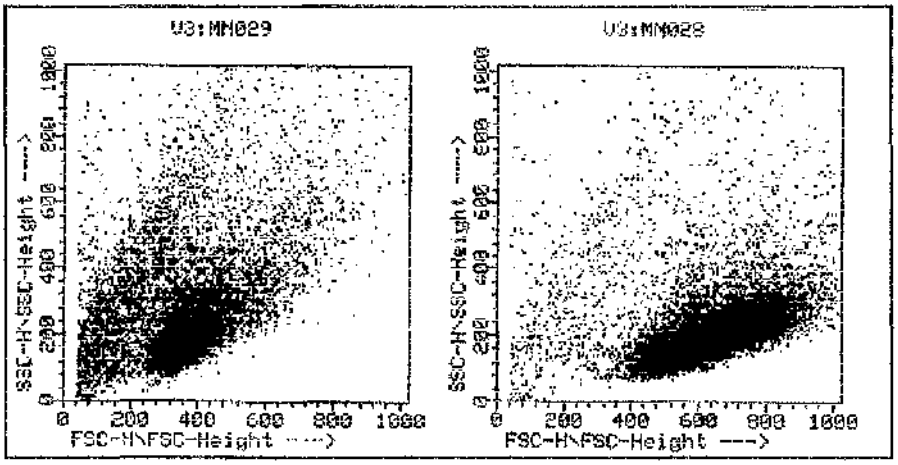
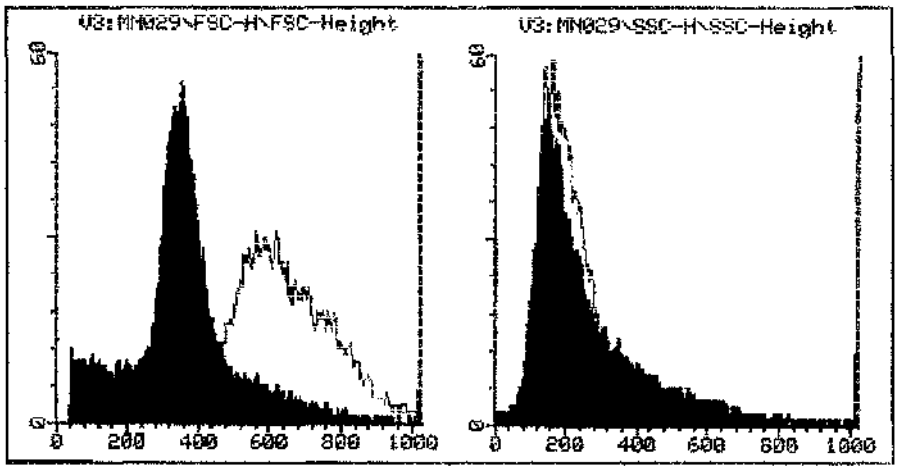


Figure 6b: Comparison of the cell size (left panel) and granularity (right panel) for Jurkat cells from a routine culture (grey opened curve) or following a culture in the presence of dbcAMP (filled black curve). The histograms were derived from data shown in figure 5a.



observed from the unchanged SSC.

5.2.1.3. The morphology of dbcAMP-treated Jurkat cells

Microscopic observation of Jurkat cells cultured with 1 mM dbcAMP and kept at 37°C revealed that a large proportion of these cells displayed polarized or irregular shape (figure 7-A and 7-C). For the sake of consistent identification three categories were defined as follows: **Polarized** cells with anterior leading edge, tapered body and uropod or with a visible constriction ring (figure 7-A); **Round** cells with circular contour with possible 'veil' or spike present (figure 7-B); and **irregular** cells that do not conform to either of the other two categories (figure 7-C).

Typical results from two experiments aimed at a quantitation of the extent of polarization in dbcAMP-cultured Jurkat cells are shown in table 1. The polarization of such cells was reversed following the wash at room temperature. However, full repolarization was achieved upon reincubation of the cells at 37°C for 30 min. The presence of FCS in the repolarization medium did not increase the proportion of repolarized cells. In contrast, FCS induces an increase in polarized morphology in PBL (Wilkinson, 1986). Thus, the process of polarization in dbcAMP-cultured Jurkat cells appears to be both temperature dependent and constitutive. The temperature dependence, and cooling of the sample prior to the glutaraldehyde treatment, may explain the discrepancy in experiment 1 between the polarization obtained directly from the culture and after the reincubation.

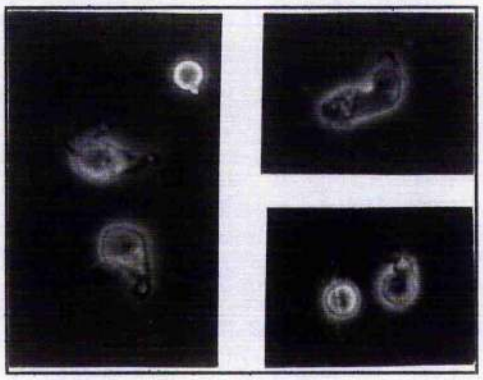
The need for incubation at a physiological temperature for the polarization and the constitutive nature of this process in dbcAMP-cultured Jurkat cells has been demonstrated. Furthermore, it was shown that the wash prior to the reincubation will not affect the final extent of polarization. Such wash may increase the reproducibility of the repolarization results as the various factors and toxins, that may accumulate in the medium during the prolonged culture, are removed.

Table 1: Morphology of dbcAMP-cultured cells treated repolarized under various conditions. The cells were washed twice at room temperature in HBSS/MOPS, resuspended in 1 ml HBSS/MOPS (with or without 10% FCS) and then were incubated at 37°C in waterbath for 30 min. The cell morphology was recorded as polarized (Pol), irregular (Irr) or round (Rnd).

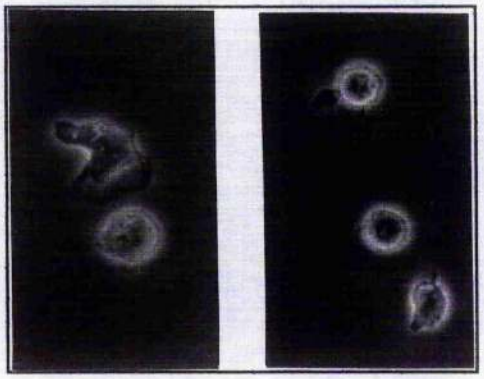
Test conditions	Experiment 1			Experiment 2		
	Pol	Irr	Rnd	Pol	Irr	Rnd
Cells from culture	37.3	19.0	43.7	58.0	27.0	15.0
Cells after wash	2.3	3.7	94.0	2.0	2.0	96.0
Incubation - 10% FCS	70.0	22.0	8.0	55.0	24.3	20.3
Incubation + 10% FCS	69.3	21.3	9.4	ND	ND	ND

Figure 7: Three categories defining Jurkat cell morphology. Jurkat cells were cultured for 3 days in the presence of 1 mM dbcAMP, and then were washed in HBSS/MOPS and repolarized at 37°C for 30 min. Finally, the cells were fixed with glutaraldehyde and photographed under phase contrast (x400). Polarized cells (A) with anterior leading edge, tapered body and uropod (left) or with a visible constriction ring (right), round cells (B) with a smooth circular contour (left) including a possible 'veil' (upper right) or spike (B) and irregular cells (C) that do not conform to either of the other two categories. The latter two categories are contrasted with a polarized cell.

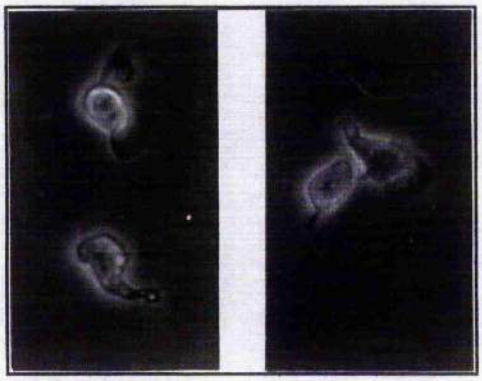
A.



B.



C.



5.2.1.4. The cell-cycle distribution of dbcAMP-treated Jurkat cells

The distribution of Jurkat cells in the cell cycle was determined by the FACS analysis of propidium iodide stained DNA content of individual cells. The cells in the G_0/G_1 phase contain only one set of chromosomes (1 arbitrary DNA unit). The cells in S phase are actively synthesizing DNA in preparation for the division and thus contain a variable amount of DNA. Finally, the cells in M/G_2 phase are just about to undergo the mitotic division. These cells therefore contain two complete sets of chromosomes (2 arbitrary DNA units).

The distribution as determined by FACS is shown in figure 8, where the intensity of propidium iodide staining on the 'FL-3' axis of the dot-plot is drawn against the relative cell size represented by the FSC. The right panel shows the distribution of cells taken from a routine culture while the left one represents Jurkat cells cultured for 3 days with 1 mM dbcAMP. The vertical clusters at 200 and 400 'FL-3' units indicate cells at G_0/G_1 and M/G_2 phases respectively.

Compared to the control culture, the dbcAMP-cultured Jurkat cells display a noticeable increase in numbers of cells registering within the G_0/G_1 cluster. In addition, the G_0/G_1 cells compared to M/G_2 cells display a shift towards lower FSC values consistent with smaller cell size. This observation is consistent not only with the arrest of division in dbcAMP-cultured cells but also with the apparent decrease in the size of these cells which was observed earlier.

Histograms describing the distribution of propidium iodide emission (figure 8-B) were used to determine the proportions of cells present at each of the cell cycle phases (table 2). The fraction of cells in G_0/G_1 phase increased from about 50% to 80% following the dbcAMP treatment.

Figure 8: Cell-cycle distribution of Jurkat cells cultured with (left panel) or without 1 mM dbcAMP (right panel). The DNA content of the cells was stained with propidium iodide, the fluorescence emission was detected by FACS and displayed as a dot-plot (A) as a histogram (B).

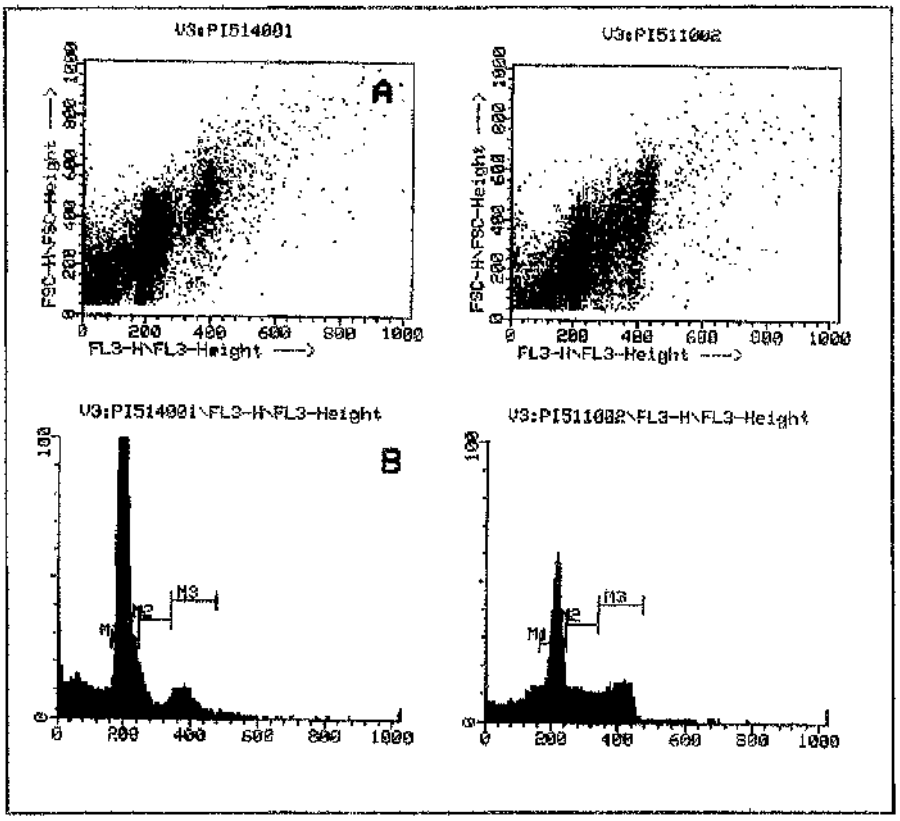


Table 2: Cell cycle distribution of Jurkat cells cultured with or without cAMP. The percentages shown were estimated based on DNA staining with propidium iodide presented in figure 8.

Cell-cycle phase	% Total cells	
	-dbcAMP	+dbcAMP
G_0/G_1	49.5	77.9
S	22.5	10.4
M/G_2	28.0	11.7

5.2.2. The characteristics of the long-term effect of dbcAMP on Jurkat cells

5.2.2.1. Time-course of acquisition of polarized morphology by Jurkat cells cultured in the presence of dbcAMP

Figure 9 shows the increase in the polarized cell population during the culture with 1 mM dbcAMP. It can be seen that the population of the round cells reached minimum after just two days of culture. The proportion of polarized cells increased most rapidly until day three and only slightly afterwards. The growth of the cells was arrested, as expected after two to three days of culture in the presence of dbcAMP.

5.2.2.2. Dose-dependent effect on Jurkat cell division of dbcAMP used in a long-term culture

The effect of various doses of dbcAMP on the division and acquisition of the polarized morphology by Jurkat cells was followed over a seven-day period (figure 10). The growth-curve in the absence of dbcAMP is described by the dotted line for the first three days. After this time, the viability of these cells decreased due to the exhaustion of the culture medium. The term 'cell growth' used below stands for the increase in the number of cells in a culture rather than for an increase in the cell size.

The rate of growth of the total cell population was lower in the presence of dbcAMP as compared to the untreated control culture. At dbcAMP concentrations between 0.25 mM and 0.50 mM, the cell numbers increased linearly as compared to the logarithmic increase observed in the untreated cultures. The slope decreased slightly at the higher dbcAMP concentration. Thus, the cell culture supplemented with 0.50 mM dbcAMP, compared to one with 0.25 mM dbcAMP, required an additional 48 hrs to arrive at the stationary phase. The addition of 0.75 mM and

Figure 9: Time-course of effect of dbcAMP, used in long-term culture, on the growth (dotted line) and polarization (solid line) of Jurkat cells. Jurkat cells were incubated with 1 mM dbcAMP at 37°C and samples were taken daily to determine the number of viable cells and their morphology. The viable cells capable of exclusion of 0.4% trypan blue stain were counted using a haemocytometer. The cells were washed, resuspended, repolarized at 37°C for 30 min and finally fixed with glutaraldehyde. The morphology was examined and quantified under phase contrast. Round cells (circle), irregular cell (square) and polarized cells (triangles).

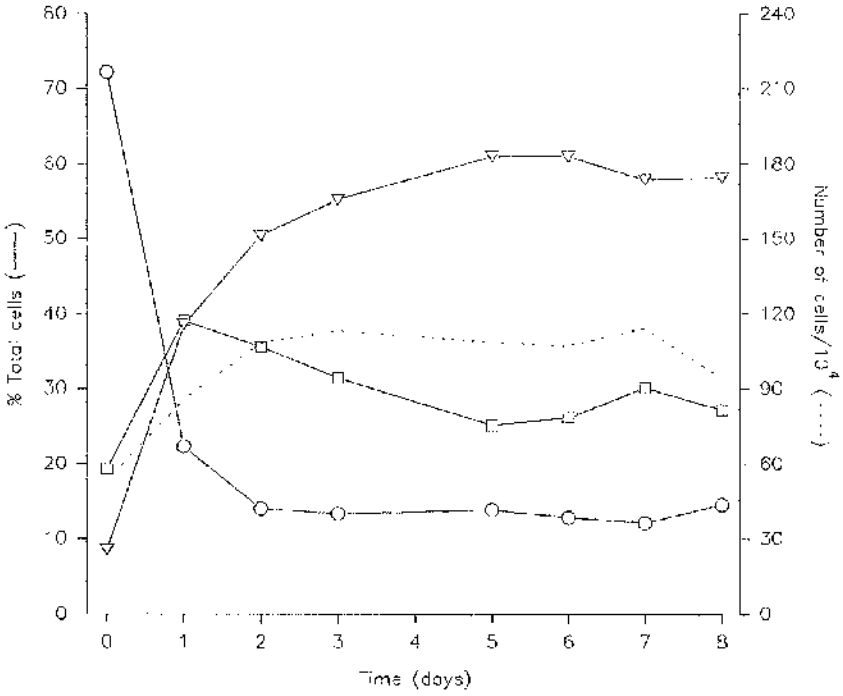
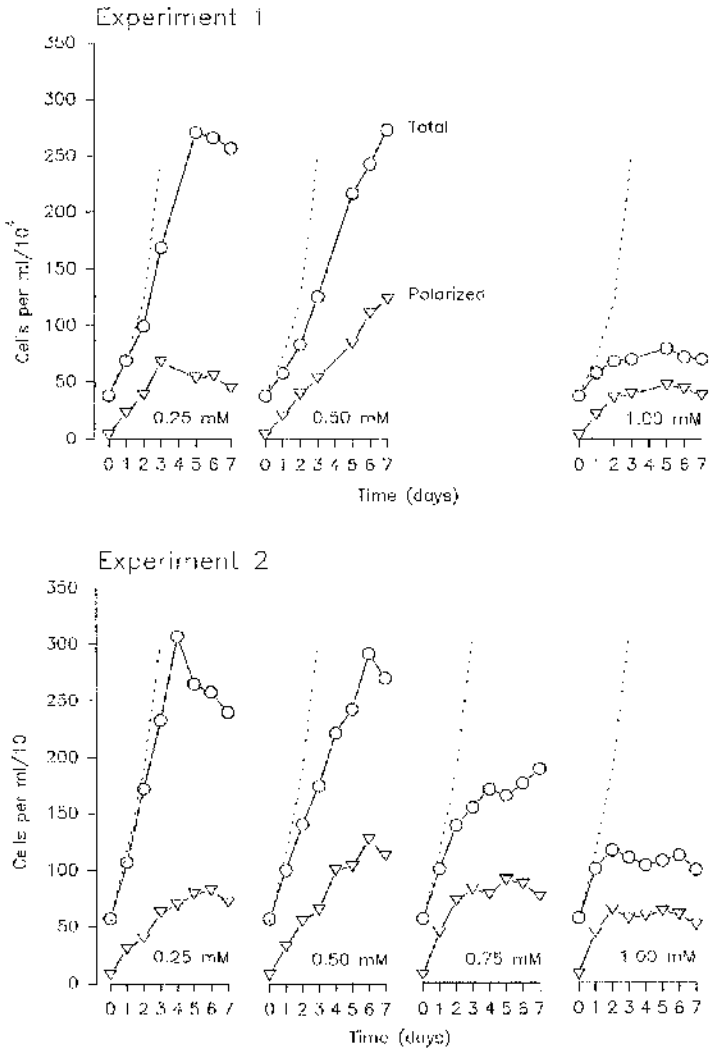


Figure 10: Dose-dependent action of dbcAMP on growth and polarization of Jurkat cells. The cells were incubated with various doses of dbcAMP at 37°C and samples were taken daily to determine the number of viable cells and their morphology. The viable cells capable of exclusion of 0.4% trypan blue stain were counted using a haemocytometer. The cells were washed, resuspended and repolarized at 37°C for 30 min. Finally the cells were fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. The percentage of polarized cells was converted to an absolute number using the total cell count. Cell growth of a culture lacking dbcAMP is shown by a dotted line. Total number of cells (circles) and number of polarized cells (triangles).



1.00 mM dbcAMP resulted in progressive decrease in the cell-growth rate. The maximum cell growth inhibition in the presence of these dbcAMP concentrations occurred after 48 hrs of treatment. However, after this time the 0.75 mM dose allowed for a sustained slow increase in cell density, while the 1.00 mM dose resulted in a complete cell-growth inhibition. Overall, the observed inhibition of the Jurkat cell-growth, in the presence of dbcAMP, was dose-dependent.

5.2.2.3. Dose-dependent effect on Jurkat cell morphology of dbcAMP used in a long-term culture

The acquisition of polarized morphology did not follow a simple pattern as did the cell-growth inhibition. This observation reflects the fact that the process of acquisition of the polarized morphology is either connected with or at least dependent on the decrease in the rate of division of the cells.

The results for 0.25 mM dbcAMP in experiment 1 and 2 gave an interesting insight into this problem. The treatment with 0.25 mM dbcAMP results only in a relatively small change in the logarithmic growth rate, resulting, at best, in a steep linear rate. In experiment 2 the number of polarized cells in such a culture increased noticeably, however the rate of acquisition of the polarized morphology decreased steadily. Experiment 1 was initiated at a lower cell density of $3.8x10^5$ cell/ml which resulted in a two-day lag phase of slower cell division. This non-dbcAMP-induced decrease in the rate of cell-growth synergized with the low dbcAMP concentration to produce an increase in the numbers of polarized cells comparable with 0.50 mM dbcAMP. After the cell reestablished in the near-logarithmic cell-growth the number of polarized cells levelled off indicating that actively dividing cells were unable to acquire the polarized morphology.

A concentration of dbcAMP of 0.50 mM and higher was necessary for the numbers of polarized cells to increase in a manner that paralleled

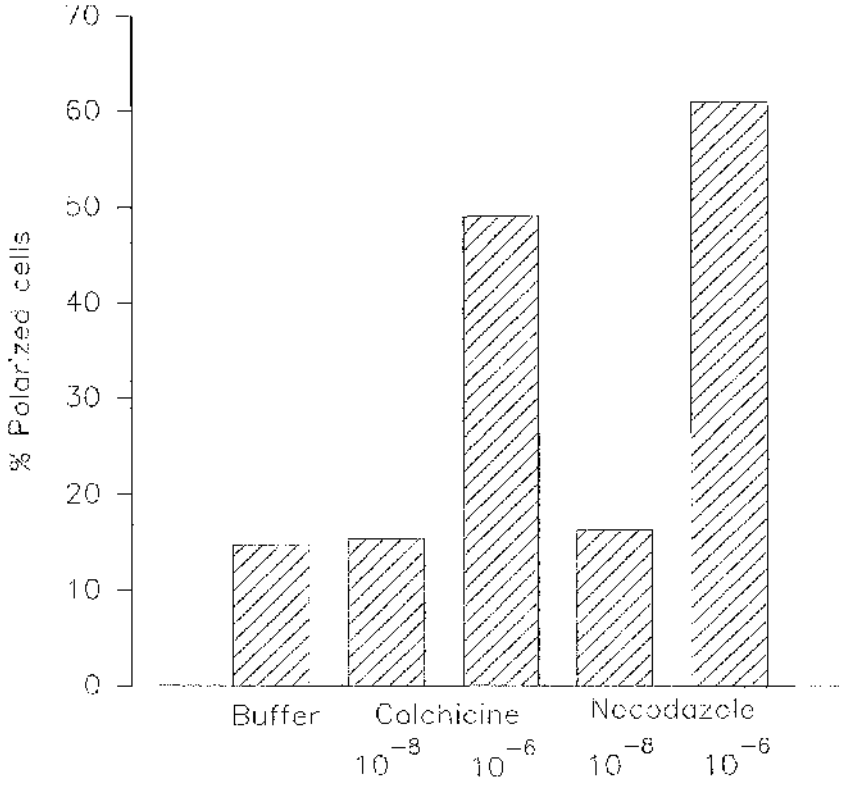
the growth of total cell population. At these concentrations, the differential effect of dbcAMP on the cell growth and on the acquisition of polarized morphology resulted in a dose-dependent increase in the proportion of the polarized cells within the total cell population.

5.2.2.4. Efficiency of the cAMP treatment in the acquisition of locomotory morphology

The maximum increase in the proportion of polarized cells was observed within the first 48 hrs of culture with dbcAMP. After this time, the proportion increased only slightly or remained constant. Interestingly, even in cell cultures whose growth had been arrested for several days, the proportion of polarized cells always remained relatively constant between 50% and 70%, depending on the experiment. This observed incomplete change to polarized morphology may be explained in three ways: (1) a proportion of cells in routine culture was resistant to dbcAMP-induced shape change so that their ability to divide was impaired while the systems needed for the locomotory morphology remained inactive; (2) the cAMP-induced block is not absolute but rather decreases the rate of progression through G_1 allowing a proportion of the population to enter the S phase (Coffino and Gray, 1978); and finally (3) a steady state was established where the cells exhibited a reversible conversion between the polarized and round morphology.

Although the contribution of (2) is possible, (3) appears to be most likely based on a result obtained using microtubule depolymerizing agents nocodazole and colchicine (figure 11). The polarized morphology was observed in 50% to 60% of normal Jurkat cells treated with these non-specific agents compared to 50% to 70% obtained following culture with dbcAMP. It does not seem probable that a homogeneous population of Jurkat cells would vary in their sensitivity to microtubule depolymerization. Thus, the apparent partial effect may be explained by a steady state involving the polarized and round morphology. In parallel with the results

Figure 11: Effect of 10^{-6}M colchicine and 10^{-6}M nocodazole on the morphology of normal Jurkat cells. The cells were washed and exposed to the drugs for 1 hr at 37°C. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast.



obtained with dbcAMP-cultured Jurkat cells, Wilkinson (1986) showed that under 50% of lymphocytes direct from blood can be induced to assume the locomotory morphology following a treatment with 10^{-5}M colchicine compared to under 5% seen in untreated blood cells. A similar observation was made by Keller *et al.* (1984) who induced polarization in over 80% of neutrophils. Also, the motility of chronic lymphocytic leukaemia lymphocytes from patients with high white blood cell counts could be induced by colchicine although these cells responded very poorly to FCS or PMA in a short term assay (Wilkinson *et al.*, 1988). Thus, the integrity of the microtubule network appears to stabilize the round lymphocyte morphology although the systems involved in the motility may be permanently active.

Based on the above preliminary experiments, it was judged that the most reproducible system, with respect to the proportion of polarized cells, would be obtained following a complete arrest of the cell culture growth. This was achieved with the minimum of 1.00 mM dbcAMP. At this concentration the polarized cell population reached equilibrium within 3 days of dbcAMP treatment. In subsequent sections, such cells will be referred to as 'dbcAMP-cultured Jurkat cells'.

5.2.2.5. Locomotory capacity of dbcAMP-cultured Jurkat cells

The correlation between the extent of polarization and the locomotor capacity of PBL has been established (Wilkinson, 1986). To ensure that polarization reflects an increase in the locomotion of Jurkat cells, a series of collagen invasion experiments was performed. A new method using chromium-labelled cells was used. The percentage of radioactivity retained by the gel correlated with the percentage of cells which had entered the collagen gels. In a series of three experiments it was determined that on average 47.1% ($\pm$ 2.2%) of dbcAMP-cultured Jurkat cells invaded the gels as compared to 4.4% ($\pm$ 1.8%) of cells rounded up at 4°C. When these cells were examined by the polarization assay, 59.1% ($\pm$ 2.2%) cells were

polarized and 24.2% (± 3.0%) were irregular. The polarization assay offers a 'snapshot' of motile activity of the constitutively motile dbcAMP-cultured Jurkat cells and therefore a proportion of cells with the potential to enter the collagen gel matrix. Although many of these randomly motile cells would do so, a small proportion would be expected to exit or remain on the surface. Thus, the slight discrepancy between the gel invasion and the polarization assay.

Jurkat cells from regular culture were examined in the same way. Interestingly, 31.5% (± 3.8%) of these cells invaded gels although only 9.1% (± 3.9%) were found to have assumed a polarized and 11.0% (± 2.4%) an irregular morphology in the polarization assay. A possible explanation implicated a non-specific adhesion to the surface of the collagen gel. Such an effect surely would be noticeable also at 4°C. However, at this temperature, only 3.3% (± 1.0%) of normal Jurkat cells were retained by the collagen gels. In a related explanation, a 'veil' or a membrane protrusion extended by the non-polar cells would attach them to the collagen matrix. Indeed, these structures are dependent on the fluidity of the plasma membrane and thus would be present only at the physiological temperature. To examine this possibility, the top layer of collagen was digested off using collagenase. In two independent experiments, 13.0% (± 4.7%) of the dbcAMP-cultured and 19.6% (± 0.9%) of normal Jurkats were removed in a 5-min collagenase treatment. This observation suggests that the normal Jurkat cells, retained by the collagen gels, have in fact entered them. The reason for the discrepancy between the polarization assay and the gel invasion of the normal Jurkat cells has not been further investigated. However, it is possible (and time-lapse video recordings support this suggestion) that when the temperature of the medium is increased from room temperature to 37°C, these cells undergo a short burst of locomotor activity, enter the gel and then round up. Although many cells may be able to do so over a period of time, only very few will appear to be active at any given time as reflected by a polarization assay.

5.2.2.6. Surface-marker expression in dbcAMP-cultured Jurkat cells

The phenotype of Jurkat cells cultured with 1 mM dbcAMP for 3 days was compared to that of Jurkat cells removed from a routine culture (figure 12a-c). The FACS profiles for six major T cells markers indicate that the Jurkat cells are $CD3^+$, $CD4^+$, $CD8^-$, $CD45RO^+$ ($UCHL1^+$), $CD45RA^-$ ($2H4^-$), and $CD25^-$. The most interesting observation was the increased expression of the CD3 molecule on the surface of the cells cultured with dbcAMP. This resembled the extent of CD3 expression detected on the surface of the HUT-78 T cell line (figure 17a) which constitutively displays polarized morphology.

The time-course of the increase in the CD3 expression was examined in an attempt to detect a correlation with the increased locomotion of dbcAMP-cultured Jurkat cells. Two batches of Jurkat cells were cultured in parallel where one was supplemented with 1 mM dbcAMP and the other reflected normal culture conditions used to maintain the cell line. Samples were removed daily from each culture flask and were stained with phycoerythrin-conjugated αCD3 for FACS analysis (figure 13). While the routine culture showed an unchanged level of surface CD3, the dbcAMP-cultured cells displayed a significant increase in surface CD3 after only an overnight culture. The higher level of CD3 was detected on these cells for the duration of the 7-day experiment.

5.3. SUMMARY

In conclusion, the above experiments showed that a long-term culture of Jurkat cells in the presence of dbcAMP caused a dose-dependent inhibition of cell division as demonstrated by the increase in cell population present in G_0/G_1 phase of cell cycle. In conjunction with this inhibition, the majority of these cells became smaller, polarized, motile and express higher levels of CD3.

Figure 12a: Expression of CD3 and CD25 on the surface of Jurkat cells cultured without (A) or with (B) 1 mM dbcAMP or on HUT-78 cells (C). The cells were stained with fluorescein- or phycoerythrin-conjugated monoclonal antibodies and were analyzed on FACScan. The grey open curve represents negative control.

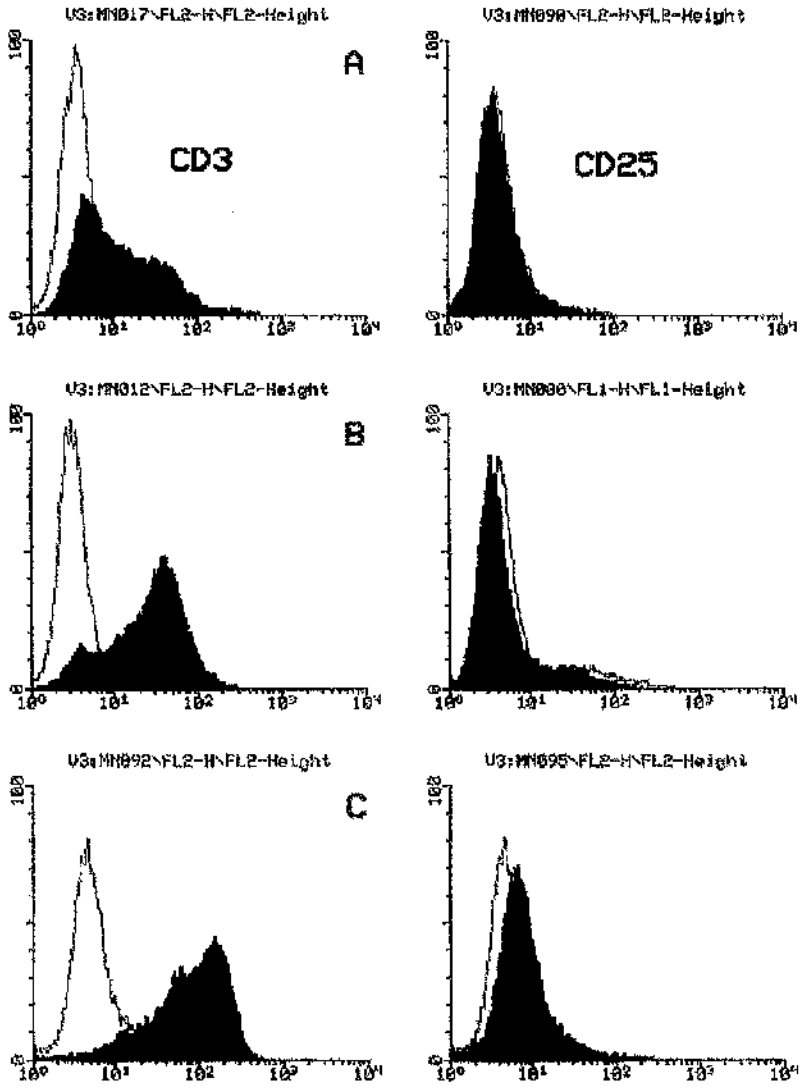


Figure 12b: Expression of CD4 and CD8 on the surface of Jurkat cells cultured without (A) or with (B) 1 mM dbcAMP or on HUT-78 cells (C). The cells were stained with fluorescein- or phycoerythrin-conjugated monoclonal antibodies and were analyzed on FACScan. The grey open curve represents negative control.

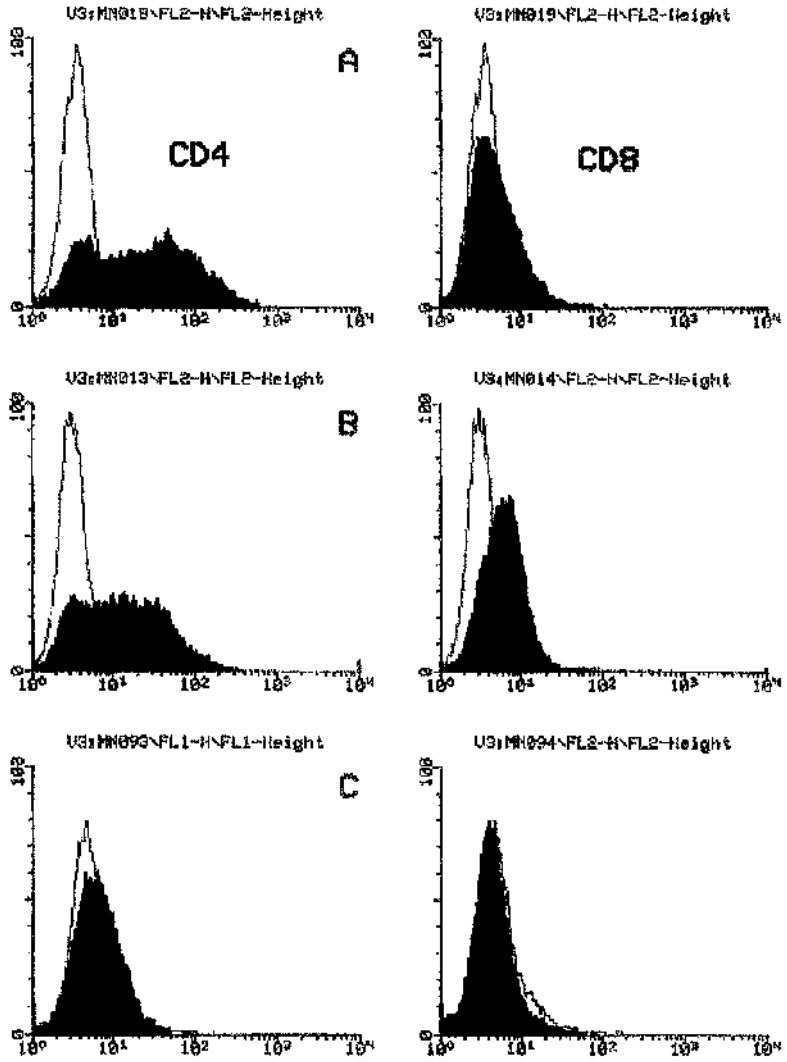


Figure 12c: The expression of CD45R0 and CD45RA on the surface of Jurkat cells cultured without (A) or with (B) 1 mM dbcAMP. The cells were stained with fluorescein- or phycoerythrin-conjugated monoclonal antibodies and were analyzed on FACScan. The grey open curve represents negative control.

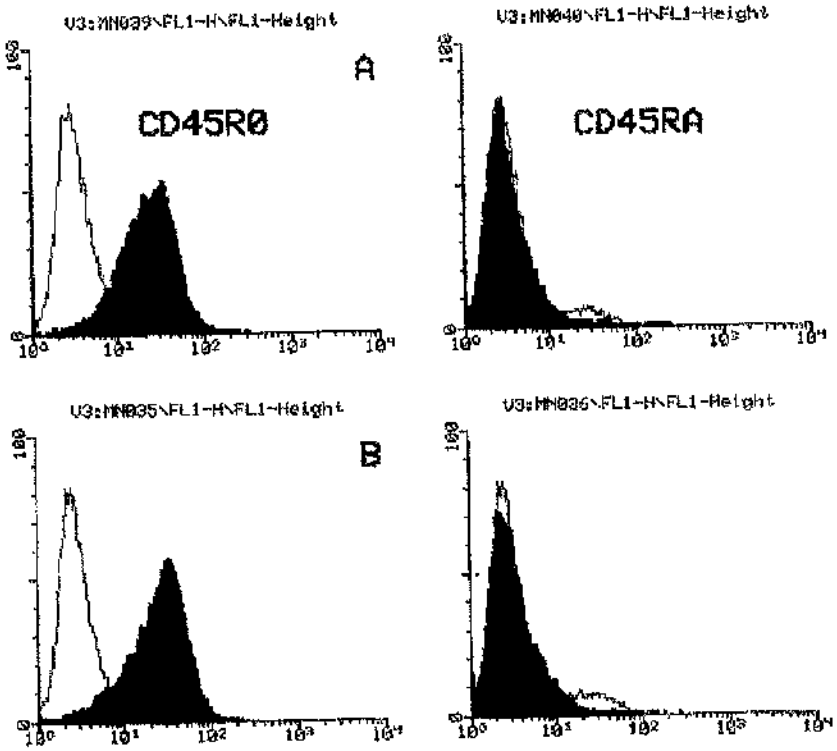
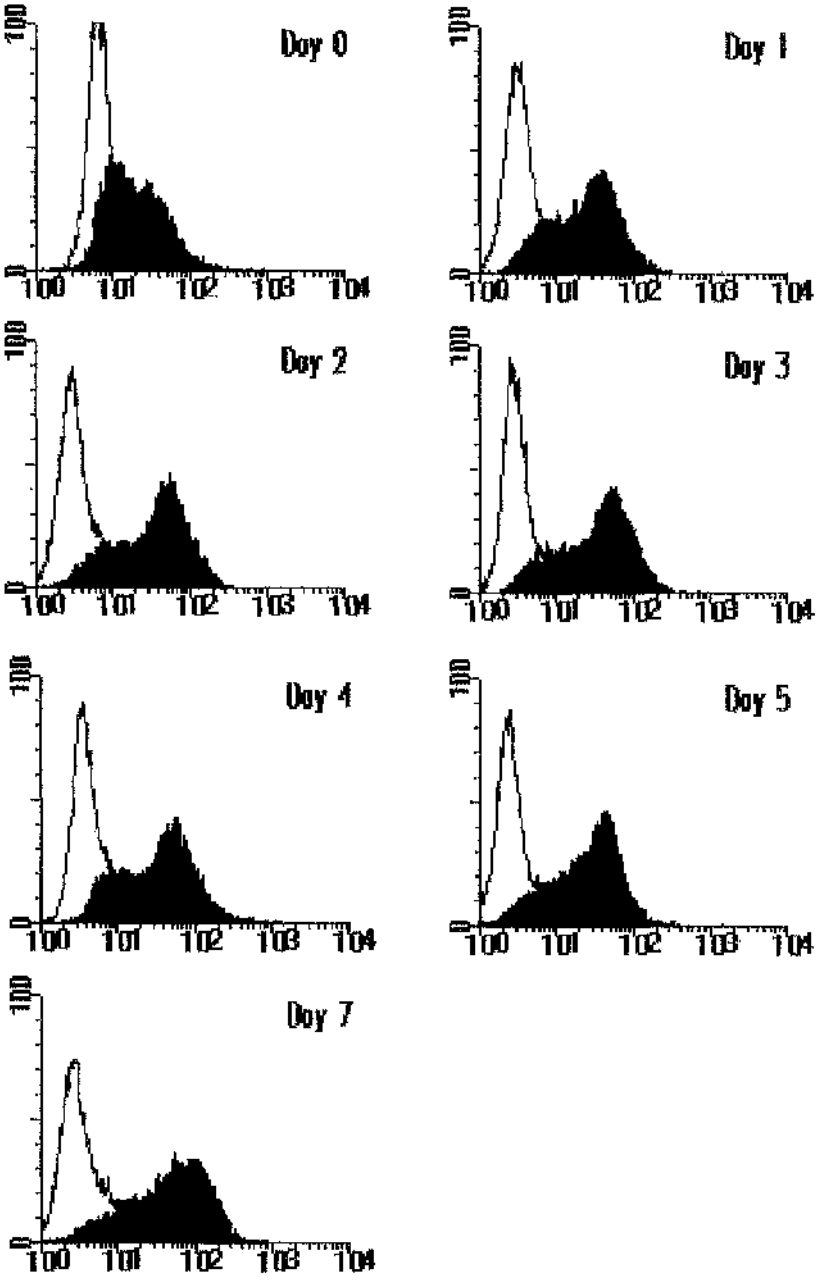


Figure 13: Effect of culture with 1 mM dbcAMP on the expression of CD3 on the surface of Jurkat cells. The cells were removed from the culture daily, stained with phycoerythrin-conjugated monoclonal antibody and analyzed on FACScan. The grey open curve represents negative control.



6. CHARACTERISTICS OF THE REPOLARIZATION PROCESS IN dbcAMP-CULTURED JURKAT CELLS FOLLOWING WASHING

6.1. BACKGROUND

In the previous set of experiments, the Jurkat cells were shown to adopt a polarized morphology when cultured in the presence of 1 mM dbcAMP. The change in the morphology of such cells occurred within 3 days of culture. The dbcAMP-cultured Jurkat cells rounded up when washed at room temperature but repolarized when placed at 37°C.

The kinetics of polarization in PBL stimulated with neat FCS or treated with colchicine was studied by Wilkinson (1986). The FCS-stimulated polarization of PBL was essentially complete in 5 min. In contrast, the action of colchicine was slow, requiring at least 15 min to approach maximum polarization. It was speculated that the slower rate of polarization reflected the fact that the drug needed to cross the plasma membrane to gain access to the intracellular components. The dynamics of polarization induced by FCS resembled that of a neutrophil stimulated with chemoattractant fMLP (McKay *et al.*, 1991).

The next set of experiments addressed the kinetics and other characteristics of the repolarization process in dbcAMP-cultured Jurkat cells.

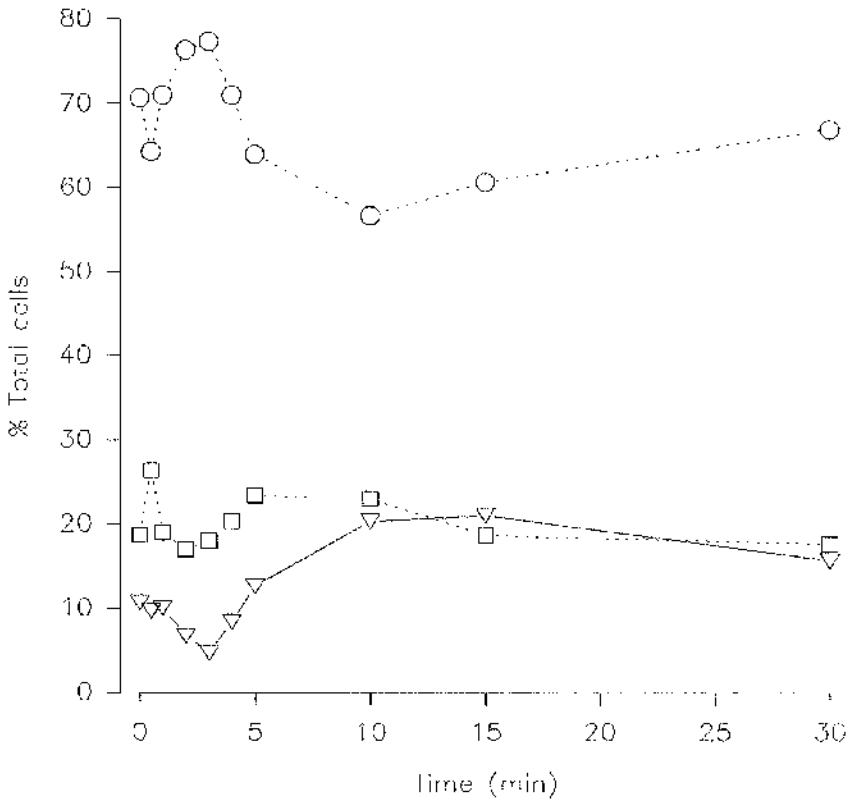
6.2. EXPERIMENTAL

6.2.1. The kinetics of the repolarization process

6.2.1.1. Time-course of repolarization of Jurkat cells from routine culture

Figure 14 shows the time-dependent variation in cell morphology observed with Jurkat cells from routine culture. The cells were washed and then incubated at 37°C. Typically, 10-20% of the normal cells were

Figure 14: Time-course of change in morphology of Jurkat cells from routine culture. The cells were maintained in RPMI 1640 medium with sodium bicarbonate supplemented with 100 mM HEPES buffer, 2 mM L-glutamine, 100 U penicillin, 100 μg streptomycin, and 10% FCS. The cultures were kept at 37°C in an incubator with a moist atmosphere containing 5% CO_2. The cells were washed in HBSS/MOPS and the pellet was resuspended in 50 μl of the medium at room temperature. The repolarization was initiated by the addition of 1 ml HBSS/MOPS preheated at 37°C and was allowed to continue for various time intervals at 37°C. The cells were finally fixed with glutaraldehyde and the morphology examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



polarized, 20-30% were irregular and 60-70% were round. In line with the previous observations, no major variations in cell morphologies were observed.

6.2.1.2. Time-course of repolarization of dbcAMP-cultured Jurkat cells

To define the time-course of shape change of Jurkat cells cultured in the presence of 1 mM dbcAMP, the cells were washed in HBSS/MOPS at room temperature and the pellet was resuspended in 50 μl of the medium. HBSS/MOPS, prewarmed in a waterbath at 37°C, was then added and the incubation was continued for various time intervals. Figure 15 shows time-course curves representing the number of cells assigned to the three different classes based on the cell morphology.

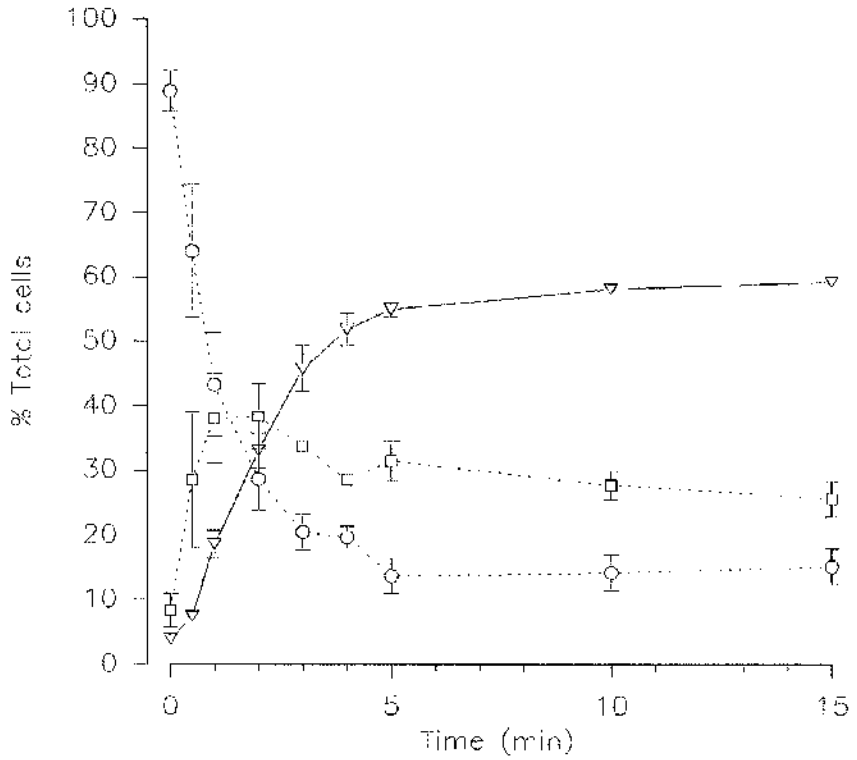
The increase in the irregular population occurred within 30 sec and preceded a similar increase in the polarized population by about 2 min. The population of round cells reached its minimum after 5 min. Similarly, the polarized population experienced the maximum rate of growth for the first 4 min. Thereafter, the number of polarized cells increased only slightly, although it continued to do so for 10 to 15 min. This appeared to be due to a slower transition of the irregular cells to the polarized morphology. After 15 min, the three populations seemed to have reached an equilibrium. Thus, the following scheme for polarization was suggested:

$$\text{Round} \underset{}{\overset{R_1}{\rightleftharpoons}} \text{Irregular} \underset{}{\overset{R_2}{\rightleftharpoons}} \text{Polarized}$$

where the rate R_1 is 3-4 times the rate R_2.

In order to ensure the full repolarization of Jurkat cells in the subsequent experiments, an incubation period of 30 min at 37°C was used to repolarize the cells using medium kept at the room temperature.

Figure 15: Time-course of repolarization of dbcAMP-cultured Jurkat cells. The routine medium was supplemented with 1 mM dbcAMP and the incubation was continued for 3 days. The cells were washed in HBSS/MOPS and the pellet was resuspended in 50 μl of the medium at room temperature. The repolarization was initiated by the addition of HBSS/MOPS preheated at 37°C and was allowed to continue for various time intervals at 37°C. The results shown are a mean of three experiments; the error bars correspond to standard deviation. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



6.2.2. Energy-dependent character of the repolarization process

The lymphocytes have very limited stores of D-glucose which is the main energy source for these cells. In the initial experiment, 100 mM 2-deoxyglucose was added to HBSS which is formulated with D-glucose (figure 16). The dynamics of the repolarization process remained unchanged. However, only 33% of the 2-deoxyglucose treated cells repolarized compared to 53% of the control cells. In a subsequent experiment it was shown that the Jurkat cells repolarize almost equally well in PBS lacking and containing 5.5 mM D-glucose (figure 17). However, when 5.5 mM 2-deoxyglucose was included in PBS the initial increase in polarized cells was reversed after about 5 min, resulting in a slow decrease, in polarized and irregular forms, which continued until the end of the 30 min observation period. This indicates that 2-deoxyglucose, having penetrated the cell, competes with the internally stored D-glucose and thus interferes with the production of energy necessary to maintain the polarized morphology of dbcAMP-cultured Jurkat cells.

6.2.3. Mechanism of the repolarization process in dbcAMP-cultured Jurkat cells

The general pattern of the repolarization process in dbcAMP-cultured Jurkat cells resembles that of FCS-stimulated PBL and fMLP-activated neutrophils. The rapid repolarization of the Jurkat cells may indicate that a chemotactic factor is rapidly produced by the cells which causes the rapid change in morphology. On the other hand, the temperature-sensitive biochemical processes, engaged in the cell polarization, may be rapidly activated when brought to a physiological temperature.

Figure 16: Effect of 100 mM 2-deoxyglucose added to HBSS/MOPS on repolarization of dbcAMP-cultured Jurkat cells. The routine medium was supplemented with 1 mM dbcAMP and the incubation was continued for 3 days. The cells were washed in HBSS/MOPS and the pellet was resuspended in 50 μl of the medium at room temperature. The repolarization was initiated by the addition of the different media preheated at 37°C and was allowed to continue for various time intervals at 37°C. The cells were finally fixed with glutaraldehyde and the morphology examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

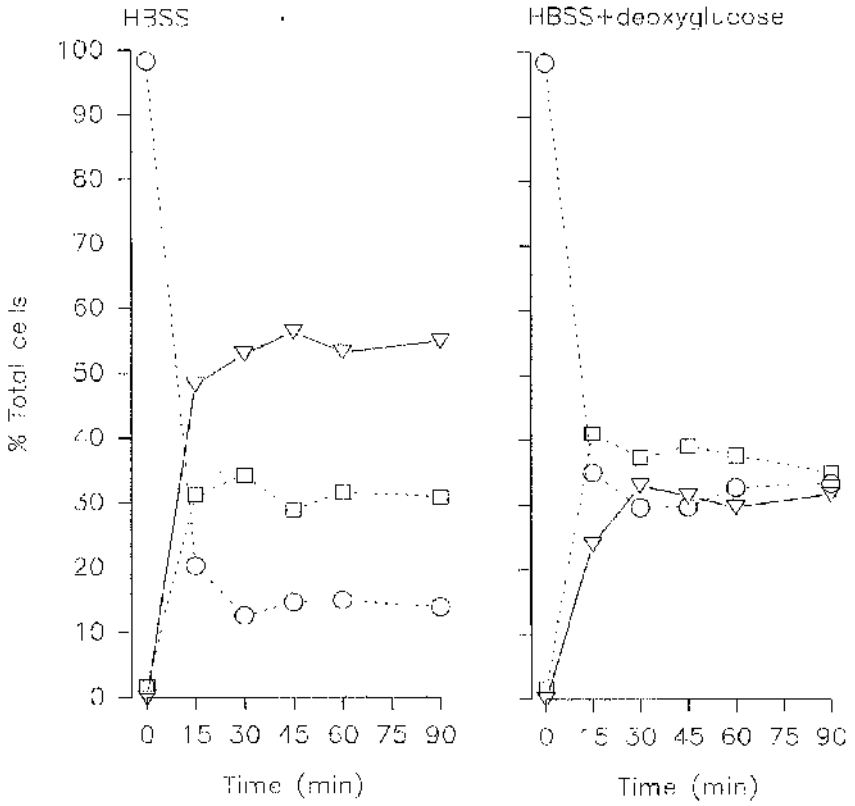
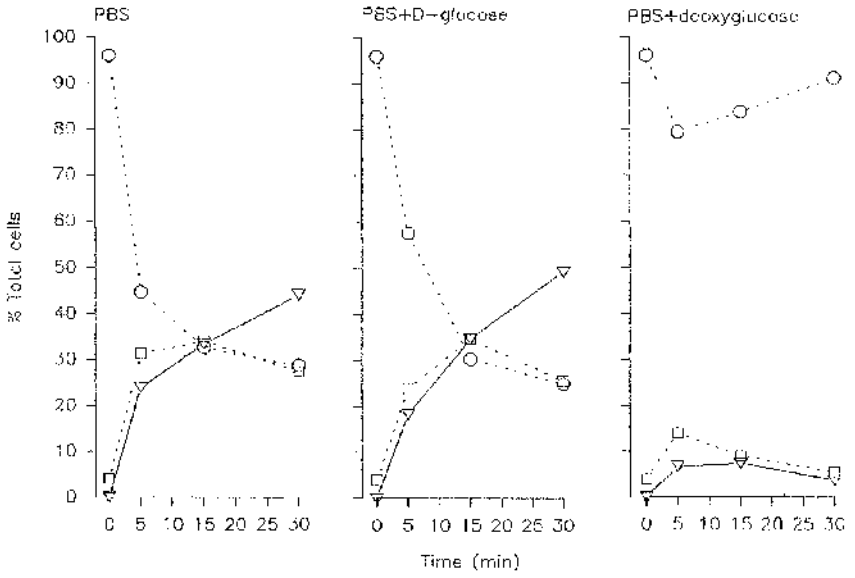


Figure 17: Effect of 5.5 mM D-glucose or 5.5 mM 2-deoxyglucose on repolarization of dbcAMP-cultured Jurkat cells. The routine medium was supplemented with 1 mM dbcAMP and the incubation was continued for 4 days. The cells were washed in HBSS/MOPS and the pellet was resuspended in 50 μl of the medium at room temperature. The repolarization was initiated by the addition of the different media preheated at 37°C and was allowed to continue for various time intervals at 37°C. The cells were finally fixed with glutaraldehyde and the morphology examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

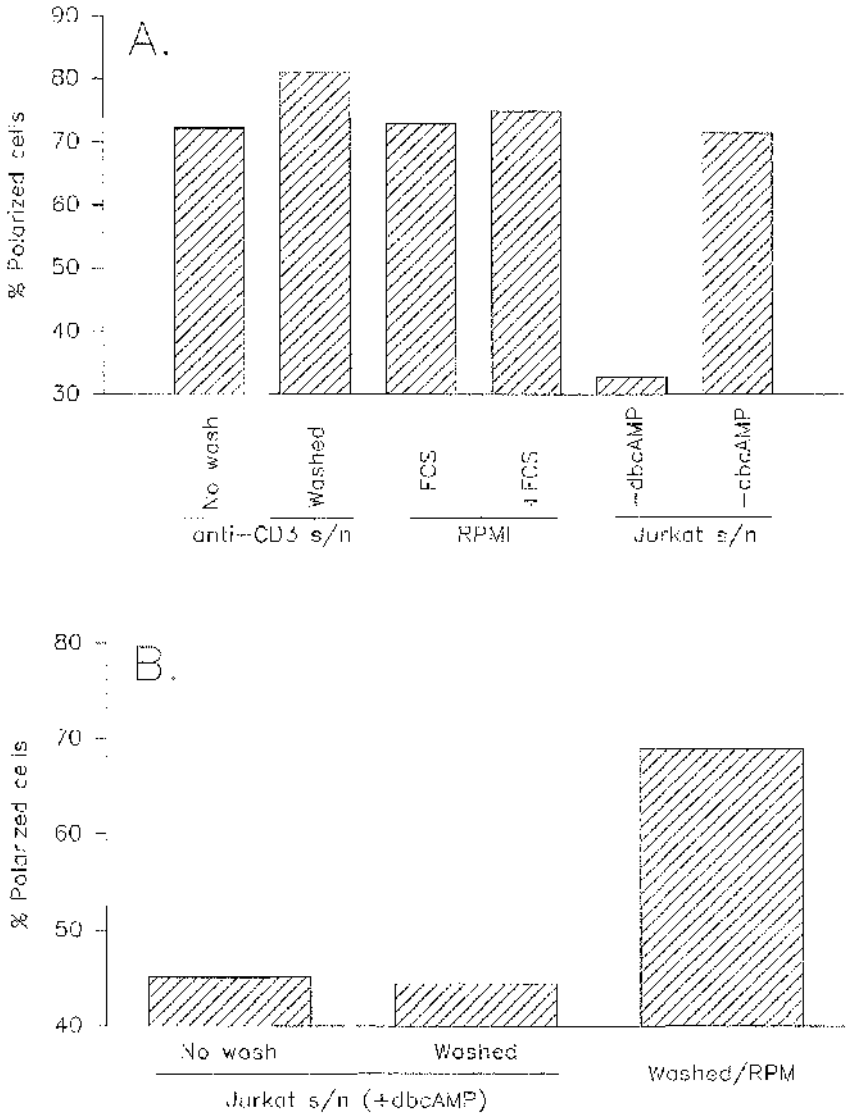


6.2.3.1. Involvement of a soluble factor in the repolarization of dbcAMP-cultured Jurkat cells

The possibility that this change is induced by a soluble chemotactic molecule released into the growth medium during the culture was examined. To obtain an independent evidence of such a chemotactic factor, αCD3-activated PBL were subjected to various media, including supernatants obtained from dbcAMP-cultured Jurkat cells. Figure 18-A shows the effect of these media and supernatants on PBL cultured for 3 days in the presence of αCD3 included in the RPMI (10% FCS) medium. The αCD3-containing supernatant in which the PBL had been cultured, fresh medium with or without 10% FCS, as well as supernatant obtained from routine culture of Jurkat cells gave equivalent levels of polarization of the PBL. However, supernatant from dbcAMP-cultured Jurkat cells showed a significant inhibition of polarization. A similar observation was made when Jurkat cell-culture supernatant containing dbcAMP was added back to Jurkat and the cells were repolarized (figure 18-B). Thus it was suggested that even though cAMP stimulates long-term growth-induced polarization of Jurkat cells, it may be inhibitory to the short-term polarization of PBL or Jurkat cells.

The release of chemotactic factor IL-8 has been described in mononuclear cell cultures however, the source is most likely the population of monocytes or other accessory cells (Wilkinson and Newman, 1992). Even then, hours of culture are required for IL-8 to be released and for the lymphocytes to respond by an increased polarization. Thus, it appears that the dynamics of repolarization of dbcAMP-cultured Jurkat cells reflect the temperature-sensitive nature of the biochemical processes responsible for the shape change. It appears that these biochemical mechanisms are engaged only after a prolonged culture of Jurkat cells in the presence of dbcAMP and correlate with the cell being in the G_1 phase.

Figure 18: Effect of various cell culture media and supernatants on the extent of repolarization of PBL cultured with αCD3 for 48 hrs (A) and Jurkat cells cultured with 1 mM dbcAMP for 3 days (B). The cells were washed with HBSS/MOPS and resuspended in various media as indicated. In some instances, the cells remained in the original culture supernatant. The cells were then repolarized at 37°C for 30 min, fixed and the morphology was examined and quantified under phase contrast.



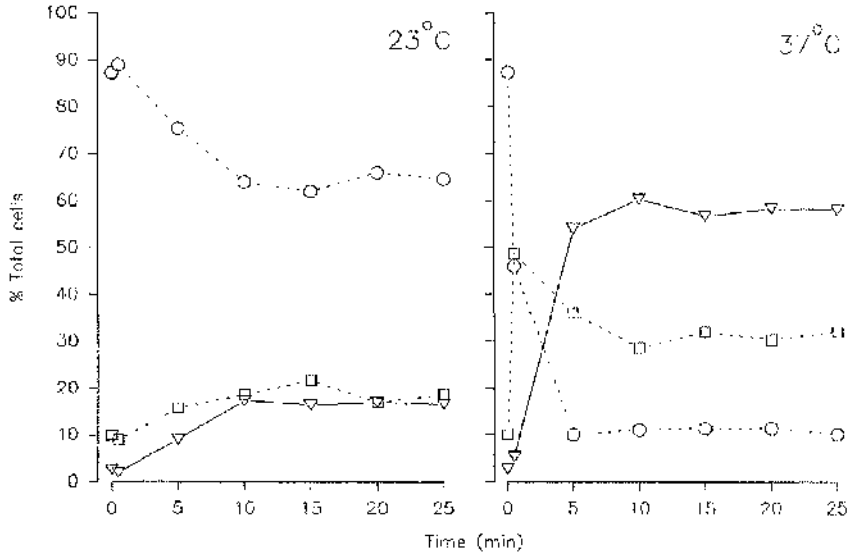
6.2.3.2. Temperature-dependent character of the repolarization process

Further examination of the time-course of Jurkat cell repolarization at 37°C and at room temperature revealed that both, the rate and the extent of repolarization, were affected by the lower temperature (figure 19). The rate of repolarization at 23°C was slower, requiring extra 5 min to reach the maximum repolarization. In addition, the proportion of polarized cells observed at 23°C was more than 3-fold lower than that observed at 37°C.

6.3. SUMMARY

In conclusion, the above experiments suggest that the process, whereby the dbcAMP-cultured Jurkat cells acquire the locomotory morphology, is energy and temperature dependent. Furthermore, this process appears to be constitutive and is inhibited upon addition of dbcAMP in a short-term assay.

Figure 19: Effect of temperature on the repolarization of dbcAMP-cultured Jurkat cells. The routine medium was supplemented with 1 mM dbcAMP and the incubation was continued for 7 days. The cells were washed in HBSS/MOPS and the pellet was resuspended in 50 μl of the medium at room temperature. The repolarization was initiated by the addition of HBSS/MOPS preheated at 37°C or at 23°C and was allowed to continue for various time intervals at 37°C or at 23°C respectively. The cells were finally fixed with glutaraldehyde and the morphology examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



7. INVOLVEMENT OF cAMP SECOND MESSENGER SYSTEM IN POLARIZATION OF dbcAMP-CULTURED JURKAT CELLS

7.1. BACKGROUND

In the previous set of experiments, the repolarization process of dbcAMP-cultured Jurkat cells was shown to be constitutive, as well as energy- and temperature-dependent. In addition, dbcAMP-containing supernatant from dbcAMP-cultured Jurkat cells was shown to interfere with the repolarization process both in αCD3-activated PBL and in dbcAMP-cultured Jurkat cells. Thus, the involvement of the cAMP-based signalling system was examined in the following series of experiments.

7.1.1. Signal transduction and intracellular second messengers

Signal transduction, in general, refers to the process whereby a messenger, bound to a ligand-specific transmembrane protein receptor on the outside surface of the cell, induces a chemical change on the cytoplasmic surface of the membrane. Such a signal transduction mechanism may take a shape of ligand-gated ion channels, second messenger systems, and receptors with integral enzyme activity.

Second messenger systems respond to the binding of a ligand to its receptor on the cell membrane by activation of an enzyme on the cytoplasmic side. This enzyme generates second messengers increasing their intracellular concentration. Finally, the second messengers activate some system which modulates a variety of cell functions resulting in physiological changes.

7.1.2. Cyclic AMP as a second messenger

Cyclic AMP was the earliest second messenger identified by its involvement in the control of glycogenolysis in liver cells stimulated with

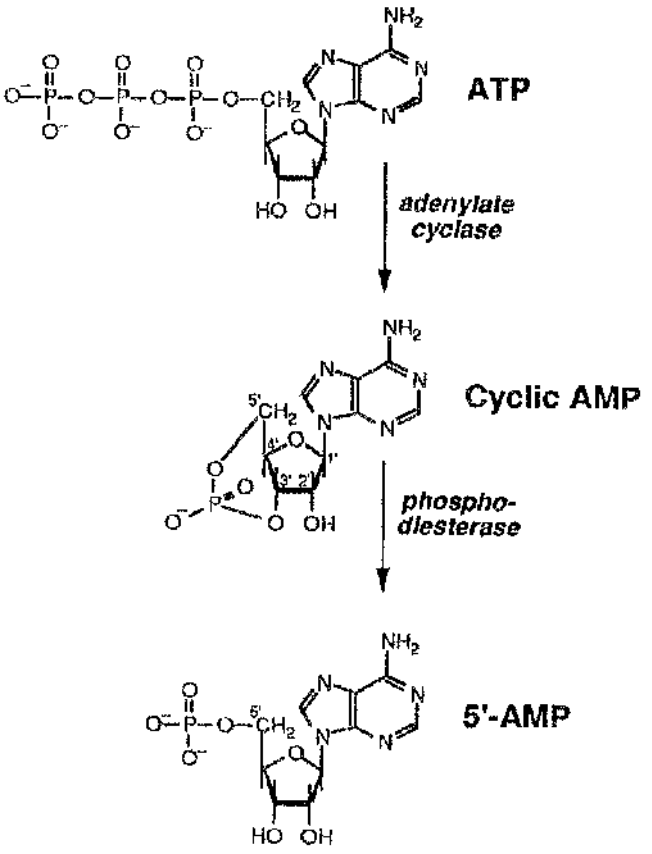
adrenaline (Sutherland and Rall, 1958). The system responsible for the maintenance of cAMP levels was eventually identified as one involving adenylate cyclase, which uses ATP to synthesize cAMP, and phosphodiesterase, which hydrolyzes the 3'-phosphate ester to give 5'-AMP (figure 20).

The involvement of a second messenger, such as cAMP, is generally accepted if two criteria are fulfilled: (1) the first messenger increases the concentration of the second messenger inside the target cell, and (2) increasing the concentration of the second messenger inside the cell mimics the effect of the first messenger. The first criterion can be readily tested using sensitive assays. In order to test the second criterion, polar analogues of cAMP were developed which are not only more cell permeable but are also resistant to breakdown by phosphodiesterases. There are also pharmacological agents available which elevate cAMP inside the cell by interfering with the second messenger biosynthetic pathway at two key points. Thus, caffeine and IBMX inhibit the breakdown of cAMP by phosphodiesterases while the plant terpenoid forskolin directly activates adenylate cyclase. Alternatively, PGE_2 can be used to stimulate this enzyme via a receptor-activated pathway. Another indirect method involves the irreversible activation of the G_s α subunit by cholera toxin. This G protein then constitutively activates adenylate cyclase which in turn produces a massive increase in the intracellular cAMP concentration.

7.1.3. G protein couples a surface receptor to adenylate cyclase

The simplest model for the second messenger system would physically associate the intracellular effector enzyme with the ligand-specific receptor. However, adipose cells were used to show that five different hormones stimulate a single adenyl cyclase by combining with separate and independent binding sites (Birnbaumer and Rodbell, 1969). The physical autonomy of the receptor and the adenyl cyclase was confirmed when transfer of an active receptor from a cell with inactive

Figure 20: Structure of cAMP and mechanism of its formation and breakdown (from Hardie, 1991).



adenyl cyclase conferred ligand-responsiveness to a cell with a defective receptor but an active adenylate cyclase (Orly and Schramm, 1976).

In early experiments, GTP was shown to be required for adenylate cyclase activity (Rodbell *et al.*, 1971). In addition, adenylate cyclase activity was stimulated by GTP and its non-hydrolyzable analogues (Londos *et al.*, 1974). Eventually it was shown that a distinct factor, a GTP-binding protein present in a detergent membrane extract, was responsible for the stimulatory actions of GTP in the GTP-dependent hormonal stimulation of adenylate cyclase (Ross *et al.*, 1978).

7.1.4. Classification of GTP-binding proteins

Signal-transducing GTP-binding proteins known today are classified into two groups. One is the high-molecular weight or heterotrimeric GTP-binding proteins (G proteins); the other contains the low-molecular weight, monomeric GTP-binding proteins of the *ras* superfamily (small G proteins). The general mechanism of action of all of these GTP-binding proteins is similar. The GTP-bound form of these proteins is an active conformation. The intrinsic GTPase activity is required to hydrolyze GTP to GDP and thus to shift the G protein to an inactive form. Interconversion of GTP- and GDP-bound form is controlled by two mechanisms. First, the conversion of GDP- to GTP-bound form is stimulated by an exchange-promoting protein. In small G proteins this activity is regulatyed by the exchange factors (GDP dissociation stimulators), while in G proteins the activated receptor assumes the role of the exchange-promoting protein. Second, the conversion of GTP- to GDP-bound form is accelerated by GTPase-activating protein in small G proteins (Kaziro *et al.*, 1991), while G proteins use their intrinsic GTPase activity. The small G proteins are involved in different areas cell biology, shared by all cell types: the Ras proteins represent major control check in signal transduction and cell proliferation; the Rho proteins appear to regulate organization of the cytoskeleton network; and the Rab proteins are

involved in the control of intracellular membrane traffic (Chavrier *et al.*, 1993).

7.1.5. Types of G proteins

G proteins are protein complexes involved in receptor-regulated signal transduction affecting the adenyl cyclase, the transducin-activated cyclic GMP phosphodiesterase, ion channels, phospholipases and other effectors (Gilman, 1987). The best known G proteins are the ubiquitous G_s and G_i which, respectively, stimulate and inhibit adenylate cyclase; G_t (transducin), a tissue specific G protein that regulates retinal cyclic GMP phosphodiesterase; and G_o which appears to regulate Ca^{2+} channels (Gilman, 1987).

7.1.6. Structure and function of G proteins

G proteins are heterotrimers composed of three distinct subunits: 39-46-kDa G_α, 37-kDa G_β and 8-kDa G_γ (Hepler and Gilman, 1992). G_β and G_γ exist as a tightly bound complex $G_{\beta\gamma}$ functioning as a unit which can associate with different G_α. Thus, G_α determines the G protein specificity. The G_α have a high affinity binding site for guanine nucleotides GTP and GDP. The GDP-bound G_α associates with $G_{\beta\gamma}$ and is inactive. G proteins interact with the receptor molecule via unique amino acid sequences on both the receptor and the G protein. The ligand-activated cell-surface receptors associated with G proteins appear to share a common motif of seven membrane-spanning domains (O'Dowd *et al.*, 1989). The activation of the receptor induces an exchange of GDP for GTP causing the GTP-bound G_α to dissociates from $G_{\beta\gamma}$. The free G_α then binds to and regulates the effector enzyme. The G_α also has an intrinsic slow-rate GTPase activity; as a result GTP is hydrolyzed to yield GDP and free inorganic phosphate and thus binds to $G_{\beta\gamma}$ and becomes deactivated. The cycle of G protein activation continues as long as the

receptor is occupied.

7.1.7. Structure of G protein α-subunit

Three regions within G_α fold to produce a GTP-binding site (Bourne *et al.*, 1991; Masters *et al.*, 1986). Indeed, the amino acid sequence GAGES at position 47 in α_s (6 in a small GTP-binding protein **ras**) is essential for normal GTPase activity; mutation within this sequence in **ras** results in inhibition of regulation by GTPase-activating protein (Gilman, 1987; Bourne *et al.*, 1991) while analogous mutation in G_α results in decrease of GTPase activity (Freissmuth and Gilman, 1989; Masters *et al.*, 1989). The carboxyl terminus of G_α appear to be at least in part responsible for receptor-G protein interaction (Hamm *et al.*, 1988) and thus posses the principal determinants of specificity for G protein-effector interactions (O'Dowd *et al.*, 1989; Masters *et al.*,1988; Woon *et al.*, 1989); the association of G_α with $G_{\beta\gamma}$ is thought to involve the amino terminus of G_α (Neer *et al.*, 1988).

7.1.8. Susceptibility of G proteins to bacterial toxins

A number of studies suggested that the cellular target protein modified by pertussis toxin activity is a G protein involved in the regulation of the adenylate cyclase complex (Codina *et al.*, 1983). Hormone-stimulated, but not basal, levels of cAMP were found to be supraoptimal within pertussis toxin-treated organ explants or tissue-cultured cell lines (Katada *et al.*, 1982). On the other hand, pertussis toxin-treated lymphocytes were unable to increase intracellular cAMP in response to PGE_2 (Parker and Morse, 1973). However, murine lymph node lymphocytes treated with pertussis toxin showed normal basal levels of cAMP and a normal response to PGE_2 (Spangrude *et al.*, 1985a).

The conserved arginine at position 201 of α_s is the site of ADP-ribosylation catalyzed by cholera toxin. Modification of α_s with cholera

toxin constitutively activates the G protein by inhibiting its GTPase activity. On the other hand, ADP-ribosyltransferase, a subunit of pertussis toxin, penetrates cells and uses endogenous nicotinamide-adenine dinucleotide to ADP-ribosylate a cysteine near the carboxyl terminus of α_i, α_t, and α_o (West *et al.*, 1985; Sunyer *et al.*, 1989). The α_s, which lacks a cysteine residue in this position, is not affected. ADP-ribosylation by pertussis toxin appears to cause uncoupling of G proteins from activated receptors (Ui, 1984) resulting in the inhibition of the relevant agonist-activated pathways.

7.1.9. General activators of G proteins

Fluoride, in the millimolar concentration range, is a general activator of G proteins. AlF_4^- formed from F^- and Al^{3+} binds to GTP and thus mimics γ-phosphate of GTP, converting GDP-bound inactive G protein to an activated form. In a number of cell types addition of NaF to pre-labeled cells or isolated membranes has been shown to stimulate the formation of inositol phosphates. In addition, non-hydrolyzable analogues of GTP, such as GTPγS, have been used for sustained activation of G proteins.

7.2. EXPERIMENTAL

7.2.1. Effect of DMSO on repolarization of Jurkat cells

The application of drugs non-soluble in aqueous media called for the utilization of DMSO as a solvent. To determine the effect of this solvent on the polarization of Jurkat cells and its toxicity a preliminary experiment was conducted. It was thought important to always use DMSO at a dilution which would not interfere with the assay. Several dose-response experiments showed that DMSO diluted 1:100 had a severe effect on the cell polarization and seemed to be noticeable even at the dilution of

1:200 (figure 21). In the subsequent experiments, DMSO was always diluted to at least 1:1000 to effectively eliminate its negative effect on the polarized cells.

7.2.2. Effect of cAMP and agents that modify intracellular cAMP on repolarization of dbcAMP-cultured Jurkat cells

7.2.2.1. Effect of dbcAMP on repolarization of dbcAMP-cultured Jurkat cells

The effect of the dbcAMP-containing supernatant from dbcAMP-cultured Jurkat cells on the short-term repolarization of these and of αCD3-activated PBL was explored further. First, the possibility of accumulation of an inhibitory factor in these supernatants during the cell culture was tested when fresh medium containing 1 mM dbcAMP was added to αCD3-stimulated PBL. Clearly, cAMP gradually reversed the polarization process observed in PBL repolarized in plain medium (figure 22-A). A similar observation was made with repolarized Jurkat cells exposed to 1 mM dbcAMP (figure 22-B). However, the dynamics of the response were different. The slow, almost linear, response observed with PBL was replaced by a rapid decrease in polarized morphology in Jurkat cells. The decrease in the proportion of polarized and irregular cells was reflected by an appropriate increase in the proportion of round cells. The change was virtually complete by 10 min of the time-course experiment.

The dose-response curve for the polarization inhibition by dbcAMP indicated that the minimum effective dose is between 62 and 125 μM with ED_{50} of about 750 μM (figure 23-A). The dose-response curves obtained in the presence or absence of EDTA added to the repolarization medium were virtually identical with similar ED_{50} (figure 23-B). The only notable difference was a slightly lower repolarization of Jurkat cells in the Ca^{2+}-free medium compared to the normal, Ca^{2+}-supplemented HBSS. In any case, the stimulus supplied by dbcAMP was shown to be independent of

Figure 21: Dose-dependent action of DMSO on dbcAMP-cultured Jurkat cells. The routine medium was supplemented with 1 mM dbcAMP and the incubation was continued for 3 days. The cells were washed with HBSS/MOPS and repolarized at 37°C for 30 min. 10 μl of dilutions of DMSO were added and the incubation was continued for 30 min. The cells were finally fixed and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

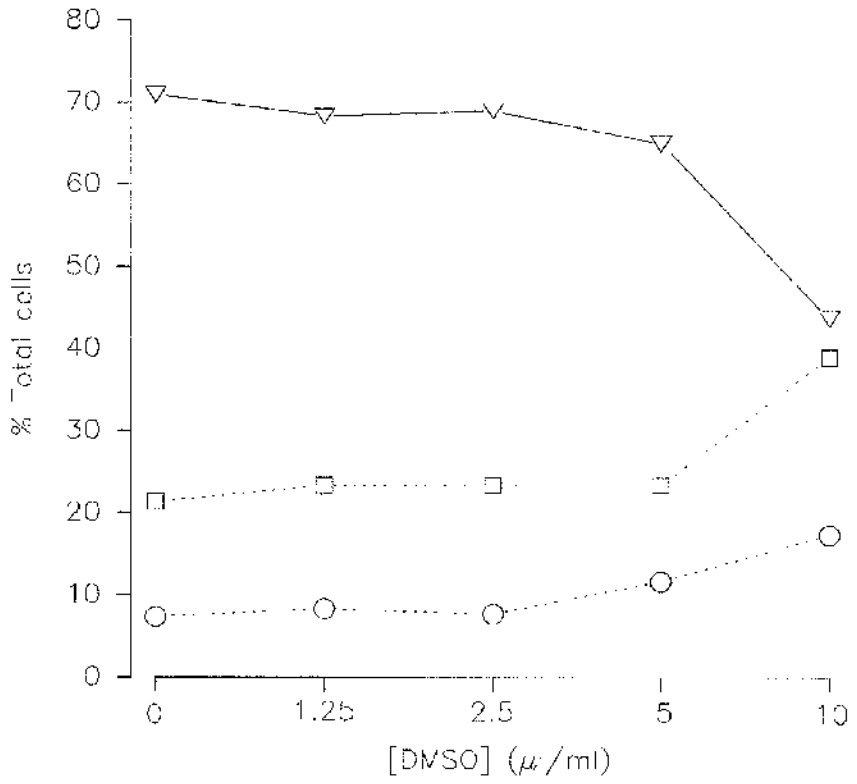


Figure 22: Time-course of the effect of 1 mM dbcAMP on PBL cultured with αCD3 for 48 hrs (A) and Jurkat cells cultured with 1 mM dbcAMP for 3 days (B). The cells were washed, resuspended in HBSS/MOPS and repolarized at 37°C for 30 min. dbcAMP was added to final concentration of 1 mM and the incubation was continued for various time intervals. The cells were finally fixed and the morphology was examined and quantified under phase contrast. (A) Round cells in the absence (open circles) or presence (full circles) of dbcAMP; (B) Round cells (circle), irregular cells (square) and polarized cells (triangles) in the presence of dbcAMP.

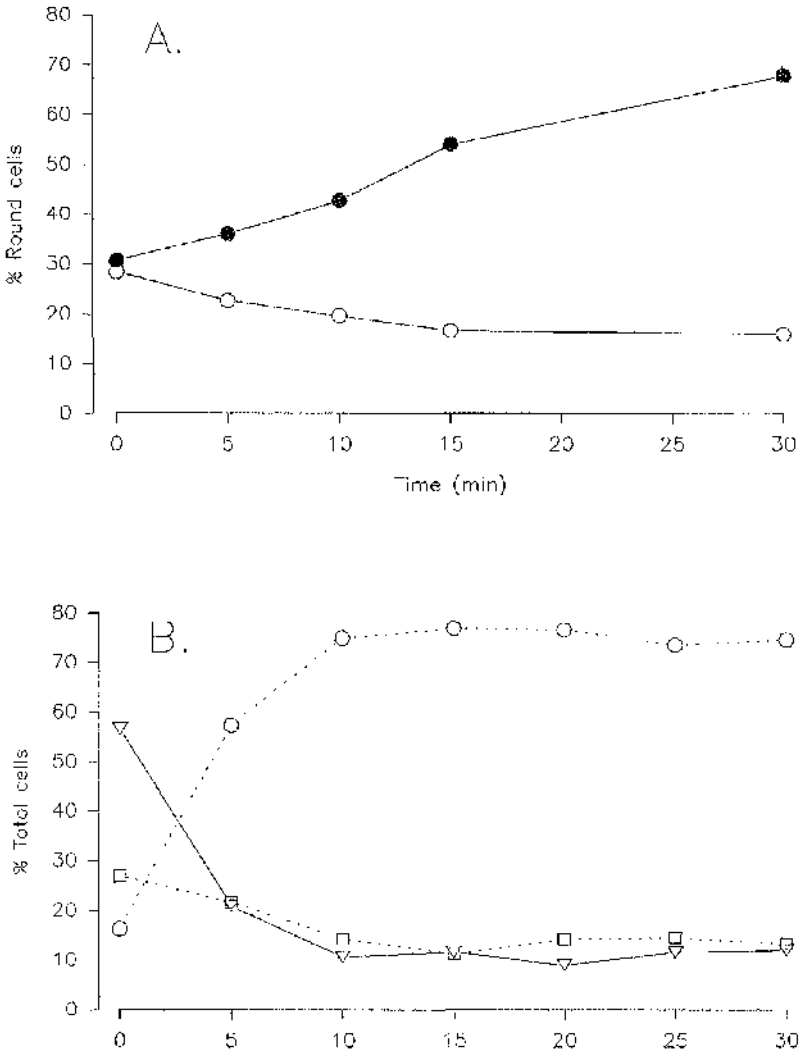
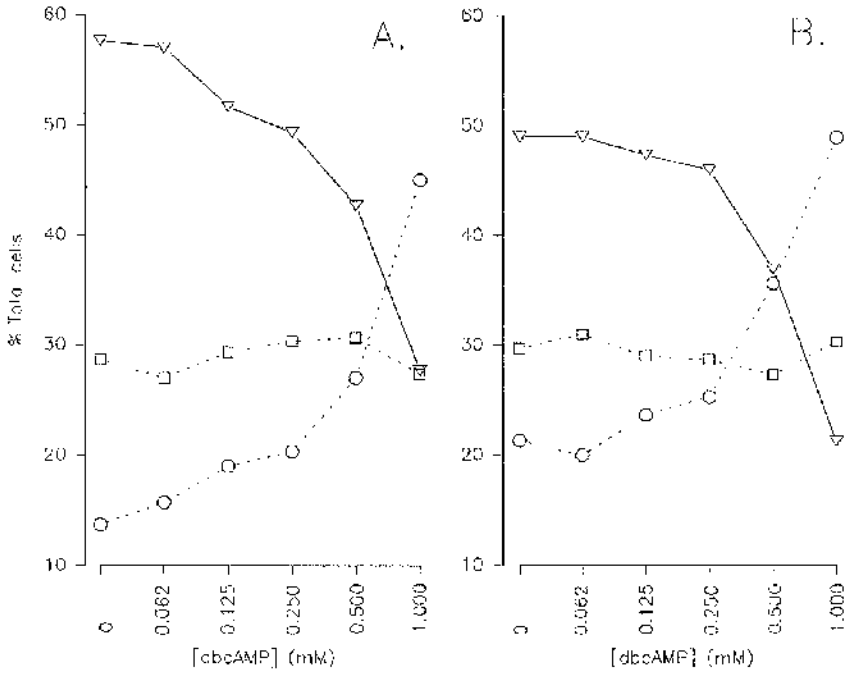


Figure 23: Dose-dependent action of dbcAMP on the morphology of Jurkat cells cultured with 1 mM dbcAMP for 3 days. The cells were washed twice with HBSS/MOPS containing Ca^{2+} (A) or in Ca^{2+}-free HBSS/MOPS supplemented with EDTA. The same medium then was used to resuspended and repolarize the cells at 37°C for 30 min. dbcAMP dilutions were added and the incubation was continued for 30 min. The cells were finally fixed and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



the presence of extracellular Ca^{2+}.

7.2.2.2. Effect of increase in intracellular cAMP concentrations on PBL morphology

Experiments with αCD3-activated PBL revealed that forskolin, PGE_2 and IBMX induced a gradual decrease in the proportion of polarized cells (figure 24-A to 24-C). The maximum change in morphology by either of the drugs separately was achieved by 30 min. The percent inhibition[2] achieved at this time was 23% for forskolin, 19% for PGE_2 and 23% for IBMX. The change in response to PGE_2 and IBMX appears to be more permanent while the forskolin-induced rounding up was partially reversed by 60 min.

When PBL were preincubated with IBMX and then forskolin was added, a faster (within 15 min) and a sustained decrease in polarized PBL was observed (figure 25-A). The forskolin-stimulated inhibition of PBL polarization at 30 min was 29%, while the presence of IBMX increased the efficiency of forskolin inhibition to 48%.

The similarity of the sustained rounding up in response to IBMX or PGE_2 alone suggests that in the latter case, an inactivation of phosphodiesterase may play a supportive role. Although PGE_2 activates adenylate cyclase, it is possible that the signal through the PGE_2 receptor induces a concominant inactivation of phosphodiesterase.

The direct activation of adenylate cyclase with forskolin, and the resulting increase in intracellular cAMP concentrations, may activate a feedback pathway which results in an increase of phosphodiesterase activity. Thus, the observed decrease in cAMP effect on PBL morphology was reversed after 60 min while the PBL remained rounded up for up to

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[2]The formula used was:

$$\%I=(P_0 - P_{30}) / P_0 \times 100\%,$$

where %I is percent inhibition, and P_0 and P_{30} is the proportion of polarized cells at time 0 and 30 min after the agent was added.

Figure 24: Effect of 10^{-5}M forskolin (A&D), 10^{-6}M PGE_2 (B&E), and 10^{-4}M IBMX (C&F) on the morphology of repolarized PBL previously stimulated with αCD3 (A-C) or of repolarized dbcAMP-cultured Jurkat cells (D-F). PBL were cultured in the presence of 10 ng/ml OKT3 for 48 hr while Jurkat cells were cultured with 1 mM dbcAMP for 3 days. The cells were washed twice, resuspended in HBSS/MOPS and repolarized at 37°C for 30 min. 10 μl of appropriate dilutions of the drugs were added and the incubation was continued for various time intervals. The cells were finally fixed and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

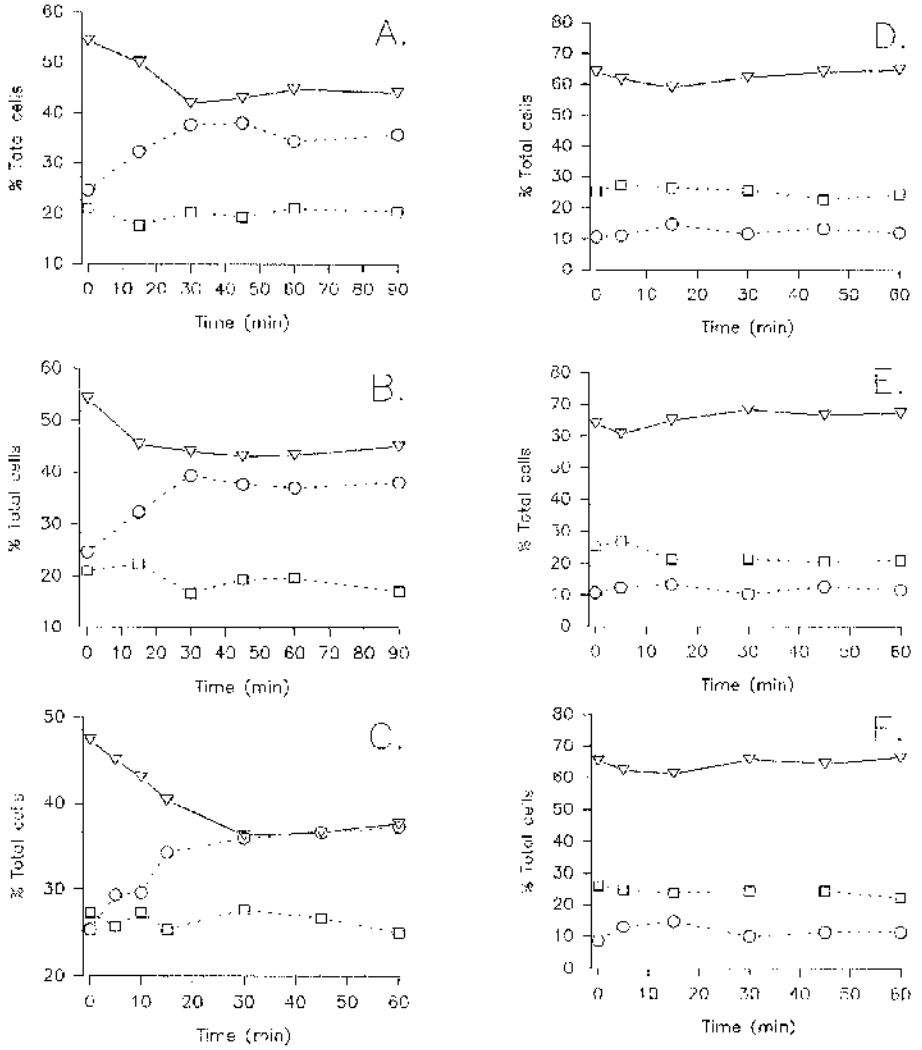
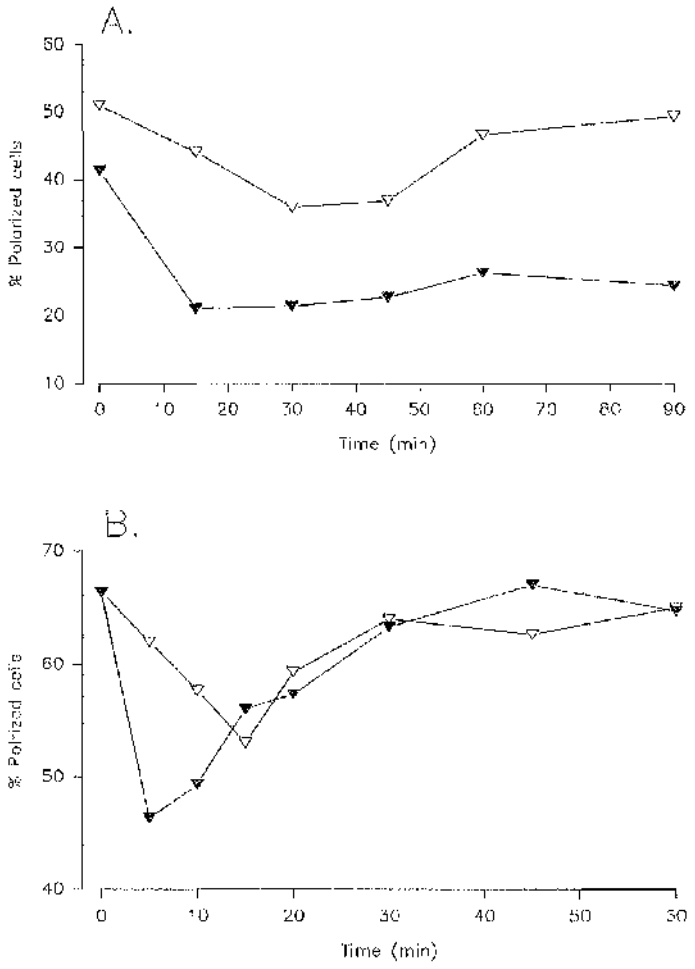


Figure 25: Effect of preincubation with 10^{-4}M IBMX on the morphological changes induced by 10^{-5} forskolin in repolarized PBL previously stimulated with αCD3 (A) and by 10^{-5}M forskolin or 10^{-6}M PGE_2 in repolarized dbcAMP-cultured Jurkat cells (B). PBL were cultured in the presence of 10 ng/ml OKT3 for 48 hr while Jurkat cells were cultured with 1 mM dbcAMP for 3 days. The cells were washed twice, resuspended in HBSS/MOPS and repolarized at 37°C for 30 min. 10 μl of 10^{-2}M IBMX were added and the incubation was continued for 1 hr. Then, appropriate dilutions of the drugs were added and the incubation was continued for various time intervals. The cells were finally fixed and the morphology was examined and quantified under phase contrast. (A) Polarized PBL in the presence (full triangle) or the absence (open triangle) of IBMX; (B) Polarized Jurkat cells in the presence of PGE_2 (full triangle) or forskolin (open triangle).



90 min when the phosphodiesterase was inhibited by IBMX.

7.2.2.3. Effect of increase in intracellular cAMP concentration on the morphology of dbcAMP-cultured Jurkat cells

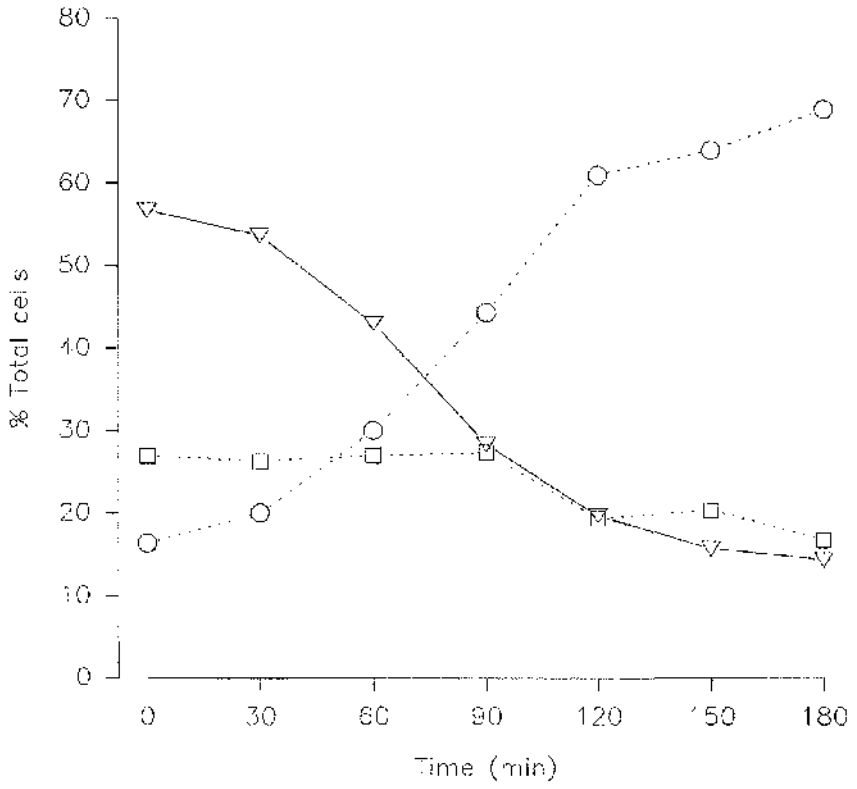
Forskolin, PGE_2, and IBMX alone, at concentrations that were active in PBL, failed to induce any change in morphology in repolarized Jurkat cells that had been cultured with dbcAMP for 4 days (figure 24-D to 24-F). The background effect of DMSO did not allow for a further increase in the drug dose.

The preincubation of Jurkat cells with IBMX yielded a response to 10^{-5}M forskolin and 10^{-6}M PGE_2 (figure 25-B). The dynamics of the response, as observed by the reduction of the polarized Jurkat cell population, were noticeably different for the two drugs. The onset of the response to PGE_2 was apparent and maximal by 5 min, while 15 min were necessary for forskolin to reach the maximum effect. The time-delay in the action of forskolin was most likely due to its need to cross the plasma membrane before it can act on the intracellular enzyme. In contrast, PGE_2 acts through a surface receptor, giving it a rapid access to the intracellular components. Indeed, the dynamics of action of forskolin and PGE_2 in intracellular cAMP regulation resemble the dynamics of action of colchicine and fMLP respectively in the acquisition of motility by the neutrophils.

The response to both forskolin and PGE_2 was transient, returning to the near-initial state by 30 min. Taken together, the observations suggest that Jurkat cells may have an extremely active phosphodiesterase, even after 10^{-4}M IBMX had been used.

The treatment with cholera toxin resulted in an interesting observation (figure 26). The Jurkat cells showed a gradual decline in polarized morphology with near-maximal response by 2 hrs of incubation. The inhibition at this time point was 65%. Thus, cholera toxin must induce in Jurkat cells a massive increase of cAMP, sufficient to counteract

Figure 26: Time-course of the effect of 1 μM cholera toxin on dbcAMP-cultured Jurkat cells cultured with 1 mM dbcAMP for 3 days. The routine medium was supplemented with 1 mM dbcAMP and the incubation was continued for 3 days. The cells were washed with HBSS/MOPS and repolarized at 37°C for 30 min. 10 μl of cholera toxin were added to final concentration of 1 μM and the incubation was continued for various time intervals. The cells were finally fixed and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



the hyperactive phosphodiesterase even in the absence of IBMX.

7.2.2.4. Absolute cAMP levels in Jurkat cells stimulated with forskolin, PGE_2 and IBMX

The measurement of cAMP levels was performed using a competitive method utilizing a known concentration of radiolabelled cAMP and a specific cAMP-binding protein.

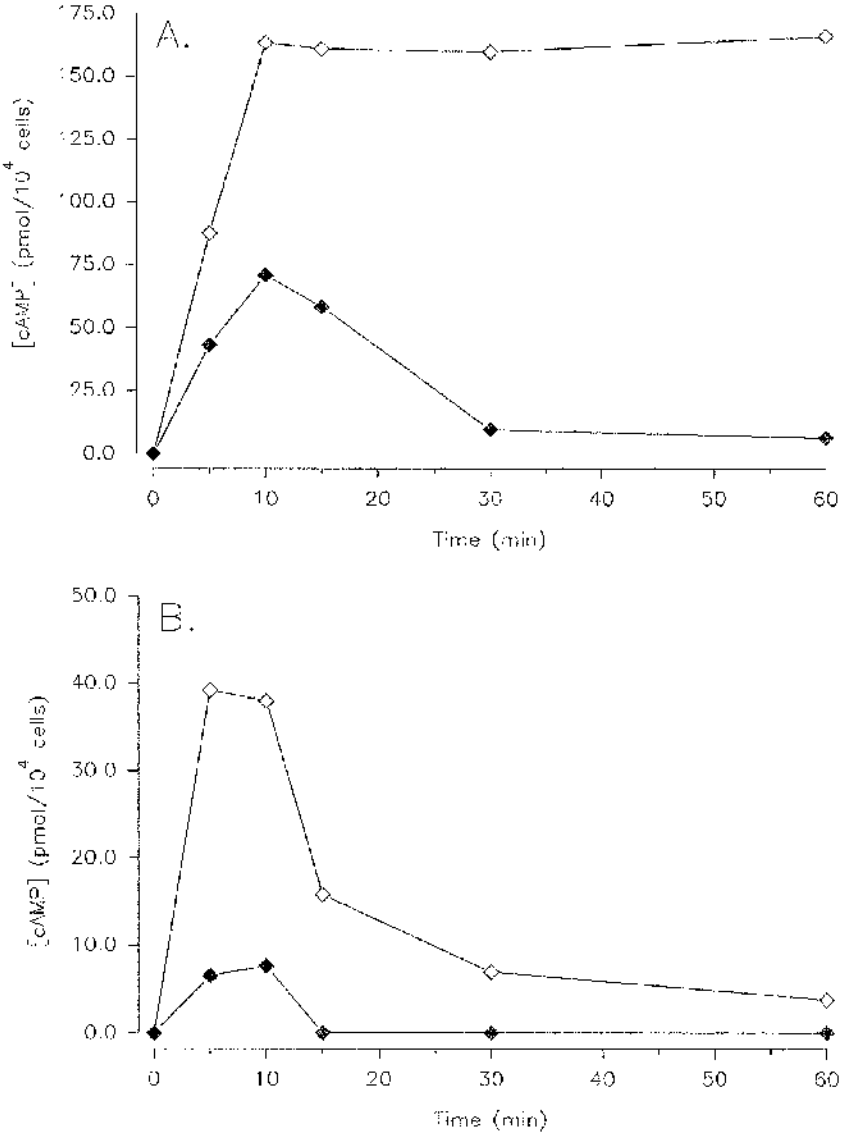
IBMX alone was shown not to have a detectable effect on the $[cAMP]_i$. The cAMP levels measured in Jurkat cells treated with forskolin and PGE_2 alone or following preincubation with IBMX are shown in figure 27.

Forskolin and PGE_2 alone induced a significantly smaller increase in $[cAMP]_i$ compared to the Jurkat cells pretreated with IBMX. Even in the absence of IBMX, forskolin induced about 8-fold more intracellular cAMP compared to PGE_2. The time-course of $[cAMP]_i$ change was different for these two agents as illustrated by the time needed to achieve maximum cAMP production: 10 to 15 min for forskolin and 5 to 10 min for PGE_2. This time-course characteristics resembled those of the polarization inhibition observed in Jurkat cells in the presence of IBMX.

The addition of IBMX resulted in a 2-fold increase in $[cAMP]_i$ induced by forskolin and about 4-fold increase with PGE_2. The dynamics characterizing the increase to maximal cAMP concentration were unaffected by the presence of IBMX. However, the inactivation of phosphodiesterase resulted in a sustained cAMP increase in Jurkat cells stimulated with forskolin. In contrast, PGE_2 induced a transient increase in $[cAMP]_i$ even in the presence of IBMX. Thus, the activation of PGE_2 receptor engages mechanisms of $[cAMP]_i$ regulation that are independent of phosphodiesterase. A downregulation of the PGE_2 receptor may be one of such mechanisms.

A sustained increase in $[cAMP]_i$, resulting from combined IBMX and forskolin action, causes a transient effect on the locomotory

Figure 27: Time-course of changes in intracellular cAMP concentration in Jurkat cells following addition of 10^{-5}M forskolin (A) or 10^{-6}M PGE_2 (B). The cells were washed, resuspended in HBSS/MOPS and repolarized at 37°C for 1 hr with or without 10^{-4}M IBMX. The appropriate concentrations of the drugs were then added and the incubation was continued for various time intervals. At each time point, cAMP was extracted and assayed. Concentration of cAMP in cells preincubated in the absence (full diamonds) or presence (open diamonds) of IBMX.



morphology of dbcAMP-cultured Jurkat cells. Thus, the rounding up must be reversed in these cells by feedback mechanism other than the activation of phosphodiesterase. The effect of forskolin may thus be reversed at the level of PKA-catalyzed phosphorylation by inducing a specific phosphatase.

7.2.2.5. Effect of cAMP-dependent protein kinase inhibitor on the effect induced by forskolin and PGE_2

Cyclic AMP exerts most of its intracellular activity through the activation of PKA. An attempt was made to determine if in fact these protein kinases are involved in the rounding up seen with forskolin or PGE_2.

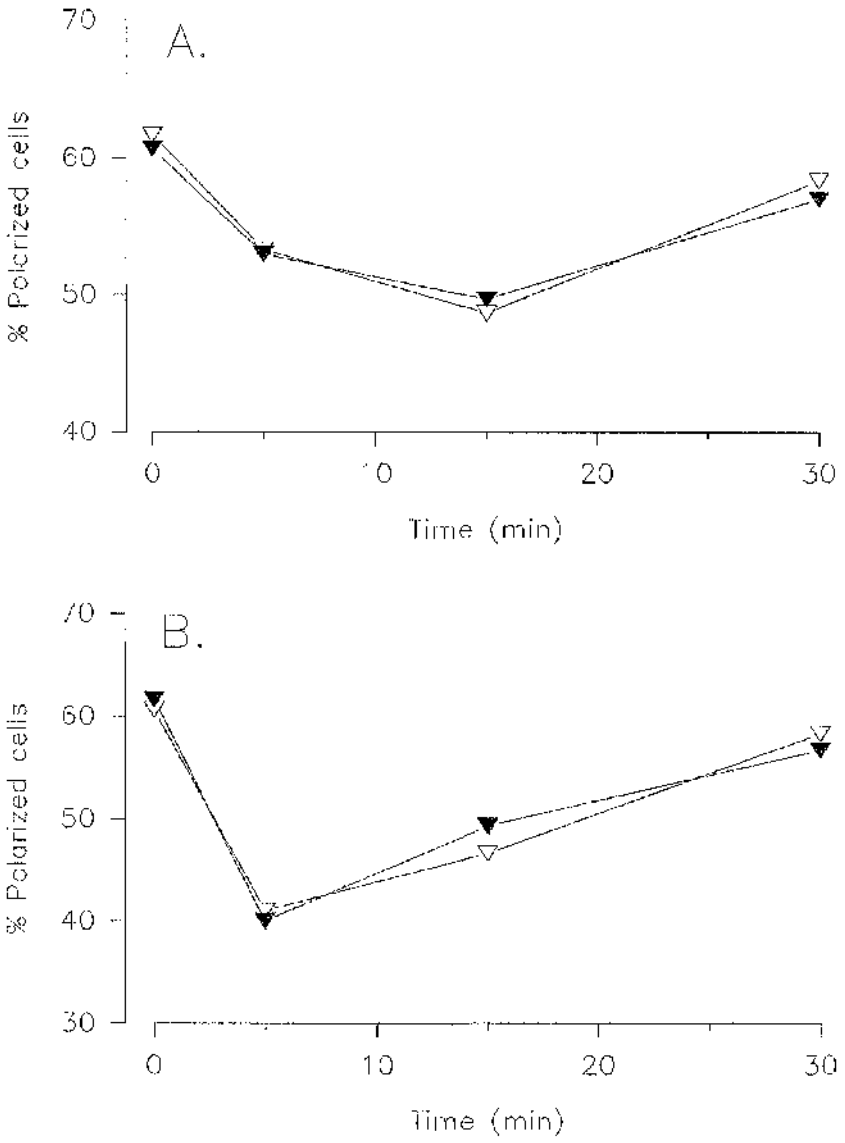
Adenosine-3',5'-cyclic monophosphothioate (Rp-isomer), a specific inhibitor of PKA, was used in an effort to diminish or eliminate the effect of forskolin or PGE_2 (figure 28). The maximum available concentration of 10^{-5} was used but no effect on the change of cell morphology was observed.

Having no positive control, it was difficult to determine if this lack of effect is due to an insufficient inhibitor concentration or, if in fact, the cAMP-stimulated rounding up is mediated by mechanisms not involving PKA.

7.3. SUMMARY

In conclusion, the above experiments showed that incubation of Jurkat cells with dbcAMP, resulted within minutes in a dose-dependent permanent reversal of polarized morphology. The action of cholera toxin required hours to achieve permanent reversal of polarized morphology, while PGE_2 and forskolin, in conjunction with IBMX, resulted within minutes in a transient polarization reversal.

Figure 28: Effect of 10^{-5}M adenosine-3',5'-cyclic monophosphothioate (Rp-isomer; cAMPS-Rp) on (A) forskolin- (B) and PGE_2-induced rounding up of dbcAMP-cultured Jurkat cells. The cells cultured with 1 mM dbcAMP for 3 days, washed twice, resuspended in HBSS/MOPS and repolarized at 37°C for 30 min in the presence of 10^{-4}M IBMX. Forskolin or PGE_2 were added to final concentration of 10^{-5}M and 10^{-6}M respectively and the incubation was continued for various time intervals. The cells were finally fixed and the morphology was examined and quantified under phase contrast. Polarized cells in the absence (open triangles) or presence (full triangles) of cAMPS-Rp.



8. EFFECT OF αCD3 ON THE MORPHOLOGY OF dbcAMP-CULTURED JURKAT CELLS

8.1. BACKGROUND

The Jurkat cells cultured in the presence of dbcAMP acquire the ability to locomote. A possible involvement of a signalling pathway, involving the adenylate cyclase, in the regulation of polarized morphology of these cells was identified. In addition, the expression of the CD3 antigen increased on the surface of dbcAMP-cultured Jurkat cells. The significance of this observation was examined in the next series of experiments where the effect of stimulation through the TcR/CD3 complex on the Jurkat cell motility was examined.

8.1.1. Structure of TcR/CD3 complex

8.1.1.1. Structure of TcR

The recognition by T lymphocytes of antigens associated with MHC surface proteins is mediated by the specific TcR αß or γδ disulphide-linked heterodimers (Yagüe *et al.*, 1985; Dembic *et al.*, 1986). Here, the former is expressed by most of the peripheral mature T cells while the latter is expressed on most of the lymphocytes residing in the tissue epithelia. The polymorphic α and ß chains are members of immunoglobulin superfamily and undergo gene rearrangement of V/J gene segments in the α chain and V/D/J gene segments in the ß chain. The TcR (Meuer *et al.*, 1983a; Oettgen *et al.*, 1984) non-covalently associates with invariant CD3 complex (Weiss and Imboden, 1987) and a dimer of ζ chains (Samelson *et al.*, 1985b; Oettgen *et al.*, 1986; Weissmann *et al.*, 1986).

8.1.1.2. Structure of CD3 complex

The CD3 complex consists of two distinct glycoproteins, CD3γ and CD3δ, and a polypeptide CD3ε (Borst *et al.*, 1982, 1983ab, 1984; Kanellopoulos *et al.*, 1983; Pessano *et al.*, 1985). The CD3 subunits have extracellular immunoglobulin-like domains and, compared to the 5 amino acid intracellular portion of TcR chains, large cytoplasmic domains of 40 to 80 amino acids (van den Elsen *et al.*, 1984; Gold *et al.*, 1986; Krissansen *et al.*, 1986; Weiss *et al.*, 1986; Clevers *et al.*, 1988). CD3 subunits exist in the receptor complex as two non-covalent heterodimers CD3γε and CD3δε (Konig *et al.*, 1990; Blumberg *et al.*, 1990; De la Herra *et al.*, 1991). The TcRαß/CD3γδε complex is formed on the surface of the endoplasmic reticulum and then is transported to the cell surface through the Golgi apparatus. The assembly of the complex appears to be regulated by CD3ω (Terhorst *et al.*, 1990).

8.1.1.3. Structure of ζ chain-associated subunit

The ζ chain is a transmembrane protein with a 9 amino acid extracellular domain, a transmembrane domain and a 113 amino acid intracellular domain (Baniyash *et al.*, 1989; Weissman *et al.*, 1988). Approximately 90% of ζ chain exists as a ζζ disulphide-linked homodimer (Baniyash *et al.*, 1988a). A small percentage of ζ chains forms a heterodimer with the η chain, an alternatively spliced form of the ζ chain (Baniyash *et al.*, 1988a; Orloff *et al.*, 1989; Jin *et al.*, 1990) or the γ chain of the multi-subunit high affinity IgE receptor $Fc_{\epsilon}RI$ (Orloff *et al.*, 1990). Although these peptides were considered to be components of CD3 complex, it appears that ζζ and ζη independently associate not only with the TcR/CD3 complex but also with other signal transducing receptors (Finkel *et al.*, 1991).

8.1.2. CD3 and ζ chain are signal transducing elements of TcR/CD3

The importance of TcR/CD3 was clearly demonstrated when the modulation of the complex from the surface of T cells resulted in their inability to be activated, followed by recovery of activation upon re-expression of CD3 (Reinherz *et al.*, 1982). Initially, the role of TcR-associated subunits in a signal transduction was deduced, primarily, from the existence of their prominent cytoplasmic domains (Weiss and Imboden, 1987). Indeed, the cell surface TcR expression and function could be restored in a ζ chain-negative murine T cell hybridoma variant reconstituted with full-length ζ chain (Sussman *et al.*, 1988). The importance of the cytoplasmic domain was underscorred when reconstitution with molecules truncated in this domain resulted in impaired cell activation (Frank *et al.*, 1990). Further evidence was obtained from experiments with chimeric receptors consisting of extracellular domains of CD4, CD8, or IL2Rα and cytoplasmic domains of ζ, η, or $FC_{\epsilon}RI\gamma$ chains (Irving and Weiss, 1991; Romeo and Seed, 1991; Letourneur and Klausner, 1991). The role of CD3ε in TcR signalling was demonstrated in a murine hybridoma which could be activated in the absence of ζ chain cytoplasmic domain (Wegener *et al.*, 1992; Hermans and Malissen, 1993).

The comparison of ζ chain- and CD3ε-mediated activation events revealed that stimulation through the different cytoplasmic domains resulted in a different patterns of protein phosphorylation (Letourneur and Klausner, 1992). Thus, two distinct and autonomous biochemical pathways appear to be activated upon stimulation of the TcR/CD3 complex. While αCD3 can signal through either signal-transduction module (Wegener *et al.*, 1992), αThy-1 and αCD2 appears to signal exclusively through the ζζ dimer (Wegener *et al.*, 1992; Moingeon *et al.*, 1992). The coupling of the various TcR/CD3 complex structures to intracellular biochemical pathways is addressed in a subsequent chapter.

8.2. EXPERIMENTAL

8.2.1. Effect of αCD3 on the morphology of dbcAMP-cultured Jurkat cells

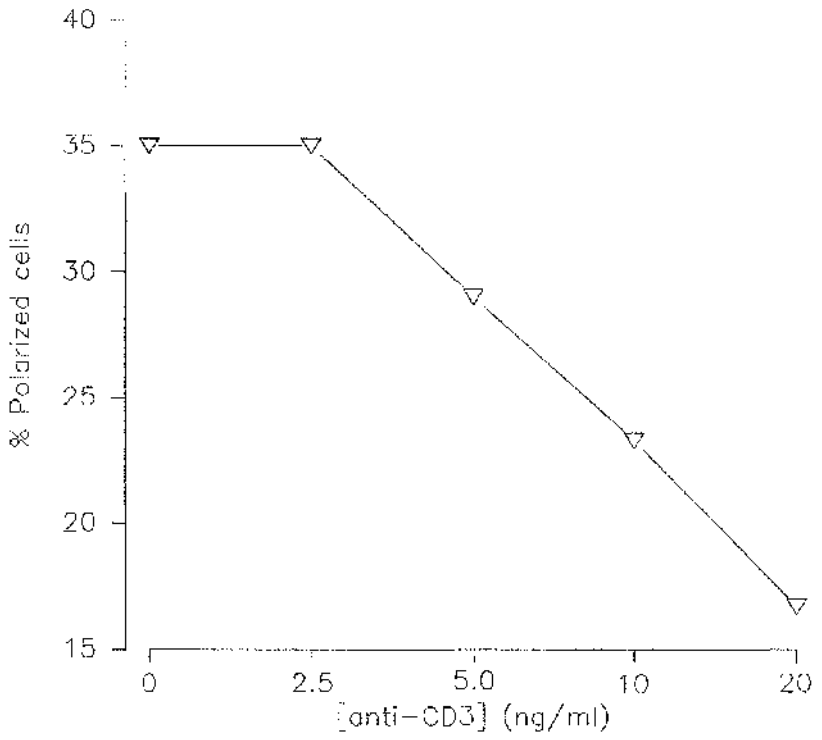
8.2.1.1. Dose-dependent effect of αCD3 on the extent of repolarization of dbcAMP-cultured Jurkat cells

The activation of normal blood lymphocytes through the TcR/CD3 complex for a minimum of 24 hrs, in the presence of monocytes, was shown to result in an increased proportion of polarized cells when compared to cells cultured only in HBSS/HSA medium (Wilkinson and Higgins, 1987a). However, no short-term effect of αCD3 on the morphology of PBL was demonstrated. To establish, whether stimulation through the TcR/CD3 complex affects the extent of repolarization of dbcAMP-cultured Jurkat cells, a preliminary dose-response experiment was performed. Surprisingly, a dose-dependent decrease in the number of polarized cells was observed after a 30 min incubation with the monoclonal antibody (figure 29).

8.2.1.2. Effect of αCD3 on the time-course of repolarization of dbcAMP-treated Jurkat cells

To examine this effect further, a time-course of repolarization was observed in the presence and in the absence of the highest αCD3 dose. The control series showed a repolarization similar to that observed previously (figure 15). However, in the cells repolarized in the presence of αCD3, the initial increase in number of polarized cells was reversed after 5-10 min, reaching a minimum at 15 min. After this time, the number of polarized cells started to recover to the levels established by the cells in the control series.

Figure 29: Dose-dependent action of αCD3 on the morphology of dbcAMP-cultured Jurkat cells. The routine medium was supplemented with 1 mM dbcAMP and the incubation was continued for 3 days. The cells were washed with HBSS/MOPS and repolarized at 37°C for 30 min. αCD3 was added and the incubation was continued at 37°C for 30 min. The cells were finally fixed and the morphology was examined and quantified under phase contrast.



8.2.1.3. Effect of αCD3 on the morphology of repolarized, dbcAMP-cultured Jurkat cells

To isolate the time-course of the effect of αCD3 from the normal dynamics of cell repolarization, the cells were repolarized for 30 min prior to the addition of the antibody (figure 30). This experiment revealed that the maximum effect of αCD3 occurs 10-15 min after addition of the antibody. After this time, the cells began the repolarization process and returned to the initial state after 90 min. A time-course of action of αCD2, antibody to another surface molecule implicated in lymphocyte activation, was shown to have no effect on the morphology of repolarized dbcAMP-cultured Jurkat cells (results not shown).

The transient effect of αCD3 could be explained in three ways. First, the CD3 molecule on the cell surface is known to be internalized as a response to the binding of αCD3. Thus, even in the presence of the αCD3 in the medium the cells would be lacking the structures necessary for the delivery of negative signals to the cells. Second, the CD3 molecule follows the cycle of internalization, dissociation and reexpression, thus depleting the extracellular αCD3. Third, the intracellular signal messengers generated as a response to the binding of αCD3 are removed after a feedback pathway has been triggered.

8.2.2. Cause of the transient nature of αCD3 effect on dbcAMP-cultured Jurkat cell morphology

8.2.2.1. TcR/CD3 internalization

The first possibility was examined using the αCD3 antibody immobilized on the surface of magnetic beads. The internalization of the monoclonal antibody was thus prevented. Figure 31 shows the result of an experiment where the time-course of action of immobilized and soluble αCD3 was examined. Although the bound αCD3 was not as effective as

Figure 30: Time-course of action of αCD3 on repolarized dbcAMP-cultured Jurkat cells. The routine medium was supplemented with 1 mM dbcAMP and the incubation was continued for 3 days. The cells were washed twice with HBSS/MOPS, resuspended in 1 ml of the medium and repolarized at 37°C for 30 min. OKT3 was added to the cells to the final concentration of 20 ng/ml and the incubation was continued at 37°C for various time intervals. The cells were finally fixed and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

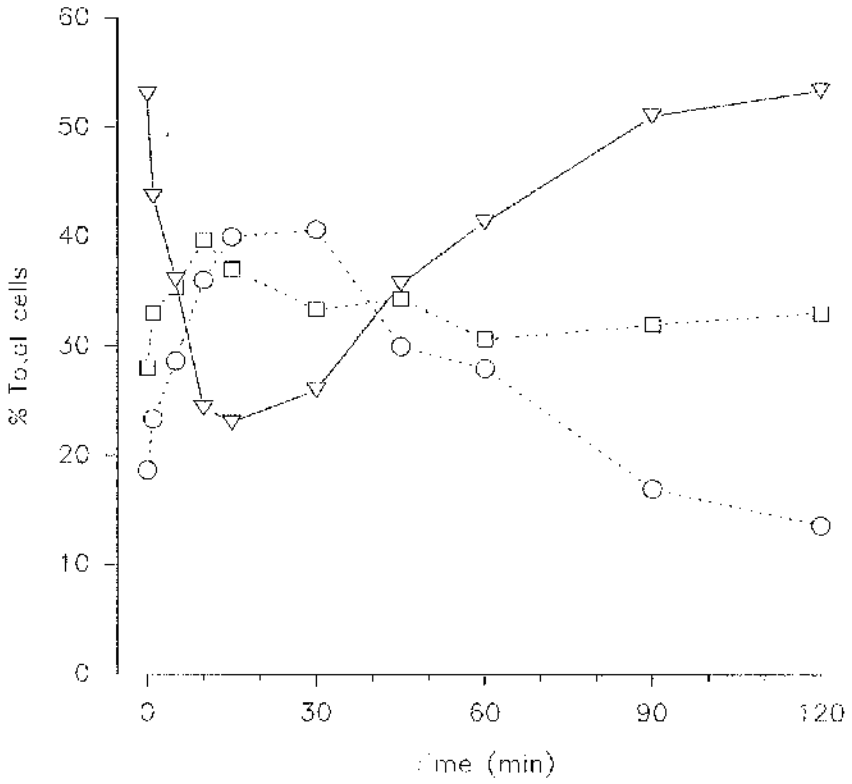
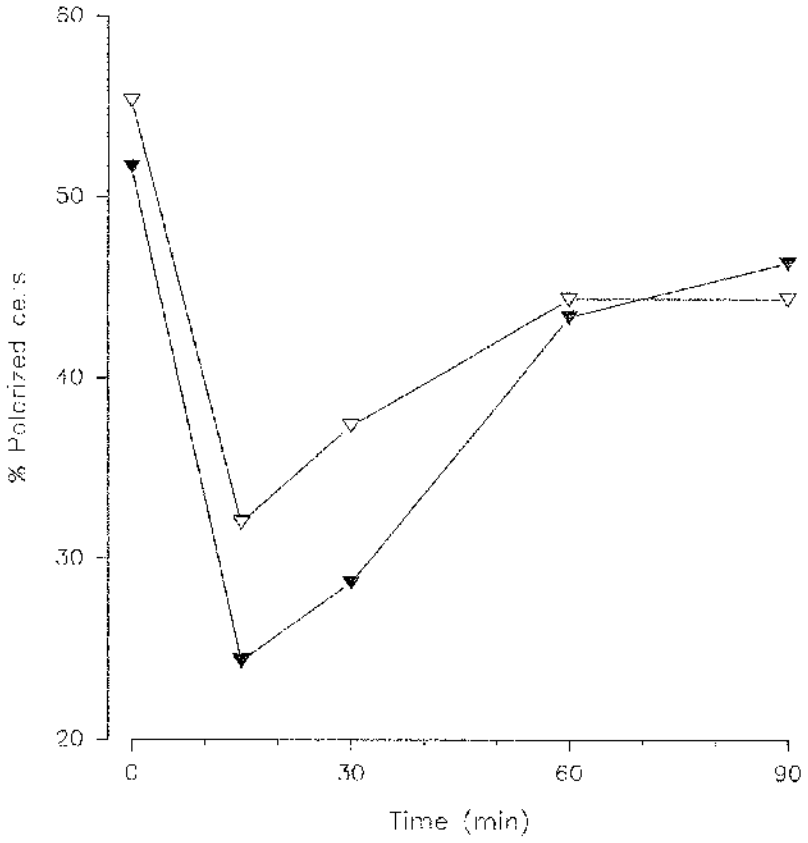


Figure 31: Time-course of action of immobilized or soluble αCD3 on repolarized dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, resuspended in 1 ml of the medium and repolarized at 37°C for 30 min. OKT3, either immobilized onto Dynabeads^{R} or in HBSS/MOPS/HSA was added to the final concentration of 20 ng/ml and the incubation was continued at 37°C for various time intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Polarized cells in the presence of immobilized αCD3 (open triangles) or soluble αCD3 (full triangles).



the soluble antibody, the transient character of its action was clearly demonstrated. The lower efficiency of the bound αCD3, when compared to the soluble antibody, may be due to slightly lower concentration of αCD3 retained by the beads. Another line of evidence against the disappearance of the surface CD3 molecule was the FACS examination of the Jurkat cells incubated with αCD3 for various time intervals (figure 32). Clearly, a short term incubation of the cells with this antibody did not significantly affect the surface CD3 density. Moreover, the CD3 molecule was still present on the cell-surface following an overnight culture.

8.2.2.2. αCD3 depletion

The depletion of the extracellular αCD3 was examined in an experiment, where dbcAMP-cultured Jurkat cells were pretreated with αCD3 and allowed to repolarize for 90 min after the addition of the antibody (figure 33). After this time, an equal dose of αCD3 was added. The cells were shown to be refractory to further stimulation by αCD3 as the proportion of polarized cells remained virtually unchanged. Thus, it appears that the stimulation of TcR/CD3 complex results in a transient generation or release of intracellular signals which interfere with the maintenance of the locomotory capacity in dbcAMP-cultured Jurkat cells. In addition, these intracellular messengers are exhaustible. The nature of such signals, known to be generated in response to TcR/CD3-mediated stimulus, was addressed in subsequent experiments.

8.3. SUMMARY

In conclusion, the above experiments showed that incubation of dbcAMP-cultured Jurkat cells with αCD3 transiently inhibited the process of repolarization and even transiently rounded up repolarized cells. Once the polarization was restored in these cells, they became refractory to a subsequent αCD3 treatment.

Figure 32: Time-course of CD3 expression on the surface of dbcAMP-cultured Jurkat cells exposed to soluble αCD3. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and resuspended in 50 μl of the medium at room temperature. The experiment was initiated by the addition of HBSS/MOPS/HSA containing 20 ng/ml OKT3 and the cells were incubated at 37°C for various intervals. Finally the cells were washed with ice-cold PBS/azide and stained with αCD3 for FACScan analysis.

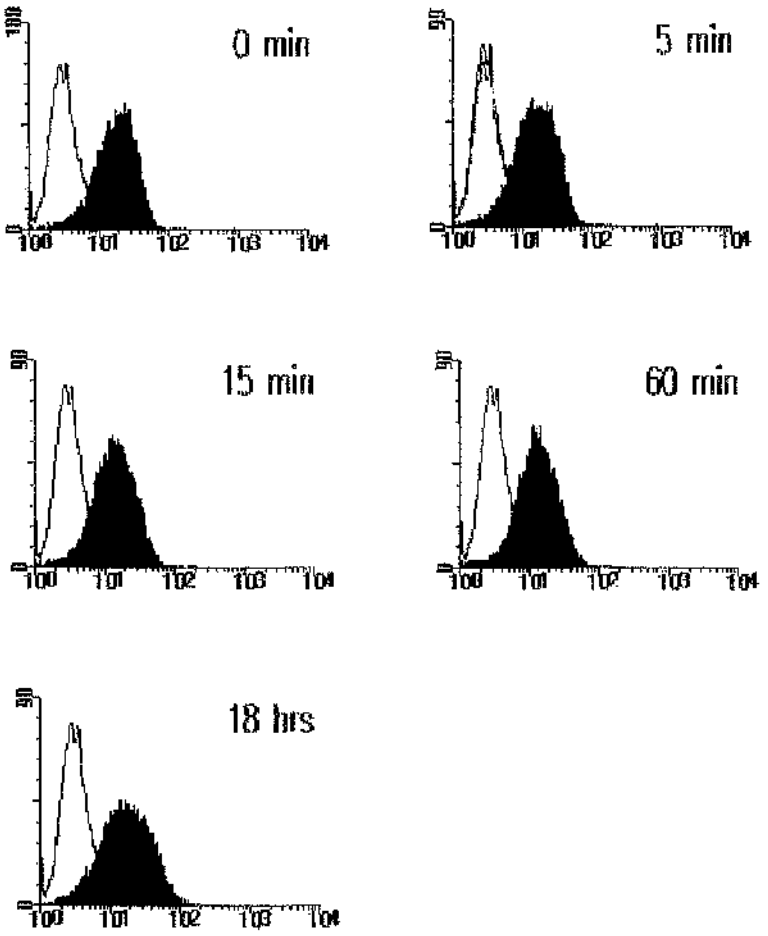
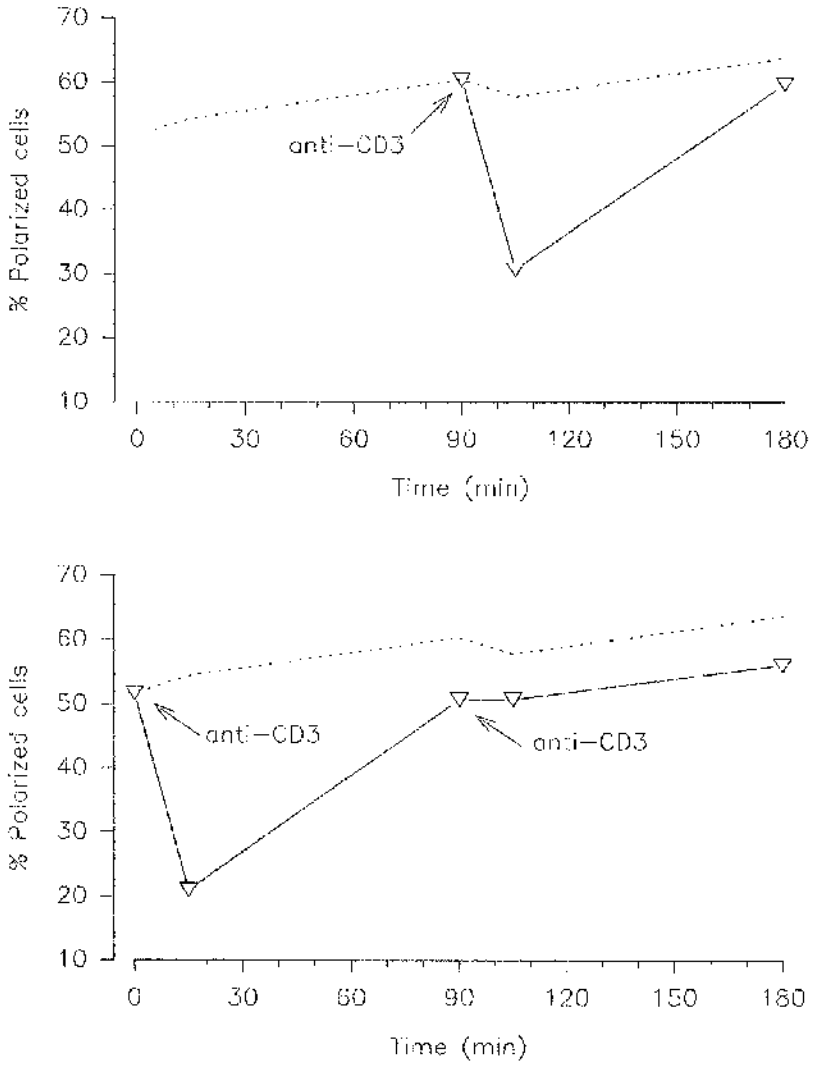


Figure 33: Effect of αCD3 on dbcAMP-cultured Jurkat cells pretreated with αCD3 for 60 min. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, resuspended in 1 ml of the medium and repolarized at 37°C for 30 min. An aliquot of 10 μl of 2 μg/ml OKT3 was added as indicated and the incubation was continued at 37°C for various time intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Polarized cells (triangle).



9. EFFECT OF MITOGENS ON THE POLARIZATION OF dbcAMP-CULTURED JURKAT CELLS

9.1. BACKGROUND

PHA was shown to increase the proportion of blood lymphocytes following a 72 hr incubation but was inactive in a 30 min assay (Wilkinson, 1986). Similarly, αCD3 was capable of inducing the locomotory activity in PBL only after a prolonged cell culture (Wilkinson and Higgins, 1987a). Thus, this effect of αCD3 and PHA is related to an activation of resting PBL resulting in the expression of new activation-associated genes (Wilkinson, 1986). In contrast, αCD3 was capable of inducing rounding up within minutes of applying this monoclonal antibody to the constitutively polarized dbcAMP-cultured Jurkat cells. In lymphocyte activation studies, PHA was reported to induce a similar increase in $[Ca^{2+}]_i$ observed following stimulation with αCD3 (Tsien *et al.*, 1982). In addition, experiments with TcR negative mutants of Jurkat cells showed that these cells could not be activated by PHA (Weis and Stobo, 1984). This suggests that the PHA signal is mediated through a pathway similar or identical to the one stimulated by αCD3. To further explore the similarities between the pathways utilized by αCD3 and PHA, the next series of experiments was performed.

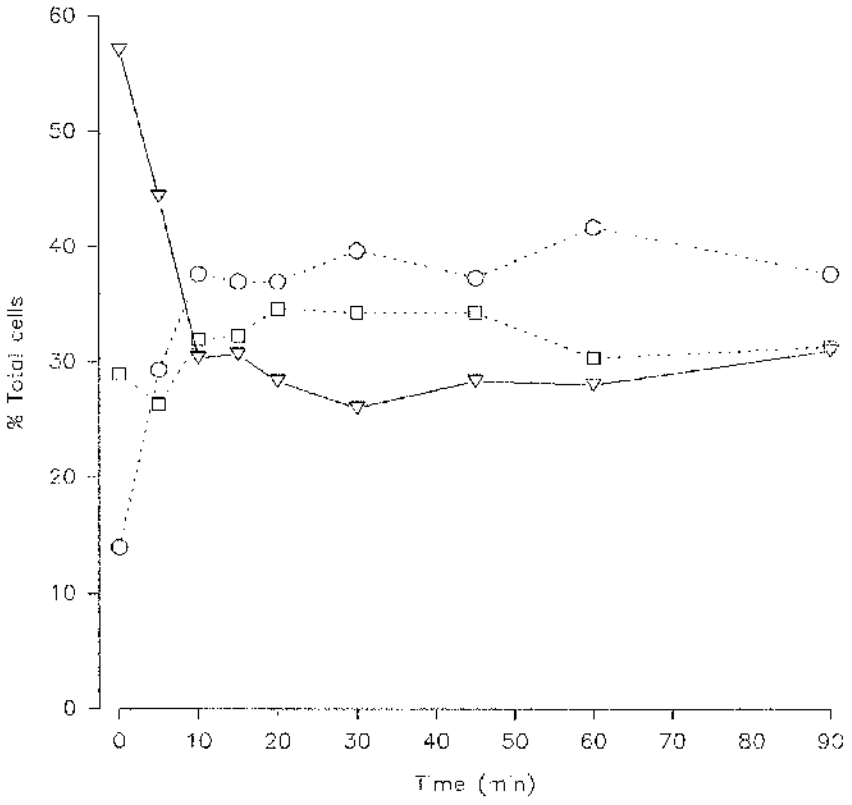
9.2. EXPERIMENTAL

9.2.1. Effect of PHA on the polarization of dbcAMP-cultured Jurkat cells

9.2.1.1. Effect of PHA on the morphology of dbcAMP-cultured Jurkat cells

The time-course of an effect stimulated by a mitogenic PHA dose (figure 34) confirmed that this mitogen induces rounding up of the

Figure 34: Time-course of the effect of 1 μg/ml PHA on repolarized dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, resuspended in 1 ml of the medium and repolarized at 37°C for 30 min. PHA was added to the cells and the incubation was continued at 37°C for various time intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



dbcAMP-cultured Jurkat cells within 15 min. The important difference between this and the αCD3-stimulated response was that PHA induced a permanent change in morphology compared to the transient effect of αCD3. The PHA effect was dose-dependent, detectable at 100 ng/ml and reaching 86% inhibition at 10 μg/ml (figure 35).

9.2.1.2. Effect of PHA on the morphology of dbcAMP-cultured Jurkat cells preincubated with αCD3

Having previously shown that Jurkat cells, pre-incubated with αCD3, become refractory to repeated stimulation with this antibody, the effect of similar pre-treatment on the PHA signal was examined (figure 36). It can be seen that the pre-treatment with αCD3 did not affect the response obtained following the addition of PHA.

9.2.2. Effect of Con A on the morphology of dbcAMP-cultured Jurkat cells

The other mitogen widely used to activate the lymphocytes is Con A. To determine if the effect stimulated by PHA is also mediated by a mitogenic dose of Con A, a time-course of its action on polarized Jurkat cells was observed (figure 37). As can be seen there was virtually no change in any of the populations defined on the basis of their morphology.

9.3. SUMMARY

In conclusion, the above experiments showed that incubation of dbcAMP-cultured Jurkat cells with PHA caused a dose-dependent and permanent reversal of polarized morphology. The preincubation of Jurkat cells with αCD3 did not interfere with the PHA effect suggesting that the two mitogens affect the Jurkat cell morphology through different pathways. Con A had no effect on Jurkat cell morphology indicating a pathway of action distinct from PHA.

Figure 35: Dose-dependent action of PHA on repolarized dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, resuspended in 1 ml of the medium and repolarized at 37°C for 30 min. Different doses of PHA were added to the cells and the incubation was continued at 37°C for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

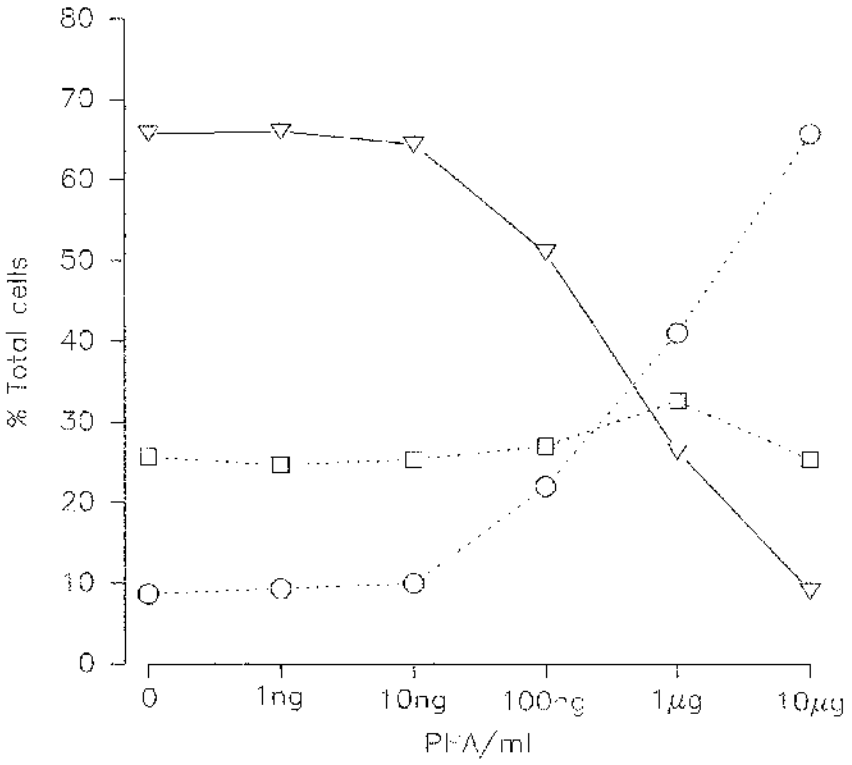


Figure 36: Effect of PHA on dbcAMP-cultured Jurkat cells pretreated with αCD3 for 60 min. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, resuspended in 1 ml of the medium and repolarized at 37°C for 30 min. An aliquot of 10 μl of 2 μg/ml OKT3 was added as indicated and the incubation was continued at 37°C. Subsequently, PHA was added to the final concentration of 1 μg/ml and the incubation was continued for various time intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Polarized cells stimulated with αCD3 and PHA (triangles), undisturbed (dotted line).

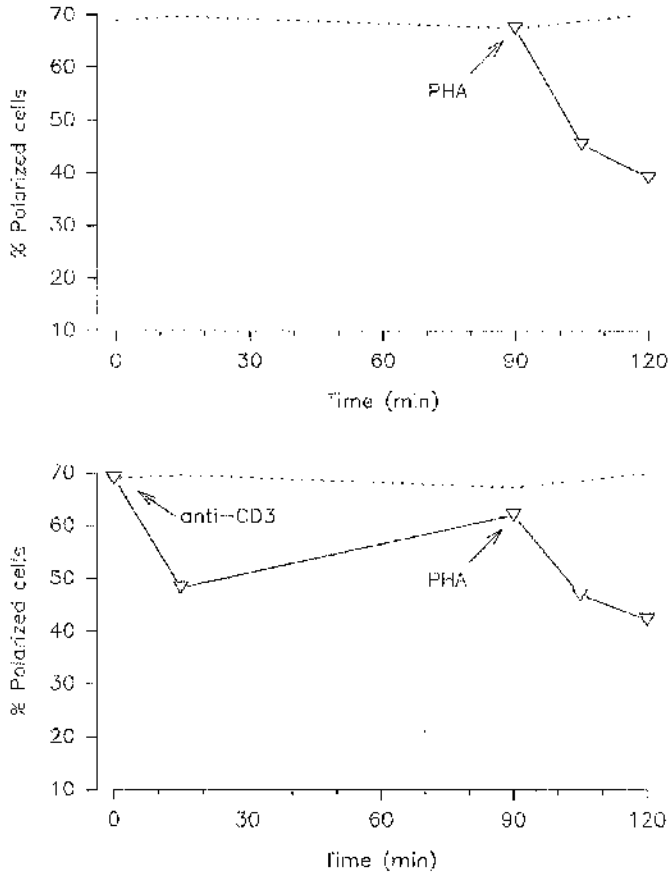
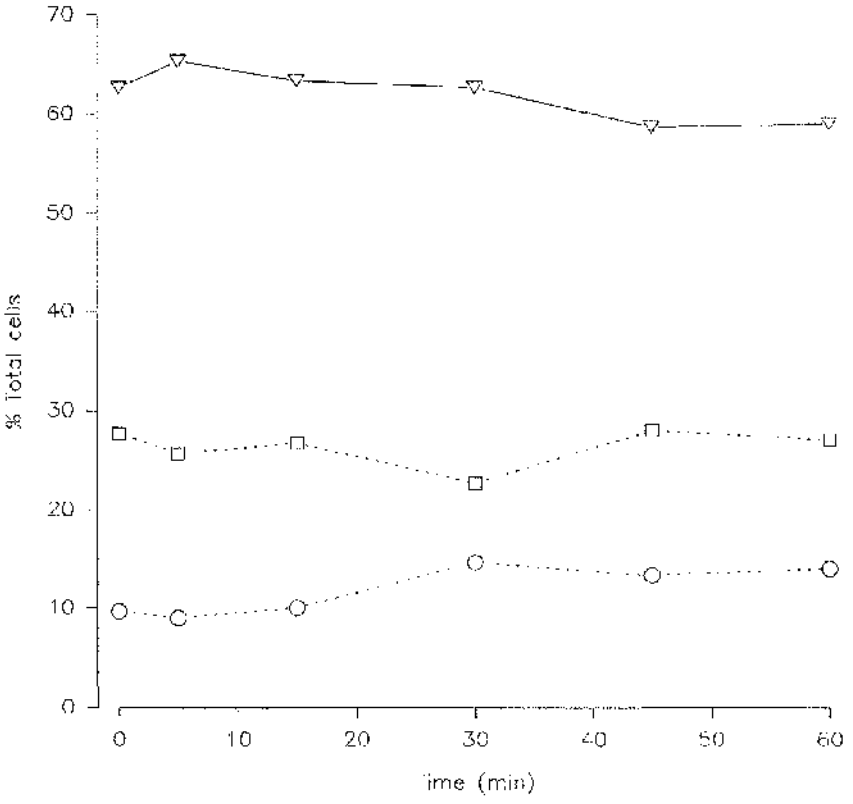


Figure 37: Time-course of the effect of 1 μg/ml Con A on repolarized dbcAMP-cultured Jurkat. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, resuspended in 1 ml of the medium and repolarized at 37°C for 30 min. Con A was added to the cells and the incubation was continued at 37°C for various time intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



10. INVOLVEMENT OF Ca^{2+} IN THE CONTROL OF THE LOCOMOTORY CAPACITY ACQUIRED BY dbcAMP-CULTURED JURKAT CELLS

10.1. BACKGROUND

Jurkat cells cultured in the presence of dbcAMP acquire locomotor morphology and become motile. The data so far have indicated that the activation of the cAMP signalling pathway results in the loss of this ability. In addition, stimulation through the TcR/CD3 complex results in a transient rounding up of the cells. The increase in $[Ca^{2+}]_i$ of Jurkat cells, stimulated by a monoclonal αCD3 or αTcR, has been documented (Weiss *et al.*, 1984b; Imboden *et al.*, 1985). To further explore the αCD3-induced change in the morphology of dbcAMP-cultured Jurkat cells, the involvement of Ca^{2+} was investigated in the next series of experiments.

10.1.1. Increase in intracellular Ca^{2+}

The concentration of intracellular Ca^{2+} is very low and in most cells $[Ca^{2+}]_i$ oscillates between 100 and 200 nM (Carafoli, 1991) while the concentration of extracellular Ca^{2+} and Ca^{2+} sequestered in specialized intracellular compartments exceeds 1 mM. Thus, a large concentration gradient drives Ca^{2+} across the plasma membrane into the cytoplasm. The plasma membrane has a limited and carefully controlled Ca^{2+} permeability. The control of $[Ca^{2+}]_i$ is performed by an importing system (Ca^{2+} channel) and an exporting system (the Ca^{2+} pump and Na^+/Ca^{2+} exchanger) of the plasma membrane.

10.1.2. Plasma membrane Ca^{2+} channels

The Ca^{2+} channels can be described as voltage-dependent or receptor-triggered. Voltage-dependent Ca^{2+} channels are regulated by

membrane potential. These Ca^{2+} channels are opened by depolarization, generally caused by increased Na^{+} permeability. These channels are present on excitable cell types, however were not found on lymphocytes (Gelfand *et al.*, 1987).

The Ca^{2+} channels identified on lymphocytes by the patch-clamp technique are voltage insensitive (Kuno *et al.*, 1986). The channels are mostly closed in resting cells but open in response to stimulation with PHA (Kuno *et al.*, 1986), αCD3 or αCD2 (Gardner *et al.*, 1989). The Ca^{2+} channels are physically separate from CD3 and CD2 surface molecules (Gardner *et al.*, 1989) and the secondary messengers mediating their opening appear to be IP_3 (Kuno and Gardner, 1987), IP_4 (Michell, 1986) or their combination (Morris *et al.*, 1987). These Ca^{2+} channels are autoregulated by Ca^{2+} which causes them to close when $[Ca^{2+}]_i$ reaches about 1 μM (Gardner *et al.*, 1989).

Ca^{2+} channels can be blocked by several organic compounds, such as tertiary amines, like verapamil and its derivatives. These agents block the channel from inside, after having entered it in the open state (Lee and Tsien, 1983).

10.1.3. Ca^{2+} pump

The active transport of Ca^{2+} is used by cells to maintain high concentration gradients of this cation across membranes delimiting cells and intracellular organelles. The sarcoplasmic reticulum membrane is highly differentiated for the active transport of Ca^{2+} and fragments of such membranes were used in characterization of Ca^{2+} transport (Ebashi and Lipmann, 1962). Ca^{2+}-dependent ATPase is the sole operator of the Ca^{2+} pump. The enzyme is a 110-kDa amphiphilic protein (MacLennan, 1970) with a globular head protruding from the cytoplasmic surface of sarcoplasmic reticulum and nonpolar segments crossing the lipid bilayer.

The enzyme sequentially binds two Ca^{2+} ions within the protein crevice (Inesi *et al.*, 1980, 1988) where they are shielded from the

membrane phospholipid surface charge (Scofano *et al.*, 1985). The ATP interacts with the catalytic site of ATPase on the cytoplasmic side resulting in translocation of the ions, phosphorylation of the catalytic site and subsequent reduction of the Ca^{2+} pump binding constant for Ca^{2+} (Inesi, 1985; Pickart and Jencks, 1984). Thus, Ca^{2+} ions are released in the lumen, the phosphoenzyme undergoes hydrolytic cleavage and a new cycle begins (Inesi, 1987; Khananshvili and Jencks, 1988).

10.1.4. Dynamics of the increase in $[Ca^{2+}]_i$

Cytosolic Ca^{2+} concentrations can be mobilized from either intracellular stores or from the extracellular compartment. In most cells, activation by Ca^{2+}-mobilizing agonists leads to two distinct phases of Ca^{2+} mobilization. Initially Ca^{2+} is mobilized from the intracellular stores and this is followed by an influx of extracellular Ca^{2+} (Rasmussen and Barrett, 1984). This latter activity is responsible for a sustained increase in $[Ca^{2+}]_i$ and also helps to replenish the depleted intracellular stores.

The role of intracellular Ca^{2+} in the TcR/CD3-mediated lymphocyte activation, suggested by the effectiveness of Ca^{2+} ionophores, was examined in quin-2-loaded Jurkat cells stimulated with PHA, αCD3 (Weiss *et al.*, 1984b), or αTcR (Imboden *et al.*, 1985). Indeed, the increase in $[Ca^{2+}]_i$ during T cell activation consisted of two distinct phases. The addition of soluble monoclonal antibodies to Jurkat cells induced within 1 min $[Ca^{2+}]_i$ increase from the basal level of 80-100 nM to a peak value of 350-750 nM (Weiss *et al.*, 1984b; Imboden *et al.*, 1985). The initial transient increase occurred independently of the presence of extracellular Ca^{2+} thus implicating the mobilization of intracellular Ca^{2+} (Imboden and Stobo, 1985). Following this rapid increase, the $[Ca^{2+}]_i$ fell by 10 min to a new plateau of 200-250 nM and remained elevated above baseline levels for at least 30 min (Imboden and Stobo, 1985). Similarly, a cloned bovine insulin-specific T hybridoma (Shapiro *et al.*, 1985) and tetanus toxoid-specific T cells (Nisbet-Brown *et al.*, 1985), physiologically activated with

appropriate antigen-pulsed AC, responded by an increase in $[Ca^{2+}]_i$ within 10 min. PMA slightly lowers basal $[Ca^{2+}]_i$ but does not prevent TcR/CD3-mediated $[Ca^{2+}]_i$ increase in Jurkat (Imboden *et al.*, 1985) as well as in other T cell lines and T cell clones (O'Flynn *et al.*, 1985; Weiss *et al.*, 1984a; Oettgen *et al.*, 1985).

10.1.5. IP_3 is a second messenger for release of Ca^{2+} from intracellular store

Based on the hypothesis first put forward by Michell (1975), the mobilization of Ca^{2+} from intracellular stores was shown to correlate with the appearance of a second messenger generated during the breakdown of phosphoinositides (Fain and Berridge, 1979ab).

The response to the stimulation of the cell surface, resulting in phosphatidylinositol changes, was first observed in brain cells (Hokin and Hokin, 1958). In these cells, acetylcholine, acting via muscarinic receptors, stimulated a specific incorporation of an isotope into the minor membrane phospholipid PI[3]. A mechanism called the 'PI-PA' cycle was shown to be responsible for this incorporation. Thus, on stimulation with the ligand, PI is hydrolysed to DAG and IP in a reaction catalyzed by a Ca^{2+}-dependent (Ryu *et al.*, 1987ab) phosphoinositide-specific PLC (Dawson, 1959; Kemp *et al.*, 1961b) and DAG is phosphorylated by ATP to form PA (Hokin and Hokin, 1959). Once the ligand is removed, PA is converted to PI by sequential action of CTP-PA cytidyl transferase and PI synthase (Agranoff *et al.*, 1958; Paulus and Kennedy, 1960).

The cleavage of $PI(4,5)P_2$ by PLC (Kemp *et al.*, 1961ab;

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[3]Inositol-containing phospholipids constitute 2-8% of the total membrane phospholipid and are preferentially localized on the internal leaflet of the plasma membrane. Although inositol can be phosphorylated to a total of 66 possible arrangements of inositol phosphates (Majerus *et al.*, 1988) there are three major *myo*-inositol containing phospholipids: PI, PI(4)P, and $PI(4,5)P_2$ (phosphoinositides).

Thompson and Dawson, 1964) leads to the formation of soluble IP_3, which diffuses into the cytoplasm, and lipophilic DAG, which remains associated with the membrane (Majerus *et al.*, 1986). A single enzyme hydrolyzes all three phosphoinositides (Wilson *et al.*, 1984). A wide variety of receptors in numerous tissues has been reported to be coupled to the activation of PLC, including the TcR/CD3 complex of T lymphocytes (Rhee and Choi, 1992). Indeed, αTcR-stimulated Jurkat cells rapidly produce significant elevations in IP_3, IP_2 and IP (Imboden and Stobo, 1985).

Since the discovery that IP_3 can release Ca^{2+} from intracellular stores (Streb *et al.*, 1983), this property of IP_3 has been confirmed in a wide variety of tissues (Berridge, 1987; Berridge and Irvine, 1989). Imboden and Stobo (1985) have been able to show that purified IP_3 was able to release Ca^{2+} from an intracellular ATP-dependent pool in permeabilized Jurkat cells.

10.1.6. Intracellular IP_3-specific receptors

The specificity of the IP_3-specific receptor has been demonstrated in various tissues where IP and IP_2, the other two products of phosphoinositide hydrolysis, were unable to trigger the release of intracellular Ca^{2+} (Streb *et al.*, 1983; Irvine *et al.*, 1986). The initial identification of specific IP_3-binding proteins was achieved using radioligand binding assays utilizing membranes from various sources (Ferris and Snyder, 1992). The native molecular weight of the IP_3-binding protein is about 1 MDa (Supattapone *et al.*, 1988b); however, a purified receptor gives a single 260-kDa SDS-PAGE band suggesting a tetrameric structure of the native protein (Maeda *et al.*, 1991).

Ca^{2+} ion was found to be a very potent inhibitor of $[^3H]IP_3$ binding, with half-maximal inhibition at approximately 300 nM (Worley *et al.*, 1987). The ability of Ca^{2+} to inhibit IP_3 binding was reconstituted by the addition of detergent extracted membranes to the purified receptor (Danoff

et al., 1988). The inhibition is mediated through a separate, membrane bound 15-kDa monomeric protein designated calmedin (Danoff *et al.*, 1988). The [^{3}H]IP_3 binding and IP_3-activated $^{45}Ca^{2+}$ flux was monitored in homogeneous preparations of IP_3 reconstituted into lipid vesicles; from such studies it was concluded that IP_3 activates release of Ca^{2+} from intracellular stores through direct binding to a ligand-activated Ca^{2+} channel (Ferris *et al.*, 1989).

The phosphorylation of the IP_3 receptor appears to provide a major mechanism of regulation. IP_3 receptors were found to be major substrate for PKA; solubilized and purified IP_3 receptor can be stoichiometrically phosphorylated by this protein kinase (Supattapone *et al.*, 1988b). In fact, these receptors were recognized as Purkinje cell-specific phosphoprotein (Walaas *et al.*, 1986; Weeks *et al.*, 1988). Following reconstitution and removal of the denaturing effects of detergents, PKC and Ca^{2+}/calmodulin-dependent protein kinase also specifically and stoichiometrically phosphorylate the receptor (Ferris *et al.*, 1991). Although all of the kinases phosphorylate the IP_3 receptor on serines, the location of these residues is specific to each of the three protein kinases (Ferris *et al.*, 1991). In cerebellar membrane preparations PKA phosphorylation results in a ten-fold decrease in the potency of IP_3 for releasing Ca^{2+} (Supattapone *et al.*, 1988b). However, since PKA phosphorylation of the Ca^{2+}-transport ATPase results in enhanced accumulation of Ca^{2+} into the endoplasmic reticulum, the total amount of Ca^{2+} releasable by IP_3 following PKA activation is significantly increased (Supattapone *et al.*, 1988b). In hepatocytes, cAMP-dependent hormones increase the sensitivity of Ca^{2+} stores to IP_3 (Burgess *et al.*, 1991). Thus, the phosphorylation of IP_3 receptor in response to DAG and the subsequent increase in Ca^{2+} may provide two separated feedback systems.

The studies on IP_3-activated Ca^{2+} channels in reconstituted vesicles revealed a direct allosteric regulation of the channel by adenine nucleotides; at low concentrations (*i.e.* 1-100 μM) ATP markedly enhances the ability of IP_3 to stimulate Ca^{2+} release while high concentrations of ATP remain

ineffective (Ferris *et al.*, 1990). Using radiolabelled ATP or its analogue, a high-affinity ATP recognition site on the purified IP_3 receptor was directly demonstrated (Ferris *et al.*, 1990).

10.1.7. Intracellular IP_3-sensitive Ca^{2+} stores

It is now clear that a discrete IP_3-sensitive organelle exists in cells although the precise nature of this organelle is controversial (Rossier and Putney, 1991). In the first models, the transfer of Ca^{2+} from the extracellular space into the pool was proposed to first directly enter the intracellular pool and then enter the cytoplasm via an IP_3-sensitive channel (Casteels and Droogmans, 1981; Putney, 1986). With a more sensitive Ca^{2+} indicator, a transient increase in the $[Ca^{2+}]_i$ was detected during refilling process (Takemura and Putney, 1989; Hallam *et al.*, 1989; Shuttleworth, 1990). A phenomenon of 'Ca^{2+} overshoot' was identified in cells whose intracellular Ca^{2+} stores had been depleted in the absence of extracellular Ca^{2+}; in such cells the increase in $[Ca^{2+}]_i$ was transiently larger than in cells with intracellular Ca^{2+} pool intact (Hallam *et al.*, 1989). Thus depletion of the IP_3-sensitive Ca^{2+} stores not only increases plasma membrane permeability to Ca^{2+} but also shows that such depletion triggers an increased influx of extracellular Ca^{2+} directly into cytoplasm.

Additional evidence was obtained by depletion of the intracellular Ca^{2+} pool by the non-phorbol ester tumour promoter thapsigargin in the presence of extracellular Ca^{2+}, and in the absence of any changes in inositol phosphates (Jackson *et al.*, 1988). This toxin depletes intracellular Ca^{2+} stores by a specific action on the Ca^{2+}-transport ATPase on intracellular membranes; thapsigargin does not inhibit active Ca^{2+} transport by the plasma membrane of cells (Thastrup *et al.*, 1990). It is thought that once the active Ca^{2+} uptake into the intracellular pool is inhibited, Ca^{2+} diffuses through a 'leak' channel. In parotid acinar cells, thapsigargin induced an increase in $[Ca^{2+}]_i$ and sustained influx of extracellular Ca^{2+}; subsequent ligand stimulated IP_3 failed to induce additional Ca^{2+} release (Takemura *et*

al., 1989). Combination of thapsigargin and ligand showed synergistic $[Ca^{2+}]_i$ increase reflecting the combined result of IP_3-sensitive channel opening and inhibition of active Ca^{2+} uptake (Thastrup *et al.*, 1990; Thastrup, 1990). Thus, thapsigargin and agonists acting through various receptors activate a common Ca^{2+} entry pathway (Marier *et al.*, 1978). It is clear that the intracellular Ca^{2+} pool and plasma membrane are physically separated. However, the ability of the former to affect the plasma membrane Ca^{2+} permeability, suggested that a means of communication between the two structures is necessary. The phosphorylated product of IP_3 metabolism, IP_4, was suggested to play a significant role in the regulation of Ca^{2+} entry (Irvine *et al.*, 1988; Irvine, 1991, 1990). However, much of the evidence is contradictory and the mechanism of its action is still unclear (Irvine, 1992).

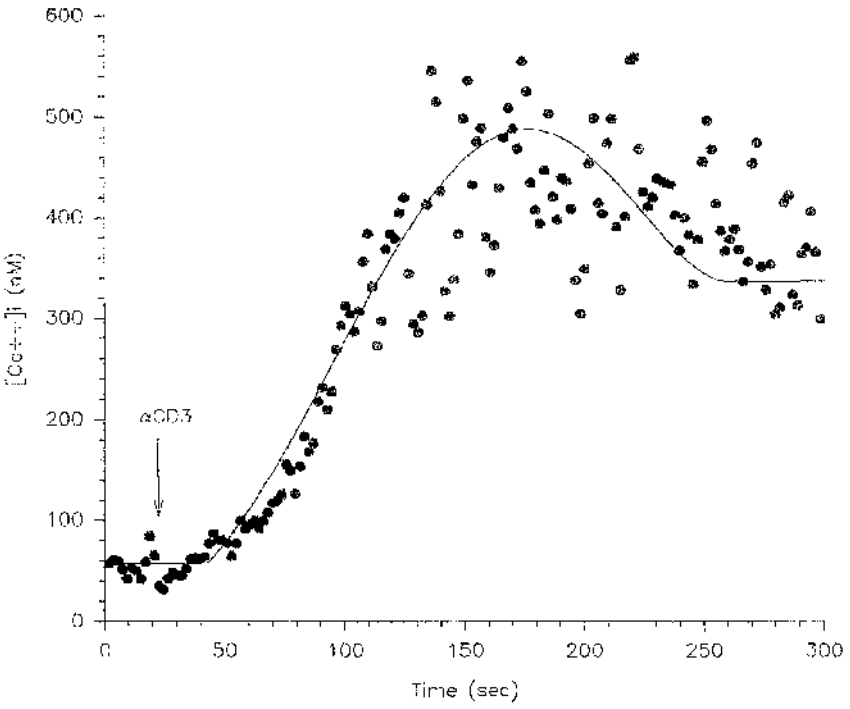
10.2. EXPERIMENTAL

10.2.1. Intracellular Ca^{2+} levels measured in dbcAMP-cultured Jurkat cells responding to various agents

10.2.1.1. $[Ca^{2+}]_i$ fluctuations in response to αCD3

The involvement of αCD3 in stimulation of intracellular Ca^{2+} increase was shown directly using a Ca^{2+} indicator fura-2 (figure 38). The Ca^{2+} concentration started to increase in about 30 sec after the stimulus was introduced and continued to do so for 2-3 min. In that time, $[Ca^{2+}]_i$ increased from 55-65 nM to 400-550 nM. Subsequently, a marked decrease in $[Ca^{2+}]_i$ was observed until a plateau of 300-350 nM was reached at 4.5-5 min of the experiment. The plateau levels in $[Ca^{2+}]_i$, induced in dbcAMP-cultured Jurkat cells by αCD3, were sustained for at least 15 min without any signs of decrease (results not shown). The observed $[Ca^{2+}]_i$ fluctuations in response to αCD3 resembled, both quantitatively and qualitatively, those published previously (Imboden and

Figure 38: Time-course of αCD3-mediated increase in intracellular Ca^{2+} in dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days and washed twice with HBSS without phenol red. The cells were then loaded with fura-2A, washed and resuspended at $2x10^6$ cells/ml in HBSS. OKT3 was added to the final concentration of 50 ng/ml and the fluorescence intensity was recorded over time at 510 nm using excitation at 340 and 380 nm. Finally, the instrument was calibrated and the absolute intracellular Ca^{2+} concentrations were calculated.



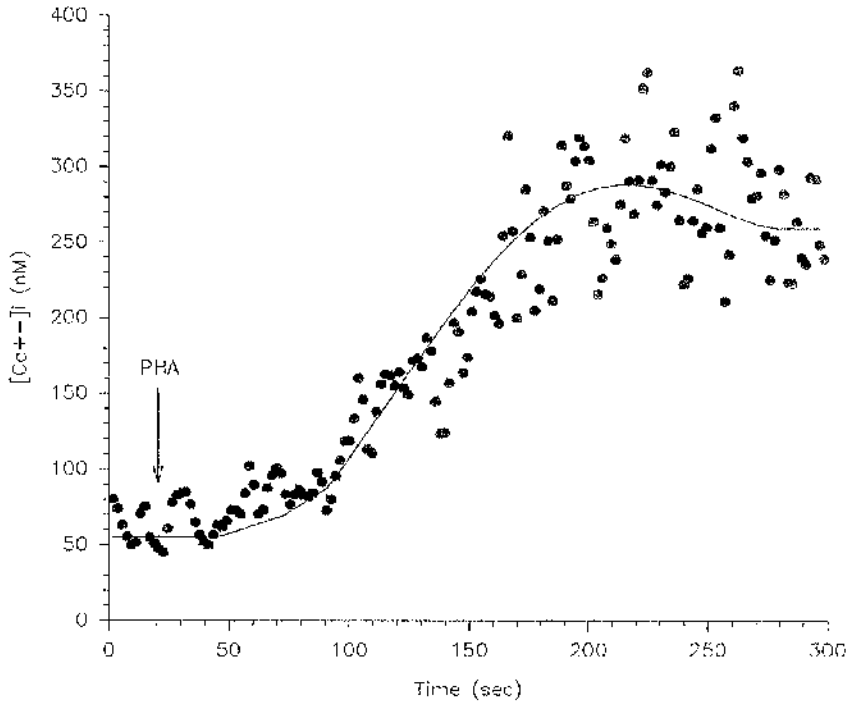
Stobo, 1985).

Although the change in the cell morphology correlates with the $[Ca^{2+}]_i$ increase, the dynamics of the shape transformation do not reflect those shown in figure 38. The rapid onset of $[Ca^{2+}]_i$ increase is contrasted with a slower progress of αCD3-mediated rounding up, which reaches maximum at 10-15 min. In addition, the plateau Ca^{2+} levels were shown to persist for at least 30 min (Imboden and Stobo, 1985), which corresponds to the time associated with substantial recovery of polarized morphology by dbcAMP-cultured Jurkat cells. Thus, it is not likely that Ca^{2+} is the messenger which directly affects the locomotory machinery. The above observations therefore suggest that (1) the increase in $[Ca^{2+}]_i$ is purely coincidental or (2) Ca^{2+} is a messenger which activates an intracellular pathway which eventually affects the locomotory machinery.

10.2.1.2. $[Ca^{2+}]_i$ fluctuations in response to PHA

The intracellular Ca^{2+} increase, induced by the mitogenic dose of PHA, was slower (figure 39) when compared to that observed with αCD3 (figure 38). Here, the maximum levels of 250-300 nM were attained after 3.5-4 min, followed by only slight decrease to pleateau levels of 200-300 nM. The lack of promient initial peak observed with αCD3, and assuming that the source of this peak is intracellular Ca^{2+} pool, PHA seems to stimulate primarily influx of extracellular Ca^{2+} in Jurkat cells. This suggestion is supported by the similarity in plateau $[Ca^{2+}]_i$ levels induced by the two mitogens. Although the relatively uniform increase in $[Ca^{2+}]_i$ by PHA is consistent with the sustained PHA-induced rounding up of dbcAMP-cultured Jurkat cells, the latter reaches maximum after 10 min compared to 4 min required by the former. This observation further challenges the possibility that Ca^{2+} is directly the regulator of changes in lymphocyte morphology. However, the two possibilities, outlined above, for the involvement of Ca^{2+} in the regulation of the locomotory capacity still cannot be delineated. Indeed, PHA may interact with multiple cell

Figure 39: Time-course of PHA-mediated increase in intracellular Ca^{2+} in dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days and washed twice with HBSS without phenol red. The cells were then loaded with fura-2A, washed and resuspended at $2x10^6$ cells/ml in HBSS. PHA was added to the final concentration of 1 μg/ml and the fluorescence intensity was recorded over time at 510 nm using excitation at 340 and 380 nm. Finally, the instrument was calibrated and the absolute intracellular Ca^{2+} concentrations were calculated.



surface structures thus activating several biochemical pathways simultameously.

10.2.2. Effect of extracellular Ca^{2+} on the response of dbcAMP-cultured Jurkat cells to αCD3 and PHA

10.2.2.1. Effect of Ca^{2+}-free media

The ability of αCD3 to affect the $[Ca^{2+}]_i$ of dbcAMP-cultured Jurkat cells has been demonstrated. The next series of experiments was used to determine the effect of extracellular Ca^{2+} on the rounding up induced by αCD3. The preliminary αCD3 dose-response experiments in Ca^{2+}/Mg^{2+}-free medium, supplemented with EDTA indicated that αCD3 at concentrations of up to 20 ng/ml OKT3 is inactive under such conditions (figure 40).

To assure that the αCD3-activated signal is affected by the absence of Ca^{2+} rather than Mg^{2+}, media containing these ions either separately or in combination were prepared. The results obtained with these media indicate that extracellular Ca^{2+} is vital for the CD3-mediated pathway (figure 41-A). The presence of EDTA in the Ca^{2+}-free medium is not necessary for the inhibition of the αCD3 effect, however it improves the efficiency of inhibition, probably by chelating the trace Ca^{2+} ions (figure 42).

To compare the PHA- and αCD3-mediated pathway further, the requirement for extracellular Ca^{2+} in the change of cell morphology induced by PHA was assessed (figure 41-B). Indeed, it was observed that PHA also requires the presence of Ca^{2+} in the test medium to mediate the change in morphology of the cells.

The presence of extracellular Ca^{2+} plays a crucial role in the αCD3-induced shape change of dbcAMP-cultured Jurkat cells. One of the possible functions considered had to do with the binding of the monoclonal antibody OKT3 to the surface CD3 molecule in a Ca^{2+}-free environment.

Figure 40: Effect of extracellular calcium on the dose-response of dbcAMP-cultured Jurkat cells to αCD3. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, and resuspended in 1 ml of the medium. The repolarization was carried out in calcium-free HBSS/MOPS supplemented with 1 mM EDTA or in regular HBSS/MOPS at 37°C for 30 min. The different concentrations of OKT3 were added and the incubation was continued for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

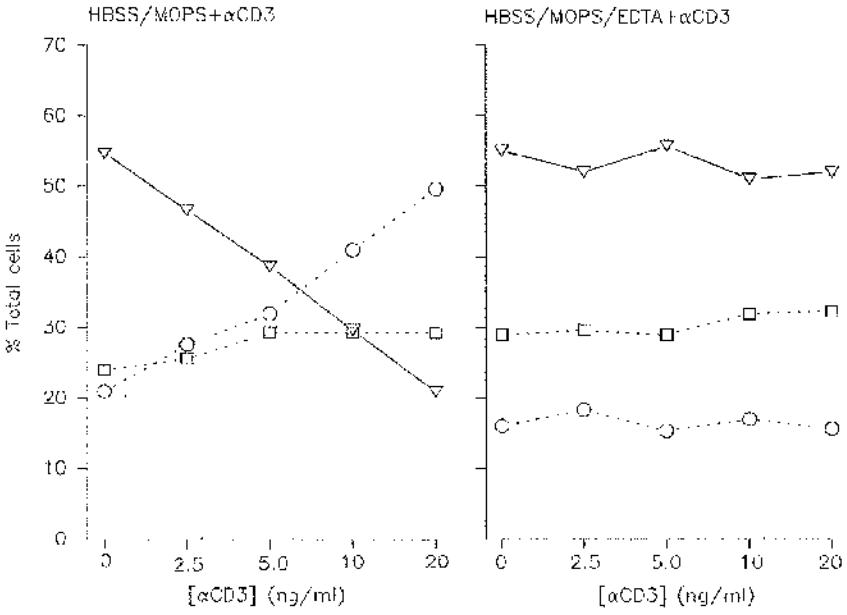
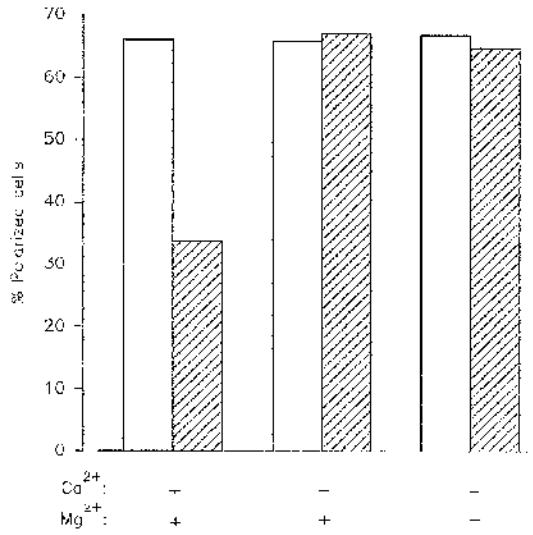


Figure 41: Effect of the absence of extracellular Ca^{2+} and Mg^{2+} on the change in morphology of dbcAMP-cultured Jurkat cells stimulated by αCD3 (A) or PHA (B). The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, and resuspended in 1 ml of the medium. The repolarization was carried out in Ca^{2+}-free HBSS/MOPS supplemented with various combinations of Ca^{2+} and Mg^{2+} at 37°C for 30 min. OKT3 or PHA was added to a final concentration of 20 ng/ml or 1 μM respectively and the incubation was continued for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Polarized cells in buffer only (open bars), in the presence of αCD3 or PHA (hatched bars).

A.



B.

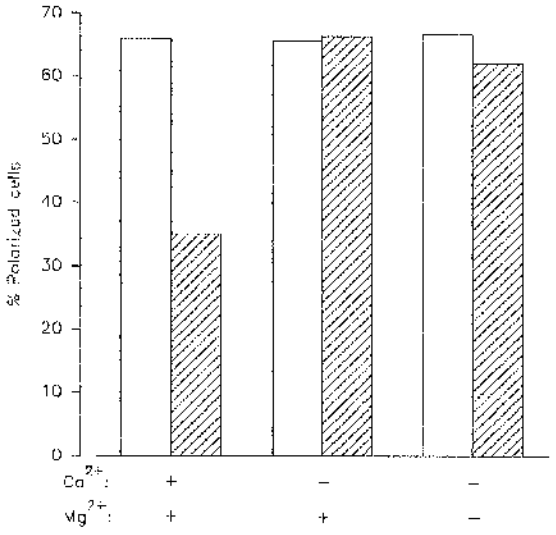
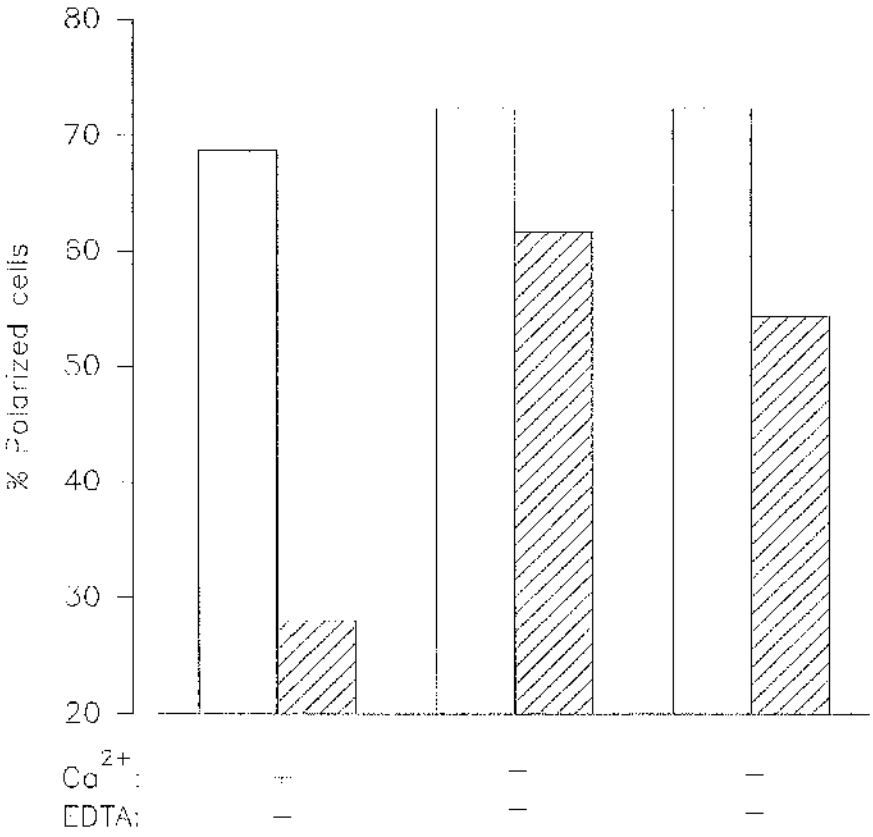


Figure 42: Effect of the presence of EDTA on the change in morphology of dbcAMP-cultured Jurkat cells stimulated by αCD3. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, and resuspended in 1 ml of the medium. The repolarization was carried out in Ca^{2+}-free HBSS/MOPS supplemented with various combinations of calcium and EDTA at 37°C for 30 min. OKT3 was added to final concentration of 20 ng/ml and the incubation was continued for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Polarized cells in the absence of αCD3 (open bars), in the presence of soluble αCD3 (hatched bars).



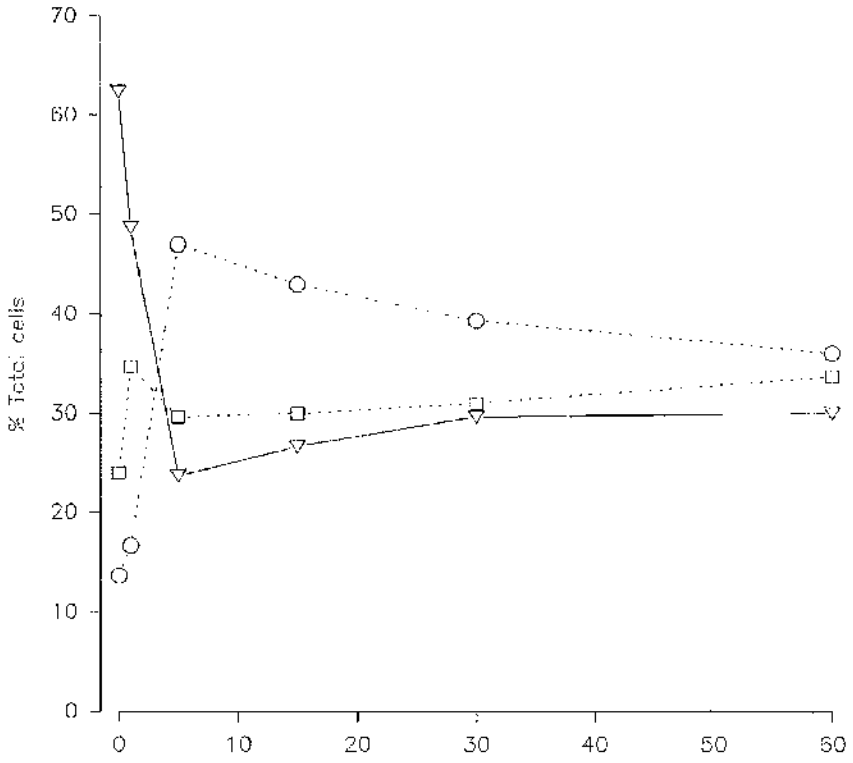
However, the staining procedure for the FACS analysis is performed using PBS which contains no Ca^{2+} in its formulation. The FACS results clearly show the ability of OKT3 to bind to CD3 in a Ca^{2+}-free environment (figure 12a-A). Further consideration was given to the conformational change, induced by the binding of αCD3 to the TcR/CD3, which may be responsible for the transmission of the signal into cytoplasm of the cell. Perhaps the conformational change, or its extent, and subsequently the intracellular signal are affected in the absence of extracellular Ca^{2+}. However, the measurements of $[Ca^{2+}]_i$, in cells responding to αCD3 in Ca^{2+}-free medium, showed a response consistent with the TcR/CD3 mediated signal. Thus, it appears that the extracellular Ca^{2+} is itself a messenger delivered during a controlled Ca^{2+} influx.

10.2.2.2. Effect of Ca^{2+} ionophore

Ca^{2+} ionophores are hydrophobic agents which specifically bind and transport Ca^{2+} ions across plasma membrane of cells. The $[Ca^{2+}]_i$ in such cells increases rapidly and irreversibly. The effect of Ca^{2+} ionophore A23187 on the morphology of repolarized dbcAMP-cultured Jurkat cells was assessed in a time-course experiment (figure 43). The second messenger role of Ca^{2+} has been shown in the context of the cell morphology regulation although a non-specific effect of the ionophore on the locomotory machinery is possible. The dynamics of the shape change suggest that a rapid increase in the intracellular Ca^{2+} is associated with a reversal of the polarization in the Jurkat cells. A slight recovery of the polarized morphology is noticeable after 15 min which may be a consequence of active removal of the intracellular Ca^{2+} by the cells.

The addition of HSA to the repolarization medium does not affect the extent of repolarization of dbcAMP-cultured Jurkat cells. However, the effective concentration of the Ca^{2+} ionophore subsequently added was decreased as it is non-specifically bound to the albumin. The concentration of the Ca^{2+} ionophore affects its ability to round up Jurkat cells

Figure 43: Time-course of action of A23187 on dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, resuspended in 1 ml of the medium and repolarized at 37°C for 30 min. A23187 was added to final concentration of 10^{-6}M and the incubation was continued at 37°C for various time intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



qualitatively and quantitatively (figure 44-A). At 10^{-6}M the ionophore produced a sustained decrease in polarization of dbcAMP-cultured Jurkat cells while in the presence of HSA, the response was much lower and transient, requiring more that 60 min to recover to the initial level of polarization. This transient effect was reproduced by lowering of the Ca^{2+} ionophore concentration in the absence of HSA (figure 44-B). Estimating from the results shown, 1 mg/ml HSA lowered the effective concentration of A23187 about 10-fold.

The data obtained with lower concentration of the Ca^{2+} ionophore seem to refute the possibility of non-specific action of A23187 on the locomotory machinery. It is more likely that the cells can reverse the effect of these lower concentrations by actively pumping the excess Ca^{2+} out of the cell. This return to normal $[Ca^{2+}]_i$ would then be reflected by the recovery of polarized morphology.

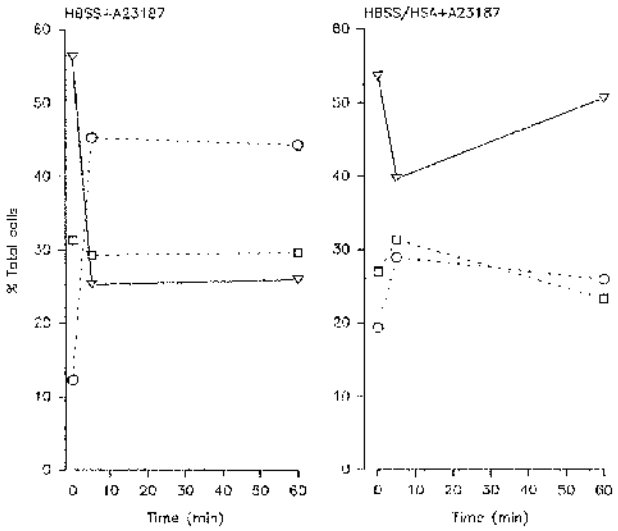
10.2.3. Involvement of intracellular Ca^{2+} on the repolarization of dbcAMP-cultured Jurkat cells

10.2.3.1. Effect of thapsigargin on cell polarization

A time-course of thapsigargin action on repolarized dbcAMP-cultured Jurkat cells resulted in rapid rounding up an interesting bi-phasic restoration of their polarized morphology (figure 45). The proportion of polarized cells decreased rapidly from the initial 67%, reaching the minimum of 24% at 5 min. In the next 5 min, the numbers of polarized cells were restored to 44% at a rate that reflected the initial decrease. After this rapid initial rate, the proportion of polarized cells recovered slowly, at a rate resembling that following αCD3 stimulation, to reach the maximum of 56% at 60 min. The final proportion of polarized cells was consistently lower than the initial one. The negative control showed marginally higher proportion of polarized cells at 60 min, compared to the observation made at time 0.

Figure 44: Effect of HSA on the time-course of action of 10^{-6}M A23187 (A); dose-dependent action of A23187 (B). The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, resuspended in 1 ml of the appropriate medium and repolarized at 37°C for 30 min. At time 0, A23187 was added to the cells at 10^{-6}M (A) or as indicated (B) and the incubation was continued at 37°C for 60 min. The cells were fixed at 5 and 60 min with glutaraldehyde and the morphology was examined and quantified under phase contrast. (A) Round cells (circle), irregular cells (square) and polarized cells (triangles); (B) Polarized cells at 5 min (open bars) or 60 min (hatched bars).

A.



B.

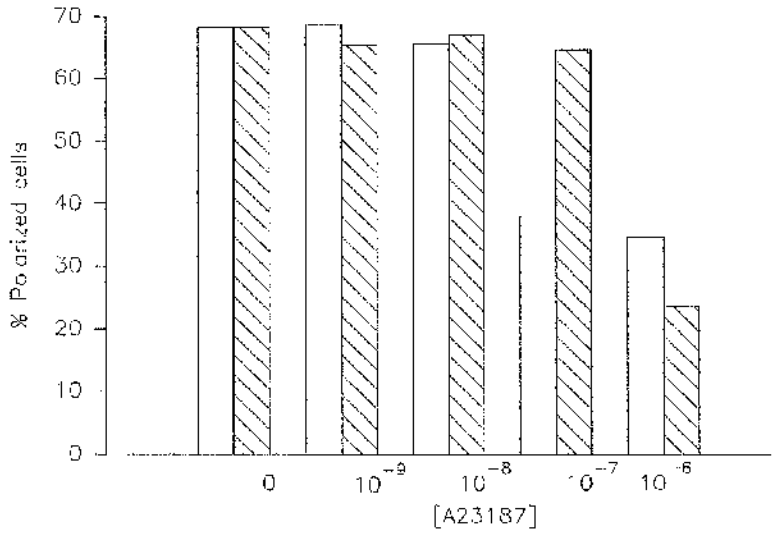
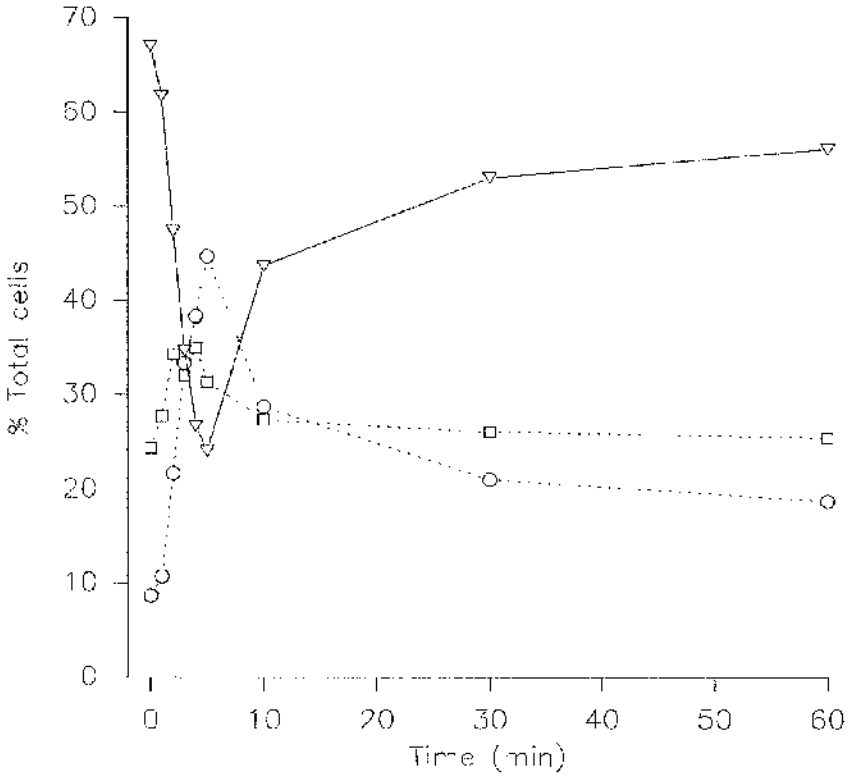


Figure 45: Time-course of the effect of 1 μM thapsigargin on repolarized dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, resuspended in 1 ml of the medium and repolarized at 37°C for 30 min. Thapsigargin was added to the cells at time 0 and the incubation was continued at 37°C for various time intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



The transient action of thapsigargin was dose-dependent with the maximum activity detected at concentrations between $5x10^{-7}$M and 10^{-6}M (figure 46).

10.2.3.2. Effect of thapsigargin on intracellular Ca^{2+} levels

The release of Ca^{2+} from intracellular stores is, under physiological conditions, mediated by IP_3-receptor operated Ca^{2+} channels. The release of intracellular stores of Ca^{2+} can be simulated, in the absence of inositolphosphates, by thapsigargin.

Direct examination of $[Ca^{2+}]_i$ in thapsigargin-treated Jurkat cells revealed a definite bi-phasic increase observed in αCD3-stimulated cells (figure 47). Clearly, the release of intracellular Ca^{2+} stores, registered as the initial peak, is linked to the induction of lower (200-400 nM) but sustained increase in $[Ca^{2+}]_i$ shown also with αCD3 and PHA. This plateau is maintained by the influx of extracellular Ca^{2+}. However, the time-course of $[Ca^{2+}]_i$ fluctuations, in response to thapsigargin, revealed differences consistent with different mode of action of these activators.

The effect of thapsigargin was noticeable in about 10 sec, compared with αCD3-stimulated increase which commenced about 30 sec after the introduction of the antibody. This difference may reflect the need of αCD3-stimulated cells to first produce sufficient levels of IP_3 prior to the release of intracellular Ca^{2+}, while thapsigargin acts immediately upon penetration into the cytoplasm.

The peak Ca^{2+} levels of 500-800 nM, induced by thapsigargin, were noticeably higher than those seen with αCD3. In addition, the variation in magnitude of intracellular Ca^{2+} 'spikes' was much larger, covering at times range between 400 and 900 nM. These observations may reflect the existence of IP_3-insensitive intracellular Ca^{2+} pools (Putney, 1988) accessed by thapsigargin but not αCD3.

Figure 46: Dose-dependent action of thapsigargin on repolarized dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, resuspended in 1 ml of the medium and repolarized at 37°C for 30 min. Different doses of thapsigargin were added to the cells and the incubation was continued at 37°C for 5 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Polarized cells (triangles).

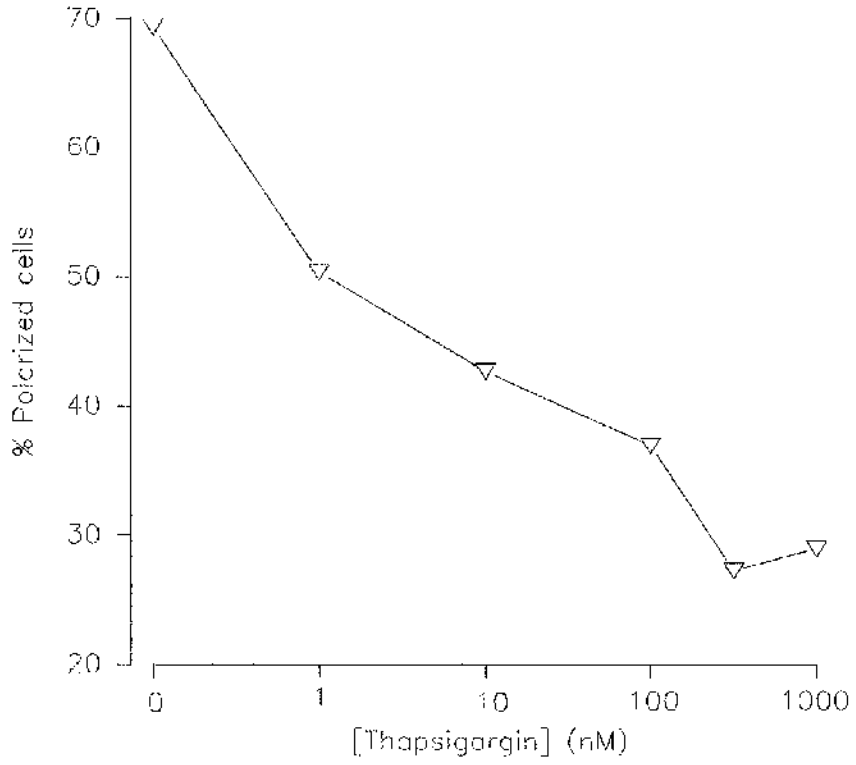
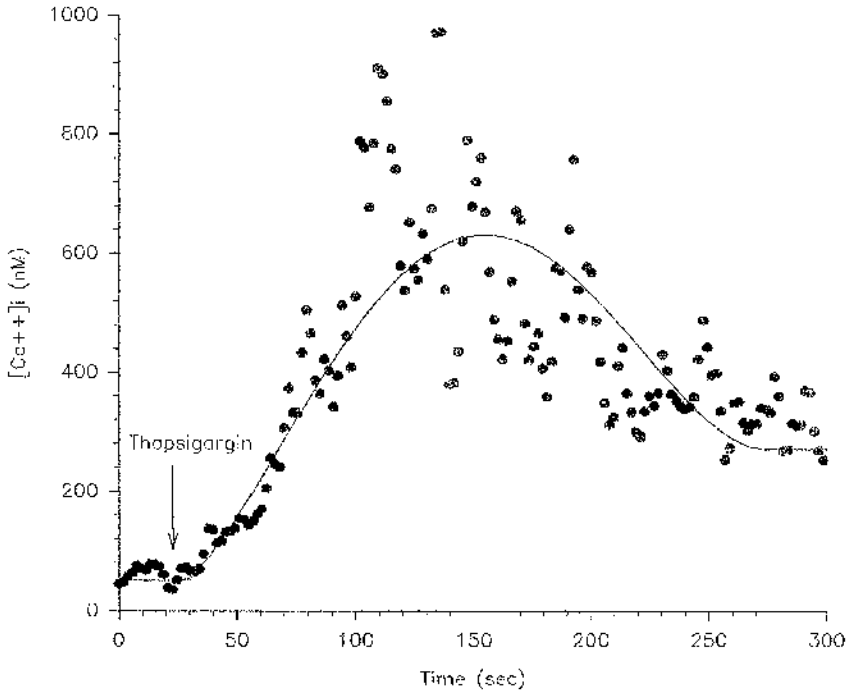


Figure 47: Time-course of thapsigargin-mediated increase in intracellular Ca^{2+} in dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days and washed twice with HBSS without phenol red. The cells were then loaded with fura-2A, washed and resuspended at $2x10^6$ cells/ml in HBSS. Thapsigargin was added to the final concentration of 1 μM and the fluorescence intensity was recorded over time at 510 nm using excitation at 340 and 380 nm. Finally, the instrument was calibrated and the absolute intracellular Ca^{2+} concentrations were calculated.



10.2.3.3. Role of extracellular Ca^{2+} in thapsigargin effect on cell polarization

The depletion of intracellular Ca^{2+} stores causes the initiation of the influx of extracellular Ca^{2+}. To determine whether this influx is necessary for the change in cell morphology, a similar time-course was performed in Ca^{2+}-free medium. The effect of thapsigargin was abolished under these conditions (figure 48). Thus, thapsigargin similarly to the αCD3 requires the presence of extracellular Ca^{2+} to affect the morphology of repolarized Jurkat cells. It may be suggested that the influx of extracellular Ca^{2+} is the key mediator of biological events resulting in the loss of the polarized morphology.

10.2.3.4. Role of intracellular Ca^{2+} stores in the effect of thapsigargin on cell polarization

The thapsigargin treatment is thought to permanently empty the intracellular Ca^{2+} stores. The thapsigargin-pretreated and repolarized cells would therefore be expected to be refractory to subsequent doses of thapsigargin. For the purpose of this experiment the dose of thapsigargin was lowered to $5x10^{-7}$M as thapsigargin at double dose of 10^{-6}M appeared to be toxic. Surprisingly, addition of thapsigargin to such pretreated and repolarized cells resulted in a response observed as transient reversal of polarization (figure 49-A). However, the initial rapid decline in polarized population, observed in normal dbcAMP-cultured Jurkat cells, was eliminated. In addition, the response was diminished to about a third of that observed in the non-pretreated cells. The recovery of polarized morphology in the pretreated cells was faster (within 30 min) and to levels slightly higher than those observed at the start of the experiment.

The evidence described suggests that the initial rapid effect of thapsigargin, observed as a 'spike' in Jurkat cell rounding up, correlates with the release of Ca^{2+} from intracellular stores. However, this release

Figure 48: Effect of the absence of extracellular Ca^{2+} on the change in morphology of dbcAMP-cultured Jurkat cells stimulated by thapsigargin. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS, and resuspended in 1 ml of the medium. The repolarization was carried out in normal or Ca^{2+}-free HBSS/MOPS. Thapsigargin was added to the cells and the incubation was continued at 37°C for various time intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Polarized cells in the presence (open triangles) and absence (full triangles) of Ca^{2+}.

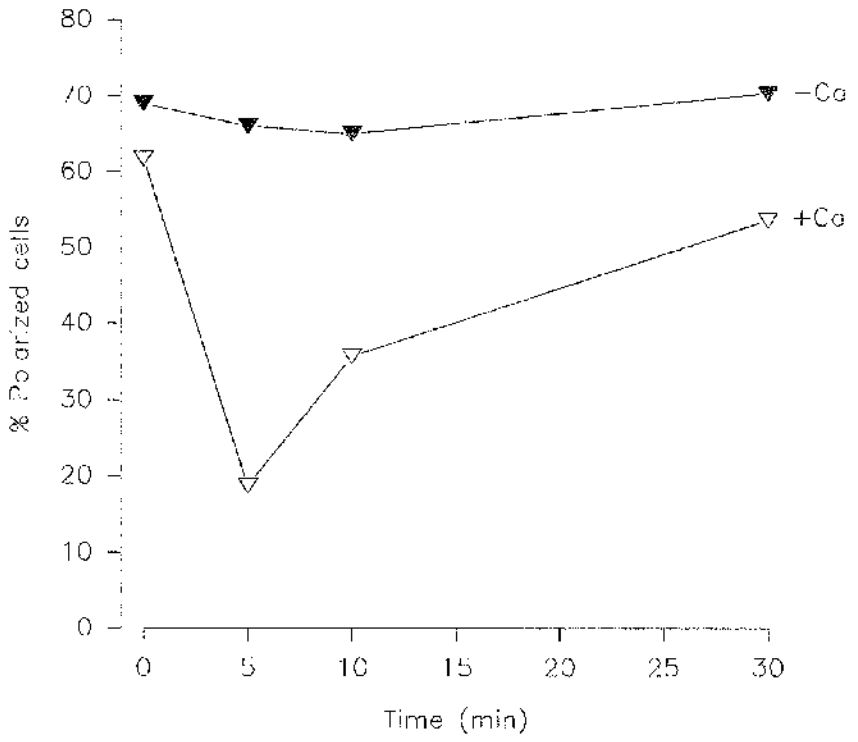
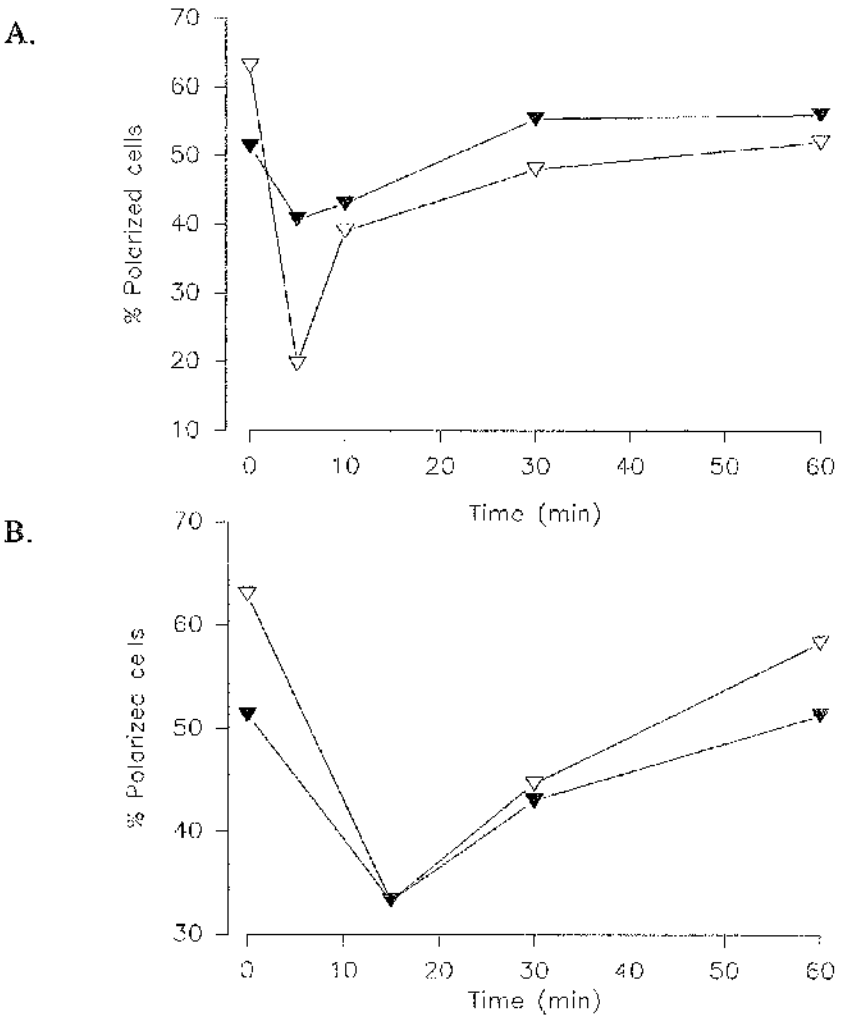


Figure 49: Effect of pretreatment of dbcAMP-cultured Jurkat cells with thapsigargin on the response to subsequent stimulation with thapsigargin (A) and αCD3 (B). The cells were cultured with 1 mM dbcAMP for 3 days, washed twice in HBSS/MOPS and repolarized for 60 min at 37°C in the presence of 500 nM thapsigargin. OKT3 and thapsigargin were added to the final concentration of 20 ng/ml or 500 nM respectively and the incubation was continued for 15 or 5 min respectively. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Polarized cells preincubated in the absence (open triangles) and in the presence (full triangles) of the agents.



is most likely amplified by the influx of extracellular Ca^{2+} as in its absence no effect on the cell morphology was seen. It is possible that transiently the $[Ca^{2+}]_i$ is sufficiently high to stabilize the microtubule network thus resulting in rapid rounding up of the Jurkat cells. The second stage, consisting of a slower rate of recovery of the polarized morphology, follows a visibly different dynamic. Thus, it is most likely caused by a different mechanism also triggered by αCD3. This mechanism requires a prolonged increase in $[Ca^{2+}]_i$ sustained by the influx extracellular Ca^{2+}. In the second stage, Ca^{2+} probably acts to activate secondary pathways which result in cell rounding up observed with αCD3 and in the latter part of the thapsigargin-stimulated response.

10.2.3.5. Role of intracellular Ca^{2+} stores in the effect of αCD3 on cell polarization

The release of Ca^{2+} from intracellular stores appears to be an important trigger affecting the influx of extracellular Ca^{2+}. Thus, the pretreatment of Jurkat cells with thapsigargin would be expected to eliminate the response to the αCD3. However, the effect of αCD3 did not seem to be affected by the pre-treatment (figure 49-B). The difference was that the initial and final proportion of polarized cells was lower in the pretreated cells which seems to be a consequence of the presence of thapsigargin. However, the magnitude of the maximum response was identical in the normal and pre-treated cells. The results suggest that the lack of Ca^{2+} in the intracellular stores does not affect the αCD3-mediated signal again implicating the influx of extracellular Ca^{2+} as the main mechanism.

10.2.4. Involvement of Ca^{2+} channels

The involvement of extracellular Ca^{2+} was further examined using verapamil, an inhibitor of Ca^{2+} channels. Verapamil on its own showed no

effect on the repolarized dbcAMP-cultured Jurkat cells at a concentration of 10^{-4}M (figure 50). Indeed, when the cells were preincubated with 10^{-4}M verapamil, the αCD3 subsequently induced the same effect as the one observed in the absence of the Ca^{2+} channel blocker (figure 51).

The effect of verapamil, at the concentration used, in blocking the influx of the extracellular Ca^{2+}, was not confirmed. Thus, two possible explanations are available: (1) the dose was insufficient to prevent the influx and (2) the Ca^{2+} channels engaged in the influx linked to the TcR/CD3 complex signal are verapamil-resistant.

10.2.5. Involvement of calcium-binding protein

The involvement of Ca^{2+}-binding protein calmodulin was examined with the help of W-7. This calmodulin inhibitor at the concentration of 10^{-4}M was found to completely reverse the polarization of the dbcAMP-cultured Jurkat cells (figure 52). The effect of this drug was no longer detected at 10^{-6}M. When the cells were pretreated with 10^{-6}M W-7 the CD3-mediated pathway was not affected (figure 53). The observations suggest that the polarization of the dbcAMP-cultured Jurkat cells is maintained by a mechanism which involves calmodulin.

10.3. SUMMARY

In conclusion, the above experiments showed that incubation of Jurkat cells with αCD3, PHA and thapsigargin caused a sustained elevation of $[Ca^{2+}]_i$. The effect of αCD3 and PHA results in rounding up of dbcAMP-cultured Jurkat cells and is dependent on the presence of extracellular Ca^{2+}. The connection between an increase in $[Ca^{2+}]_i$ and the regulation of lymphocyte morphology was established when incubation of dbcAMP-cultured Jurkat cells with A23187 and thapsigargin caused reversal of polarization. The experimental evidence suggests that the

Figure 50: Time-course of the effect of 10^{-4}M verapamil on repolarized dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 5 days, washed twice with HBSS/MOPS, resuspended in 1 ml of the medium and repolarized at 37°C for 30 min. Verapamil was added and the incubation was continued at 37°C for various time intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

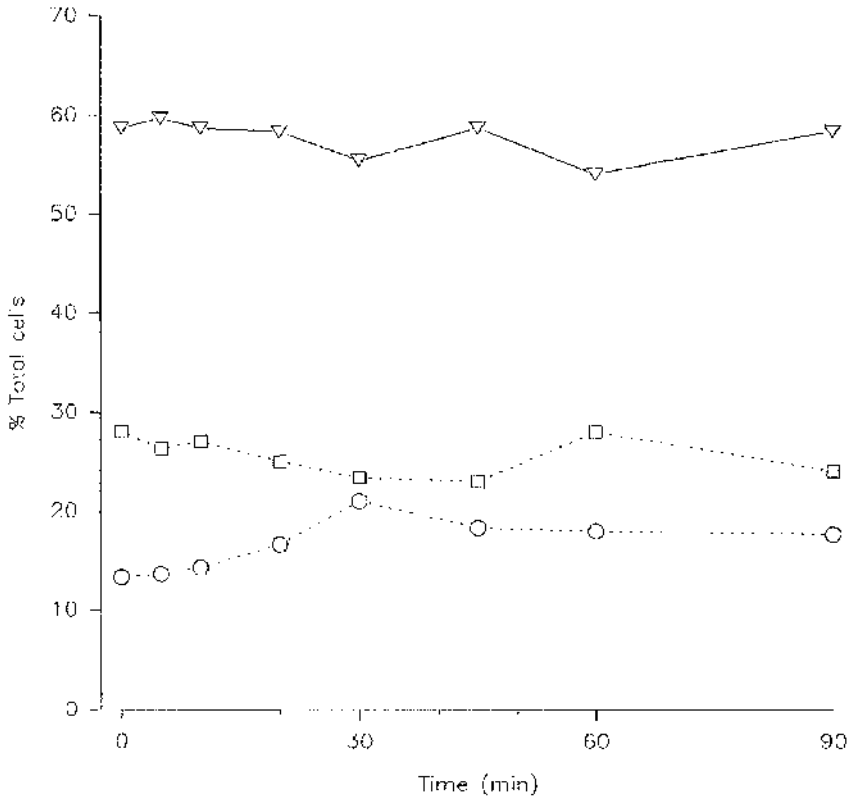


Figure 51: Effect of 10^{-4}M verapamil on the morphology change of dbcAMP-cultured Jurkat cells stimulated with αCD3. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS or calcium-free HBSS/MOPS supplemented with EGTA. Cells were then resuspended in 1 ml of the same medium. Verapamil was added and the cells were repolarized at 37°C for 30 min. After this time OKT3 was added to the final concentration of 20 ng/ml and the incubation was continued at 37°C for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Polarized cells in the absence (open bars) or presence (hatched bars) of αCD3.

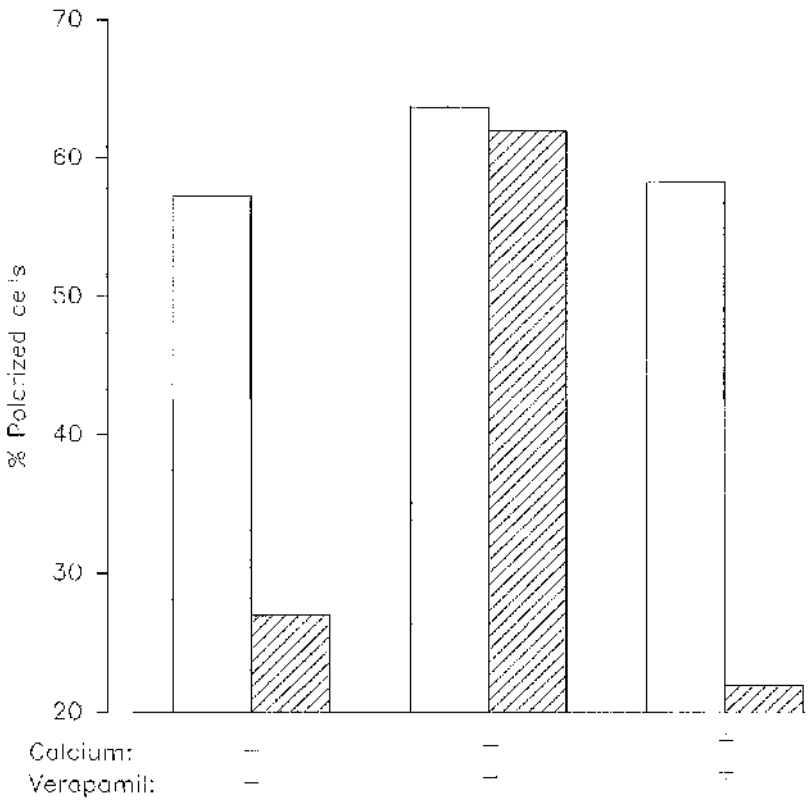


Figure 52: Dose-response of repolarized dbcAMP-cultured Jurkat cells to the action of W-7. The cells were cultured with 1 mM dbcAMP for 4 days, washed and repolarized at 37°C for 30 min. Various concentrations of W-7 were added and the incubation was continued for 80 min. The cells were finally fixed and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

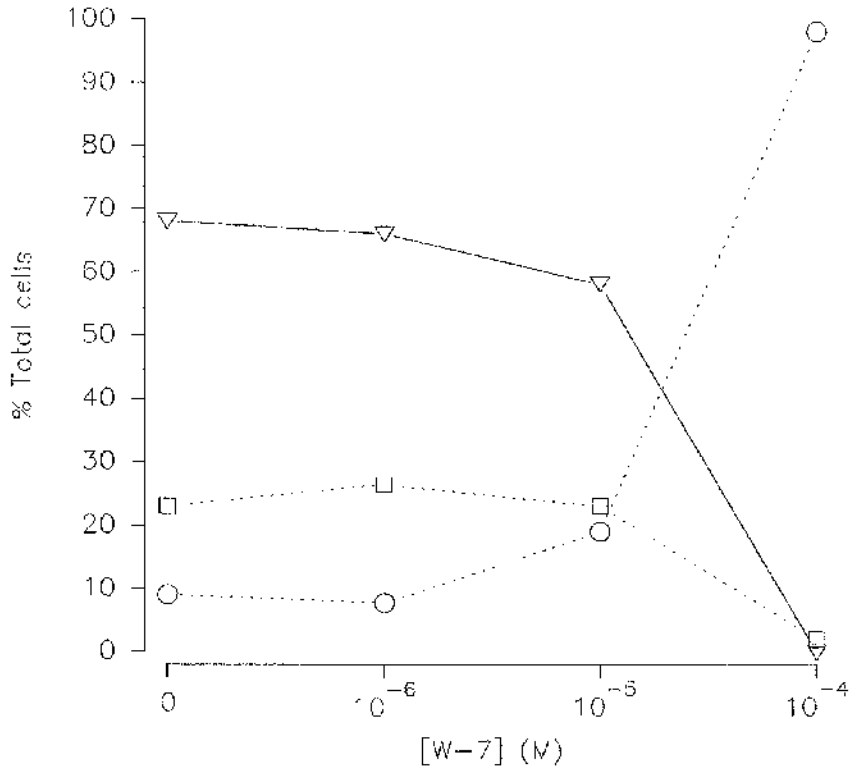
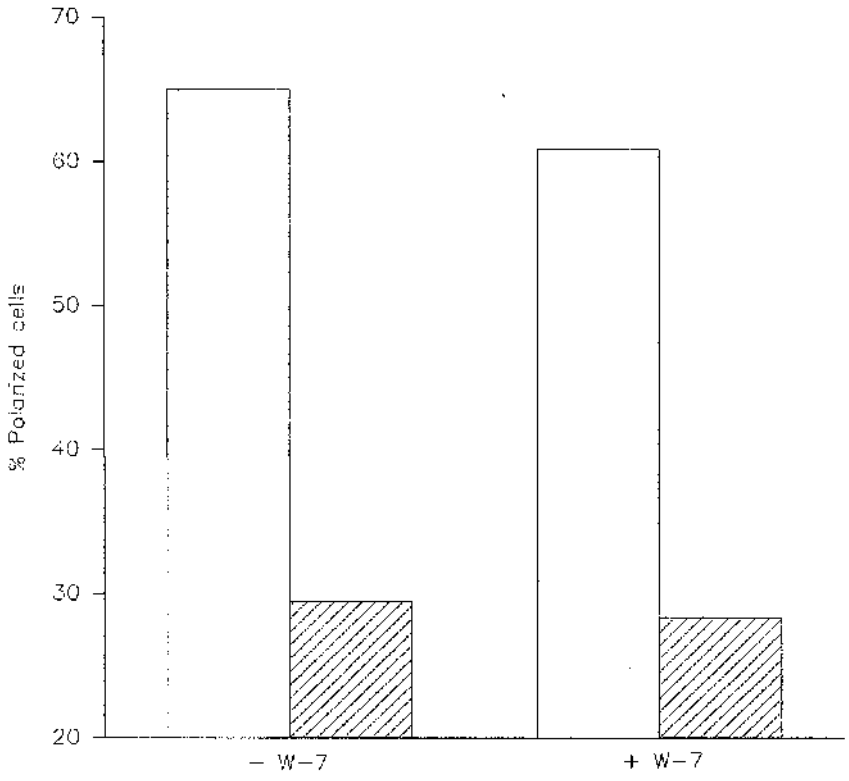


Figure 53: Effect of the pretreatment with W-7 on the morphology change of repolarized dbcAMP-cultured Jurkat cells stimulated with αCD3. The cells were cultured with 1 mM dbcAMP for 3 days and washed. W-7 was added to the final concentration of 10^{-6}M and the cells were repolarized at 37°C for 1 hr. After this time OKT3 was added to the final concentration of 20 ng/ml and the incubation was continued at 37°C for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Polarized cells in the absence (open bars) or presence (hatched bars) of αCD3.



influx of extracellular Ca^{2+} is necessary affect the cell morphology with Ca^{2+} playing most likely a role of secondary messenger.

11. INVOLVEMENT OF PROTEIN PHOSPHORYLATION IN THE REGULATION OF THE LOCOMOTORY CAPACITY ACQUIRED BY dbcAMP-CULTURED JURKAT CELLS

11.1. BACKGROUND

The polarization of dbcAMP-treated Jurkat cells is transiently inhibited by a signal through the TcR/CD3 complex. This process has been shown to require the presence of extracellular Ca^{2+} and appears to correlate with the increase in $[Ca^{2+}]_i$. The intracellular store of Ca^{2+} is released by IP_3, a second messenger generated during the αCD3-induced phosphoinositide hydrolysis. The other second messenger generated is DAG which is the physiological activator of PKC. Thus, there are two possible explanations of the locomotion-related events associated with signalling through the TcR/CD3 receptor. First, the involvement of intracellular Ca^{2+} whose elevated concentration may temporarily stabilize the polymerization of microtubules or activate Ca^{2+}-dependent intracellular enzymes such as protein kinases or phospholipases. Second, the transient production of DAG synergizing with the increase in the intracellular Ca^{2+} activates PKC.

The next set of experiments was designed to explore the effect of phorbol esters, and other agents affecting the phosphorylation state of intracellular proteins, on the locomotory capacity of dbcAMP-cultured Jurkat cells.

11.1.1. Regulation of intracellular processes by protein phosphorylation

The variations in the phosphorylation of proteins has been recognized as the most common mechanism of regulation of their function. Indeed, an immediate consequence of T cell activation is the phosphorylation of a wide range of proteins on seryl, threonyl and tyrosyl residues (Alexander and Cantrell, 1989). The protein phosphorylation at

tyrosine residues has received much attention due to its role in key regulatory mechanisms affecting growth and differentiation (Hunter and Cooper, 1985). However, 99% of protein phosphorylation occurs at serine and threonine residues and is catalyzed by kinases such as PKA, PKC, and MAP kinase (Permutter *et al.*, 1993). The phosphorylation state of proteins is kept at a dynamic equilibrium maintained by the action of kinases and phosphatases (Cohen, 1992). It is suggested that stimulation of T cells via the TcR leads to activation of a specific set of protein kinases and phosphatases, leading to phosphorylation of a specific set of cellular proteins (Alexander and Cantrell, 1989). Some of these target proteins may be important in the motor and cytoskeletal structures involved in lymphocyte locomotion.

11.1.2. PKC and its substrates

PKC was originally detected as an unidentified protein kinase present in many tissues and activated by limited proteolysis by Ca^{2+}-dependent neutral thiol protease calpain or by trypsin (Inoue *et al.*, 1977; Takai *et al.*, 1977). Subsequently it was shown that PKC could be activated in the presence of Ca^{2+} and phospholipid without proteolysis (Takai *et al.*, 1979a).

PKC when tested *in vitro* had a broad substrate specificity phosphorylating seryl and threonyl but not tyrosyl residues of many endogenous proteins in most tissues (Nishizuka, 1984). *In vitro* these substrates include a number of cell surface receptors, contractile and cytoskeletal proteins and enzymes (Turner and Kuo, 1985; Nishizuka, 1986; 1988). Among the most relevant substrates in T cell activation are CD3γ, CD3δ, and CD3ε (Samelson *et al.*, 1985ab, 1986, 1987; Baniyash *et al.*, 1988b; Breitmeyer *et al.*, 1987; Cantrell *et al.*, 1985, 1987; Minami *et al.*, 1987; Chatila and Geha, 1988; Patel *et al.*, 1987), CD4 and CD8 (Chatila and Geha, 1988; Acres *et al.*, 1986, 1987; Blue *et al.*, 1987; Hoxie *et al.*, 1988), CD45 (Autero and Gahmberg, 1987;

Schackelford and Trowbridge, 1986), IL-2rα (Gaulton and Eardley, 1986; Schackelford and Trowbridge, 1984, 1986), MHC class I (Autero and Gahmberg, 1987; Schackelford and Trowbridge, 1986), and $p56^{lck}$ (Veillette *et al.*, 1988a, 1989a).

11.1.3. Requirements for activation of PKC

Activation of PKC requires the 'unmasking' of the regulatory domain from the catalytic site. The conformational change is brought about by the interaction of PKC with co-factors. PKC interacts directly with phospahtidylserine or Ca^{2+} but not with DAG or phorbol ester in the absence of the former two co-factors. PKC showed preference for phosphatidylserine, as this phospholipid was found to be the most effective in reconstituting the PKC activity (Takai *et al.*, 1979b). Other phospholipids may show positive (*i.e.* phosphatidylethanolamine) or negative (*i.e.* phosphatidylcholine and sphingomyelin) cooperativity (Kaibuchi *et al.*, 1981) suggesting the importance of the phospholipid environment on PKC activity. The four carboxyl groups of serine are thought to ligate with Ca^{2+} so that PKC can associate with this membrane-associated complex prior to its activation with DAG (Ganong *et al.*, 1986).

Kinetic studies revealed that a small amount of DAG increased about 100-fold the affinity of PKC for Ca^{2+}, fully activating it without any change in intracellular Ca^{2+} levels (Takai *et al.*, 1979b; Kishimoto *et al.*, 1980; Kaibuchi *et al.*, 1981). PKC requires one molecule of DAG and four molecules of phosphatidylserine for complete activation (Hannun *et al.*, 1985, 1986). Thus, the active PKC is a quaternary complex consisting of phospholipid, Ca^{2+}, DAG and the enzyme.

11.1.4. Heterogeneity of PKC

There are more then one species of PKC molecule showing distinct enzymological characteristics with defined tissue expression and

intracellular localization (Nishizuka, 1988; Kikkawa *et al.*, 1989). At the present, ten subspecies of PKC have been identified in cDNA libraries from various tissues. The first group of classical or conventional PKC (cPKC) subspecies (designated α, $ß_I$, $ß_{II}$, γ) was identified during the initial screening, while the second group consists of new PKC (nPKC) subspecies (δ, ε, η[L] and θ) and the third of atypical PKC (aPKC) subspecies (ζ and λ), so called to reflect their unusual activation requirements. Full activation of the cPKCs is thought to require phosphatidylserine, Ca^{2+}, and DAG while members of nPKCs do not require Ca^{2+} for activation (Hug and Sarre, 1993). PKCζ, a member of aPKC family, exhibits a low but constitutive activator-independent kinase activity which can be further stimulated by phosphatidylserine or unsaturated fatty acids such as arachidonic acid (Hug and Sarre, 1993).

PKC was isolated also in lymphocytes (Ogawa *et al.*, 1981) and in lymphoid tissues and leukaemic cell lines (Kuo *et al.*, 1980). Peripheral T lymphocytes or leukaemic T cells express PKCα and PKCß (Altman *et al.*, 1990; Mills *et al.*, 1988; Beyers *et al.*, 1988; Koretzky *et al.*, 1989; Shearman *et al.*, 1988) where normal T cells express high levels of PKCß compared to PKCα (Shearman *et al.*, 1988) while leukaemic cell lines such as Jurkat express higher levels of PKCα (Alexander and Cantrell, 1989).

11.1.5. Structure of PKC

All of the PKC subspecies are composed of a single polypeptide where cPKC comprise four conserved (C_1-C_4) and five variable (V_1-V_5) regions (Coussens *et al.*, 1986; Kikkawa *et al.*, 1987) while the others lack C_2.

C_1 and C_2 make up the regulatory domain presumed to contain Ca^{2+}, DAG and phospholipid binding sites while C_3 and C_4 contain an ATP-binding site and show an extensive homology with many other protein kinases and thus represent the catalytic domain. The catalytic domain is fully active without Ca^{2+}, phospholipid and DAG when preferentially

cleaved from the native PKC by calpain (Kishimoto *et al.*, 1983).

11.1.6. Regulatory functions of PKC

PKC stimulation is clearly associated with the activation of T cells (Isakov *et al.*, 1986, 1987) via processes such as the induction of IL-2r and IL-2 gene expression; however PKC provides also negative feedback control over various steps in intracellular signalling, operating in both short-term and long-term responses of the cell (Nishizuka, 1986, 1988).

PKC may inhibit Ca^{2+} mobilization by blocking the receptor-mediated hydrolysis of phosphoinositides or by stimulating the hydrolysis of IP_3 by activating an IP_3 phosphatase (Connolly *et al.*, 1988). The treatment with phorbol esters was shown to inhibit (Abraham *et al.*, 1987, 1988) or attenuate (Gelfand *et al.*, 1987) mitogen-induced increase in phosphoinositide hydrolysis and $[Ca^{2+}]_i$ perhaps by uncoupling a G protein from TcR/CD3 (Mills *et al.*, 1989). In addition, PKC activates in various cell types Ca^{2+}-transport ATPase and the Na^+/Ca^{2+} exchange protein both of which serve to remove Ca^{2+} from cytoplasm (Nishizuka, 1986; Kikkawa and Nishizuka, 1986).

A second mechanism of negative feedback acts through deactivation of a receptor stimulated by its ligand. Thus, phosphorylation of EGF receptor results in the decrease in its affinity for EGF (Schlessinger, 1986) while the treatment of T cells with phorbol esters results in a down-regulation of TcR/CD3 (Isakov *et al.*, 1987; Cantrell *et al.*, 1985, 1987; Minami *et al.*, 1987; Weyand *et al.*, 1987), CD4 (Isakov *et al.*, 1987; Weyand *et al.*, 1987; Solbach, 1982; Hoxie *et al.*, 1986; Wang *et al.*, 1987) and CD8 (Isakov *et al.*, 1987).

PKC itself is also regulated by autophosphorylation (Mochly-Rosen and Koshland, 1987); by calpain-catalyzed proteolytic cleavage liberating a constitutively active catalytic domain (Kishimoto *et al.*, 1983, 1989b) which is then gradually depleted from the cell (Young *et al.*, 1987); and by an endogenous pseudosubstrate sequence that acts as a highly specific

and potent PKC inhibitor (House and Kemp, 1987).

11.1.7. Implications of PKC heterogeneity

The variable regions identified within each PKC subspecies are likely to contribute to the different properties with respect to the substrate specificity (Ido *et al.*, 1987) and activation requirements (Hug and Sarre, 1993). The PKC isoenzymes which differ in their activation requirements may thus be regulated independently (Bell, 1986). For example, the different PKC subspecies may be activated sequentially in response to DAG, arachidonic acid and lipoxin A as these phospholipid metabolites appear following the receptor stimulation. In addition, the different enzymological properties of PKC shown *in vitro* (Nishizuka, 1988; Shearman *et al.*, 1988) combined with the differential expression and localization of the different PKC subspecies may affect the activation requirements of the T cells subsets.

11.1.8. Phorbol esters

Phorbol esters are potent tumour promoters in mouse skin (Slaga *et al.*, 1982) and also induce a range of biological effects in cultured cells (Diamond *et al.*, 1980) including modulation of growth and differentiation.

Early kinetic studies in various cell types suggested that the primary site of phorbol ester action is at the plasma membrane (Weinstein *et al.*, 1979). Subsequently, phorbol esters were shown to substitute for DAG in PKC activation, first *in vitro* (Castagna *et al.*, 1982), and later in intact cells (Sano *et al.*, 1983; Yamanishi *et al.*, 1983). PKC was identified as the phorbol ester receptor based on several lines of evidence. First, the similarity between the dose-response of PKC activation and the saturation curve for PMA binding to its receptor (Castagna *et al.*, 1982). Second, the similarity between the PKC activation constant and the dissociation constant for PDB and its receptor (Kikkawa *et al.*, 1983; Dunphy *et al.*,

1981; Horowitz *et al.*, 1981; Solanki and Slaga, 1981). Finally, tissue co-distribution (Kikkawa *et al.*, 1983; Ashendel *et al.*, 1983) and co-purification (Leach *et al.*, 1983; Niedel *et al.*, 1983; Sando and Young, 1983) of the phorbol ester-binding protein and PKC activity.

The stoichiometry shows that one molecule of phorbol ester binds to one molecule of PKC in the presence of physiological concentrations of Ca^{2+} and in an apparent excess of phospholipid (Kikkawa *et al.*, 1983). The phorbol esters have an absolute requirement for Ca^{2+} and phospholipid in binding to PKC and will not bind either to the PKC or to the phospholipid, regardless of Ca^{2+} presence, unless all four components are present simultaneously (Kikkawa *et al.*, 1983). The phospholipid specificity for PDB binding resembles conditions present during physiologic PKC activation with phosphatidylserine being the most effective (Kikkawa *et al.*, 1983; Ashendel *et al.*, 1983; Sando and Young, 1983; Leach *et al.*, 1983). Using a phorbol ester photoaffinity probe it was shown that phorbol ester interacts primarily with phospholipid thus presumably activating PKC by modifying its phospholipid microenvironment (Delclos *et al.*, 1983).

The molecule of phorbol ester intercalates into the membrane phospholipid bilayer where it becomes available for binding by PKC which moves towards it from cytoplasm to form the quaternary complex by which the enzyme is activated. Thus, the PKC translocation to the membrane is suggested to be a critical event in its physiologic activation (Bell, 1986).

Although DAG and PMA stimulate PKC they mediate qualitatively and quantitatively different effects (Abraham *et al.*, 1987; Rosoff *et al.*, 1988). The activation with mitogens or αTcR antibodies may activate some PKC isoenzymes selectively; DAG activates distinct PKC isoforms (Kishimoto *et al.*, 1980; Isakov *et al.*, 1990), which then phosphorylate various targets (Houslay, 1991). Phorbol esters most likely activate them all.

The phorbol esters are much more slowly metabolized than is DAG. This is probably responsible for sustained PMA-induced PKC activation

(Kikkawa *et al.*, 1983) as opposed to the transient activation stimulated by mitogens and αCD3 (Ledbetter *et al.*, 1986, 1987; Isakov and Altman, 1987; Kraft and Anderson, 1983; Farrar and Ruscetti, 1986; Nel *et al.*, 1987; Manger *et al.*, 1987). The importance of persistent PKC stimulation in T cell activation was illustrated by the need for repeated stimulation with DAG to achieve their activation (Koyasu *et al.*, 1987).

In most unstimulated cells PKC activity is recovered from the cytosol. The term translocation, in the context of PKC, refers to the change in the intracellular site occupied by the enzyme on exposure of certain cell types to phorbol esters or natural agonists. PKC translocation was first observed in a study of subcellular PKC distribution after phorbol ester exposure (Kraft *et al.*, 1982; Kraft and Anderson, 1983). In such cells, the PKC activity was recovered in a form tightly associated with cell membranes.

11.1.9. PKC translocation and depletion

The translocation of PKC activity is fairly consistently observed in response to phorbol esters, however the results vary with natural agonists (Rana and Hokin, 1990). However, activation of PKC in T cells stimulated with phorbol esters, mitogens, αCD3, or αCD2 is accompanied by translocation of the PKC activity from cytoplasm to the plasma membrane (Ledbetter *et al.*, 1986, 1987; Isakov and Altman, 1987; Kraft and Anderson, 1983; Farrar and Ruscetti, 1986; Nel *et al.*, 1987; Manger *et al.*, 1987). Phorbol esters and Ca^{2+} act in synergy to activate and translocate PKC in T cells (Isakov and Altman, 1987).

The treatment of intact 3T3 cells with $4x10^{-7}$M PDB for 48 hrs resulted in a complete disappearance of active PKC (Rodrigues-Pena and Rozengurt, 1984).

11.1.10. PKC inhibitors

Staurosporine is a very potent PKC inhibitor of microbial origin whose IC_{50} against PKC, PKA and Ca^{2+}/calmodulin-dependent protein kinase is 0.01, 0.12 and 0.04 μM respectively (Davis *et al.*, 1989). Recently, new PKC inhibitors based on staurosporine have been developed and some became available either commercially or as an experimental sample. These reagents include Ro31-8220 (Davis *et al.*, 1989), CGP041251 (Meyer *et al.*, 1989), and GF109203X (Toullec *et al.*, 1991). Although some of these inhibitors are not as potent as staurosporine, they display superior selectivity for PKC. For example, Ro31-8220 has an IC_{50} against PKC, PKA and Ca^{2+}/calmodulin-dependent protein kinase of 0.01, 1.5 and 17 μM respectively (Davis *et al.*, 1989). An important characteristic observed with staurosporine is its high toxicity.

11.1.11. Serine-threonine phosphatases

The phosphorylation of CD3 and CD4 is reversible suggesting the involvement of phosphatases in the activation process.

The regulation of phosphatase activity is largely unknown and the phosphatase activity may differ depending on Ser/Thr sites involved. Thus, two Ser sites in CD3γ are differentially phosphorylated by selective action of PKC and phosphatases (Davies *et al.*, 1987).

It is assumed that T cells contain the four major phosphatases found in mammalian cells (Ingebritsen and Cohen, 1983). It was confirmed that T cells contain the type 1 and type 2A phosphatases and calcineurin and these are present in cytosolic and plasma membrane associated forms (Alexander *et al.*, 1988). *In vitro* experiments showed that type 2A phosphatases are the most active form of phosphatase involved in dephosphorylation of CD3γ (Alexander and Cantrell, 1989) suggesting that type 2A play a key role in reversing PKC-mediated phosphorylation events (Parker *et al.*, 1986).

11.1.12. Involvement of phosphorylation in cell locomotion

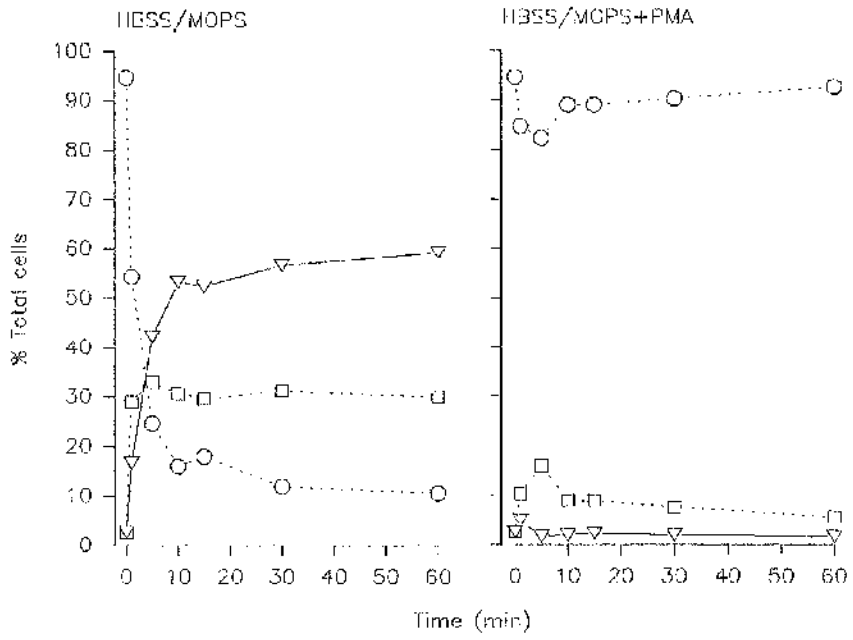
PKC appears to be involved in the locomotion of leukocytes as suggested by several lines of evidence. Phorbol esters, the activators of PKC, have been shown to induce atypical cell morphology in fresh human blood lymphocytes while an overnight culture resulted in shapes similar to the classical locomotor morphology (Wilkinson *et al.*, 1988). Studies investigating the connection between protein phosphorylation and the locomotion of neutrophils found that directional locomotion (Gallin and Wright, 1978), as well as polarization or locomotor activity (Roos *et al.*, 1987) are suppressed by PKC activators. H-7, a specific PKC inhibitor (Hidaka *et al.*, 1984), elicited within few seconds vigorous shape changes and F-actin redistribution in non-motile spherical neutrophils (Keller *et al.*, 1990). In contrast, a specific staurosporine type PKC inhibitor CGP041251, at concentrations of up to 1 μM, did not affect the morphology and F-actin localization in non-motile neutrophils (Niggli and Keller, 1993). CGP041251 inhibited front-tail polarity in fNLPNTL-activated neutrophils and this inhibition correlated with inhibition of phosphorylation of a 67-kDa protein. Staurosporine and K252a, similarly to PMA, suppress cell polarity and locomotor activity of Walker carcinosarcoma cells (Zimmermann and Keller, 1992).

11.2. EXPERIMENTAL

11.2.1. Effect of PMA on the repolarization of dbcAMP-cultured Jurkat cells

A time-course of repolarization in the presence and in the absence of 10^{-6}M PMA was determined (figure 54). Surprisingly, the normal repolarization of dbcAMP treated cells was almost completely abolished after a 10-min treatment. It can be seen that significant inhibition of repolarization was evident after only 1 min in the presence of PMA. The

Figure 54: Time-course of repolarization of dbcAMP-cultured Jurkat cells in the presence of PMA. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and resuspended in 50 μl of the medium at room temperature. The experiment was initiated by the addition of HBSS/MOPS containing 10^{-6}M PMA and the cells were incubated at 37°C for various intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



time-course of 10^{-8}M PMA action on repolarized dbcAMP-cultured Jurkat revealed that the polarization event observed in these cells was reversed noticeably 20 sec after addition of PMA (figure 55). The maximum inhibition of the repolarization was observed 10 min after the introduction of PMA which confirmed the preliminary observation. The cells, rounded up following the PMA treatment, were phase bright and resembled glutaraldehyde-fixed fresh blood cells.

The dose-response curve revealed that the full effect of PMA was achieved at 10^{-8}M while 10^{-10}M PMA had a very slight effect on the repolarized cells (figure 56). The action of PMA does not require the presence of extracellular Ca^{2+} as can be seen from the dose-response experiments in Ca^{2+}-free EDTA-supplemented medium (figure 56).

11.2.2. Effect of PKC depletion by long-term culture with PMA

11.2.2.1. Effect on PMA-induced shape change

The cells were incubated with dbcAMP in the presence or absence of 10^{-7}M PMA for 3 days. After this incubation the cells were washed twice in a medium and repolarized for 30 min at 37°C. The cell density obtained at the end of the incubation was 1.24×10^6 and 1.22×10^6 cells/ml with and without PMA respectively. Thus, the PKC depletion had no effect on the inhibition of the cell growth. Indeed, long-term culture with PMA has no effect on the activity of cAMP-dependent protein kinase (Rodrigues-Pena and Rozengurt, 1984) which is thought to be the primary enzyme to mediate the effect of cAMP (Kammer, 1988). Cells removed from the culture containing PMA did not profoundly react by shape change in response to a new challenge with PMA at concentration between 10^{-11}M to 10^{-6}M. When treated with these two extreme PMA concentrations, the populations of polarized, irregular and round cells were as follows: 25.7% and 26.7%, 60.0% and 41.00%, and 14.3% and 32.3%. Normal dbcAMP treated cells cultured without PMA show a very significant change in

Figure 55: Time-course of the effect of PMA on repolarized dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min. PMA was added to final concentration of 10^{-8}M and the incubation was continued for various time intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

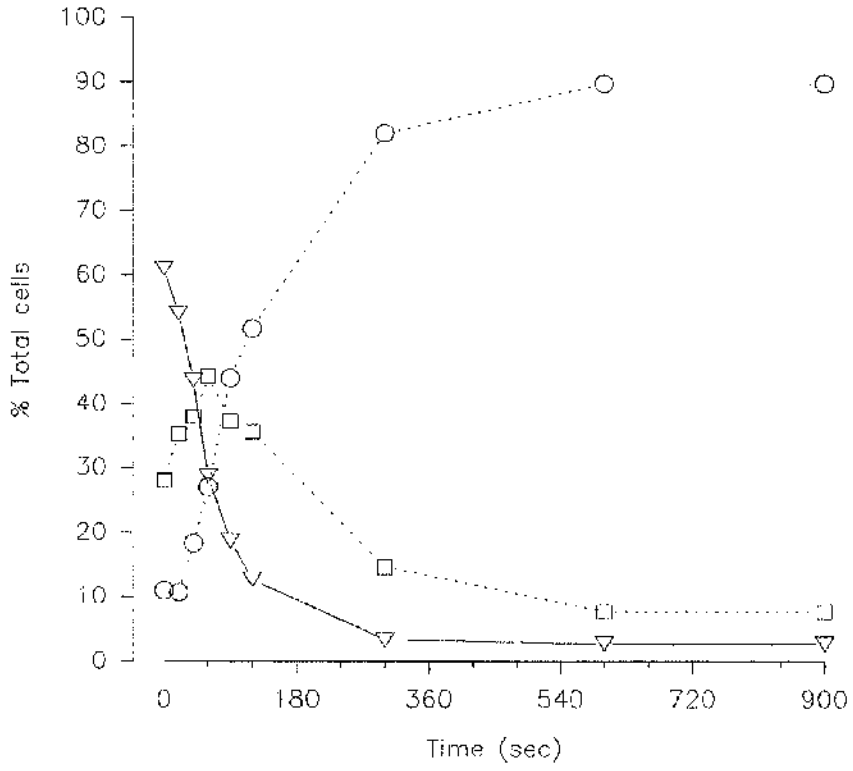
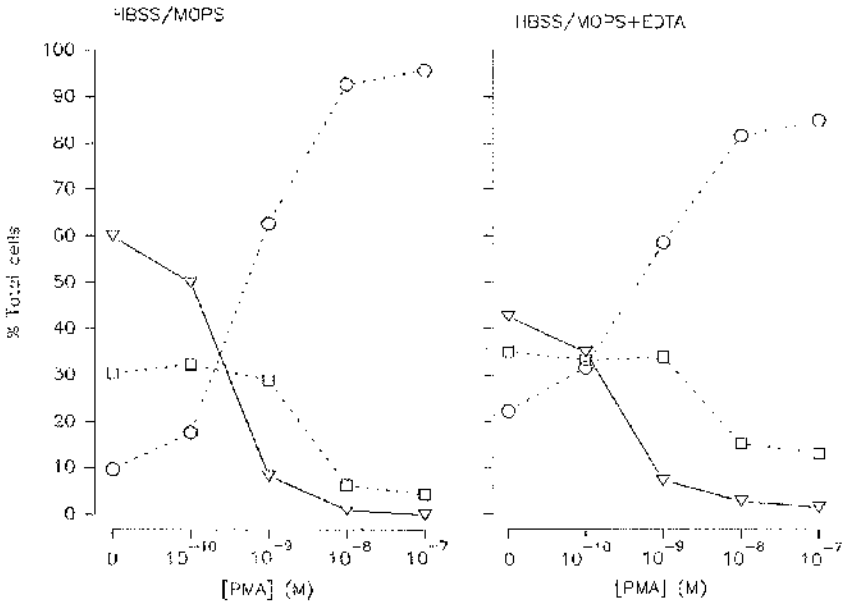


Figure 56: Dose-response of repolarized dbcAMP-cultured Jurkat cells stimulation with PMA. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min. Various concentrations of PMA were added and the incubation was continued for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

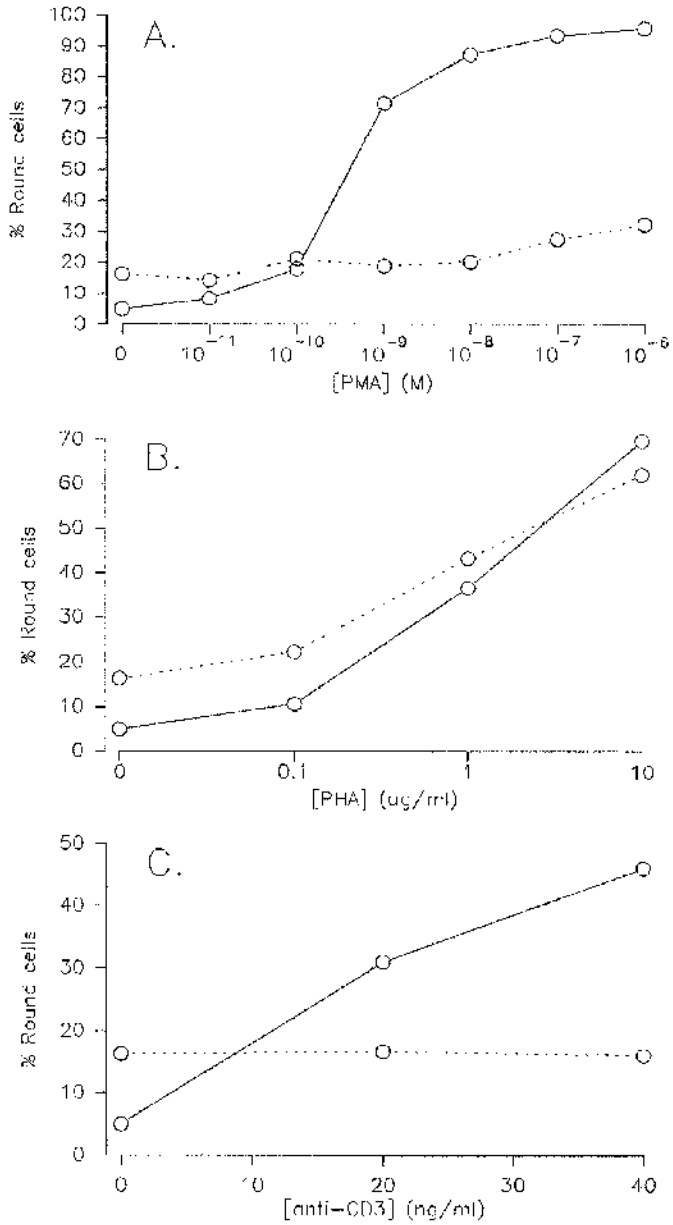


morphology under identical conditions: 65.3% and 0%, 26.3% and 4.3%, and 8.3% and 95.7% for polarized, irregular and round cells respectively. The dose-response to PMA of cells cultured with or without PMA shows the most striking difference when the changes in proportions of cells displaying the round morphology are considered (figure 57-A). It is evident that while the proportion of round cells in PMA-treated cells remained relatively constant, the proportion of round cells in identically treated normal Jurkat cells sharply increased at PMA concentration of 10^{-9}M and higher. The results indicate that, in the PMA treated cells, the most prevalent morphology is irregular. The presence of predominantly irregular cells in PMA-treated cells may by analogous to that reported by Wilkinson *et al.* (1988) using lymphocytes fresh from blood. In these experiments the irregular cells were non-motile. An explanation may lie in the strongly hydrophobic nature of PMA. It is conceivable that the PMA effect observed on the fresh blood cells (Wilkinson *et al.*, 1988) and on the PMA-cultured Jurkat cells is due to insertion of the hydrophobic molecule into the membrane lipid bilayer rather than due to its activity on PKC. Such disruption of normal membrane lipid environment and its effect on the cytoskeleton structures could result in an irregular cell shape.

11.2.2.2. Effect on αCD3-induced shape change

The PKC depleted cells were used to determine whether PKC is involved in the pathway mediated by αCD3 which leads to the reversal of Jurkat cell polarization. While the proportion of round cells in Jurkat cultures supplemented with only dbcAMP increased in a dose-dependent manner following the 15 min incubation, this proportion remained virtually unchanged in the PMA-treated cells (figure 57-B). This observation strongly suggests the crucial role played by PKC in the αCD3 effect.

Figure 57: Effect of PKC depletion on response to PMA (A), PHA (B), and αCD3 (C) by repolarized dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP with or without 10^{-6}M PMA for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min. Various concentrations of PMA, PHA, and αCD3 were added and the incubation was continued for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells previously cultured in the absence (full line) and presence (dotted line) of PMA.



11.2.2.3. Effect on PHA-induced shape change

The PHA-mediated effect on the polarized cells was not affected by the depletion of PKC (figure 57-C). Thus, it can be suggested that under these conditions PHA effect is mediated by a pathway that does not involve a PMA-sensitive PKC.

11.2.3. Effect of PKC inhibitors on repolarization of dbcAMP-culture Jurkat cells

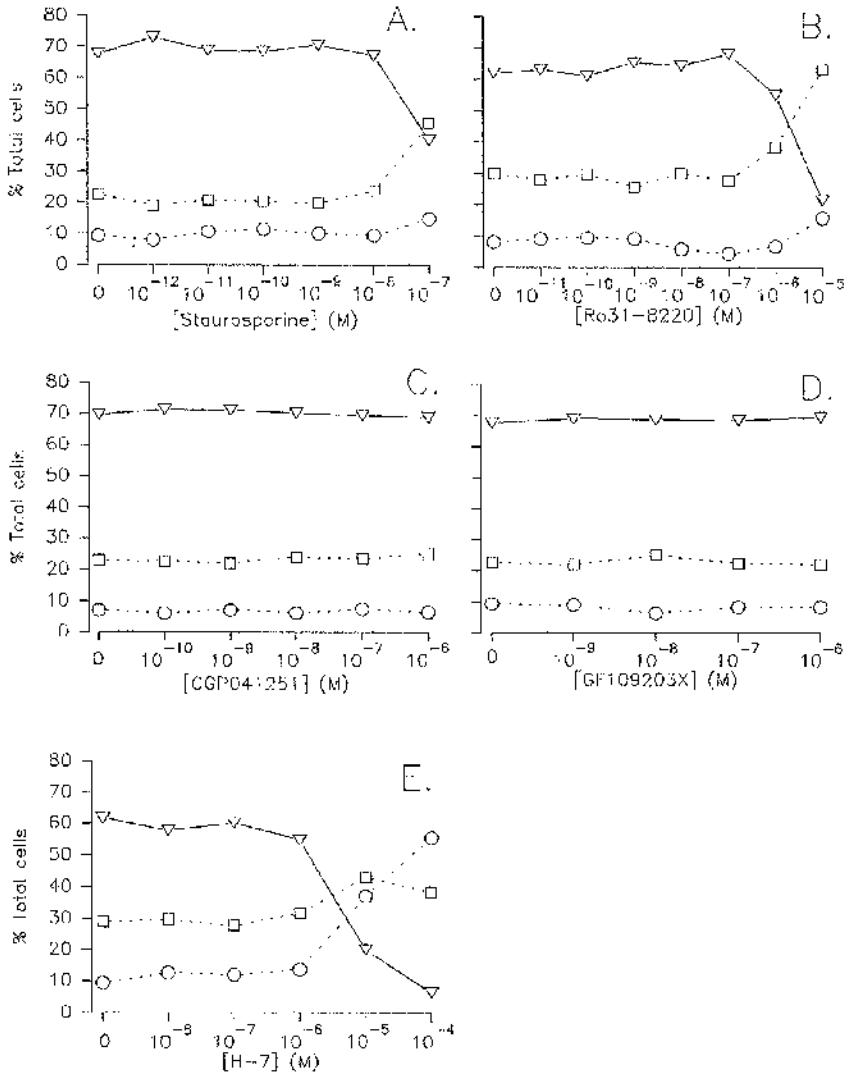
11.2.3.1. Toxicity effects of PKC inhibitors

Staurosporine and the available derivatives of this PKC inhibitor were tested with polarized dbcAMP-cultured Jurkat cells to establish their effect on the polarized cells as well as their toxic effect on the Jurkat cells in general. Staurosporine at 10^{-6}M was very toxic resulting in numerous dead cells and thus reliable counts could not be performed. A dose-response curve for staurosporine in figure 58-A indicates a rapid decrease in the number of polarized cells at 10^{-7}M dose, which was reflected by a corresponding increase in the number of irregular cells. The lack of an increase in a number of round cells would suggest that the effect of staurosporine on the morphology of the cells was non-specific and probably due to its toxicity.

A dose-response curve for Ro31-8220 in figure 58-B shows that this drug is about 100-fold less potent as an inhibitor of polarization than staurosporine in terms of dose-response. A slight effect of this drug on polarization occurred at 10^{-6}M dose while a severe inhibition of polarized morphology was seen at 10^{-5}M. At this dose, many cells appeared irregular with unusual morphology characterized by numerous protrusions or 'blebs' on the cell surface. This effect is clearly due to the toxicity of Ro31-8220 at this concentration.

The remaining two drugs, CGP041251 and GF109203X were found

Figure 58: Dose-response of repolarized dbcAMP-cultured Jurkat cells to staurosporine (A), Ro31-8220 (B), CGP041251 (C), GF109203X (D), and H-7 (E). The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min. Various concentrations of the PKC inhibitors were added and the incubation was continued for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



to have no effect at concentrations up to 10^{-6}M (figure 58-C and 58-D).

Based on the above observations, staurosporine, Ro31-8220, CGP041251, and GF109203X were used in the subsequent experiments at maximum non-toxic concentrations of 10^{-8}M, 10^{-7}M, 10^{-6}M, and 10^{-6}M respectively.

11.2.3.2. Effect of PKC inhibitors on the PMA-stimulated response

Staurosporine was used to determine the effect of PKC inhibitor in preventing the response of Jurkat cells stimulated by PMA. In preliminary experiments, preincubation with staurosporine at concentrations as low as 10^{-12}M was shown to interfere with the 10^{-6}M PMA-induced rounding up of dbcAMP-cultured Jurkat cells. In subsequent experiments, the concentrations of PMA used were ones that produce an intermediate (10^{-9}M) and full (10^{-8}M) response as seen from the dose-response experiments (figure 56).

Figure 59 shows the results of one of two experiments which illustrated the efficiency of various PKC inhibitors in preventing PMA-induced rounding up of Jurkat cells. H-7 and Iso H-7 proved to be quite ineffective and thus were eliminated from further investigations. Staurosporine used at 10^{-8}M was as effective as GF10920X or CGP041521 used at 10^{-6}M and 10^{-7}M respectively. Ro31-8220 was slightly less effective at 10^{-7}M but clearly partially inhibited the PMA-stimulated effect.

Having established that the PKC inhibitors partially inhibit the effect of PMA on the polarization, it was of interest to determine whether these agents are capable of reversing the effects of PMA. Jurkat cells were first rounded up using PMA and then the time-course of change in morphology was observed following the addition of CGP041521 (figure 60). The recovery of a polarized and an irregular morphology continued over a period of at least 60 min. In contrast, the inhibition was shown to be relatively rapid, resulting in a full effect following only a 30-min preincubation with the inhibitors. The difference in dynamics of PMA

Figure 59: Inhibition of PMA-stimulated morphology change in repolarized dbcAMP-cultured Jurkat cells preincubated with PKC inhibitors. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min in the presence of 10^{-8}M staurosporine, 10^{-7}M Ro31-8220, 10^{-6}M GF109203X, 10^{-7}M CGP041521, 10^{-6}M H-7, and 10^{-6}M Iso-H7. PMA was added to final concentration of 10^{-9}M and the incubation was continued for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast.

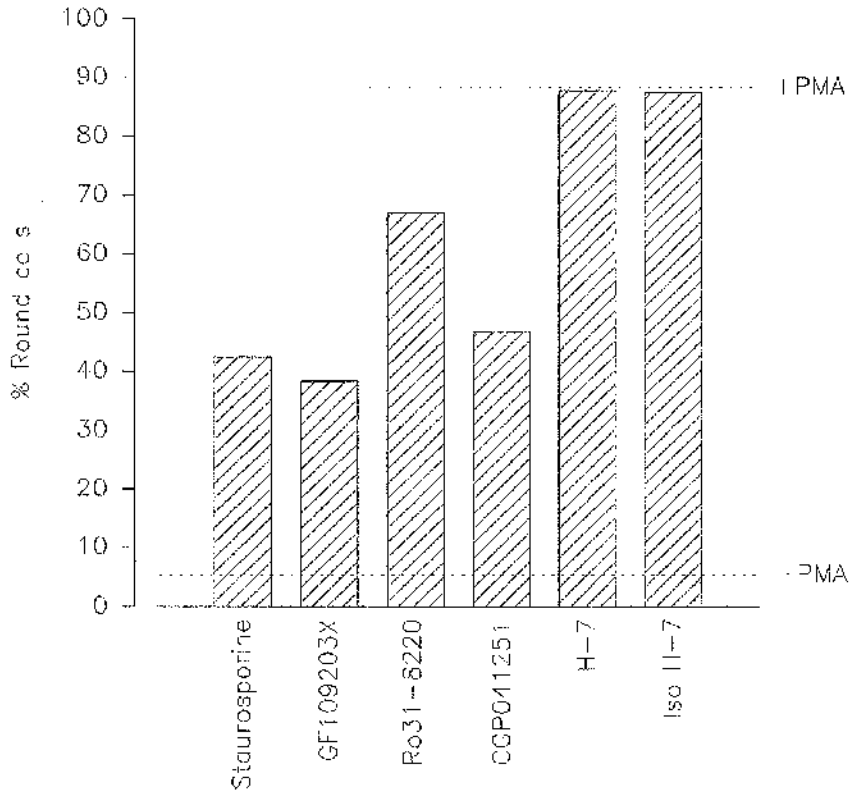
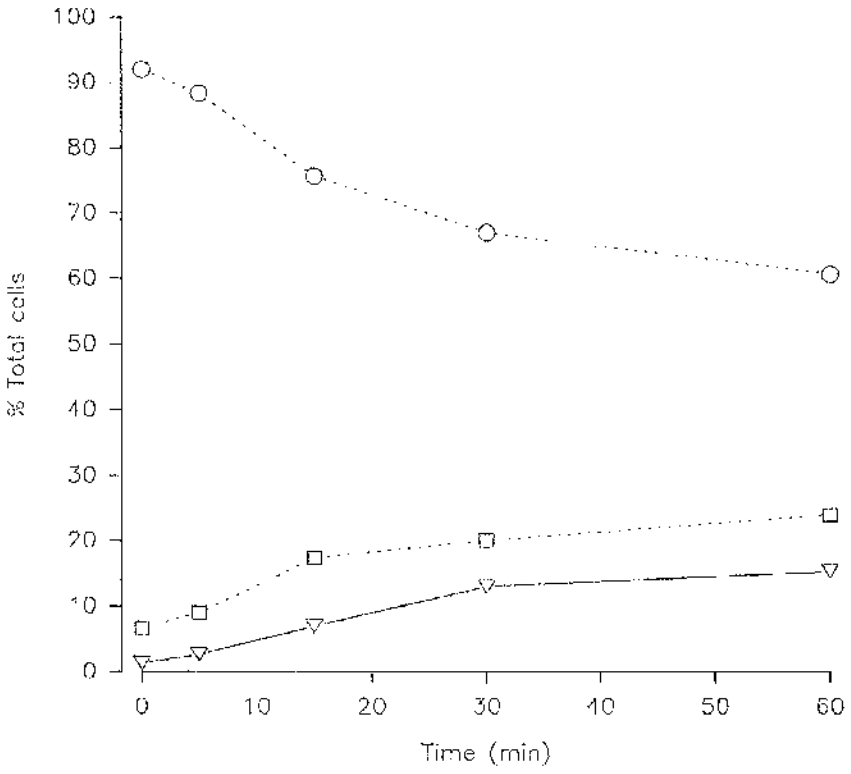


Figure 60: Time-course of repolarization of dbcAMP-cultured Jurkat cells rounded up with PMA and then treated with a PKC inhibitor. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min. Subsequently, PMA was added to final concentration of 10^{-9}M and the incubation was continued for 15 min. Finally, CGP041521 was added to final concentration of 10^{-7}M and the incubation was continued for various time intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



effect inhibition and reversal may rest in the hydrophobic nature of the phorbol ester. It can be suggested that the PKC inhibitors bind to the PKC molecule and subsequently interfere with the binding of and activation by PMA. However, these inhibitors may be inefficient in displacing PMA from PKC once the phorbol ester binds to its receptor.

11.2.3.3. Effect of PKC inhibitors on the αCD3- and PHA-stimulated response

So far, it was shown that PKC has a prominent role in the shape change of polarized Jurkat cells as shown by rounding up of cells on specific stimulation by PMA. Also, it has been established that PKC is one of the enzymes stimulated by αCD3-generated DAG. The transient character of the response to αCD3 may reflect the fast rate of DAG metabolism. Thus, specific inhibition of PKC would be expected to abolish the transient rounding up of Jurkat cells in response to αCD3. All of the PKC inhibitors tested showed a dose-dependent inhibition of the αCD3-mediated rounding up of Jurkat cells (figure 61). The minimum effective dose of staurosporine (ED_{50} 10^{-9}M) was about 1000-fold lower than that of Ro31-8220 (ED_{50} $2x10^{-7}$M) or GF109203X (ED_{50} $>10^{-7}$M). The latter two inhibitors showed virtually identical activity.

The requirement for signalling stimulated by αCD3 and PHA are similar although the change in morphology of repolarized Jurkat cells induced by these agents are qualitatively different. Having identified PKC as the key enzyme in the observed effect, a set of PKC inhibitors was used to alter the PHA-induced morphology changes (figure 62). A significant, approximately 50% inhibition was observed in the presence of 10^{-9}M staurosporine, 10^{-7}M Ro 31-8220, and 10^{-7}M CGP041251. This apparent involvement of PKC, as suggested by the effect of PKC inhibitors, is in a sharp contrast with the lack of effect of PMA-induced PKC depletion on the PHA-stimulated rounding up. A posiible explanation of this discrepancy may rest in the documented heterogeneity of PKC isoenzymes.

Figure 61: Dose-dependent inhibition of αCD3-stimulated morphology change in repolarized dbcAMP-cultured Jurkat cells preincubated with PKC inhibitors. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min in the presence of various concentrations of staurosporine, Ro31-8220, or GF109203X. OKT3 was added to final concentration of 20 ng/ml and the incubation was continued for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Staurosporine (square), Ro31-8220 (diamond) and GF109203X (circle).

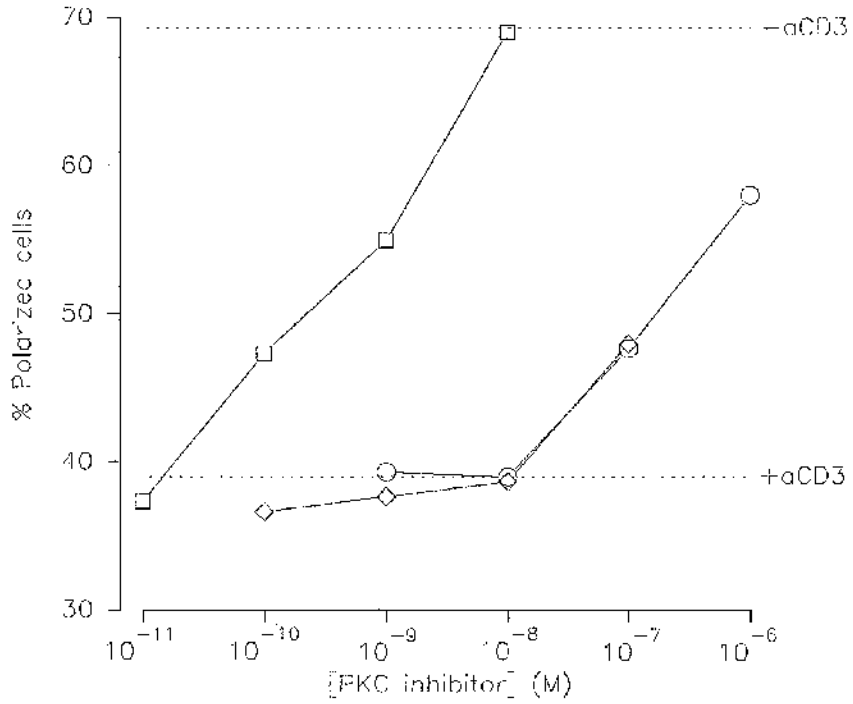
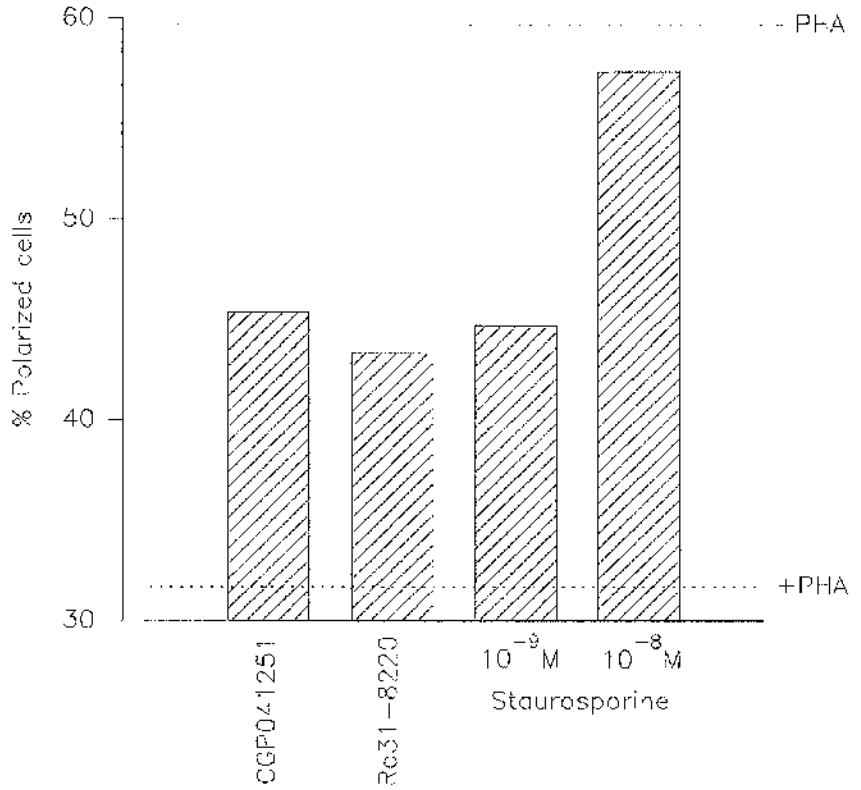


Figure 62: Inhibition of PHA-stimulated morphology change in repolarized dbcAMP-cultured Jurkat cells preincubated with PKC inhibitors. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min in the presence of 10^{-9}M or 10^{-8}M staurosporine, 10^{-7}M Ro31-8220 and 10^{-7}M CGP041521. PHA was added to final concentration of 1 μg/ml and the incubation was continued for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast.



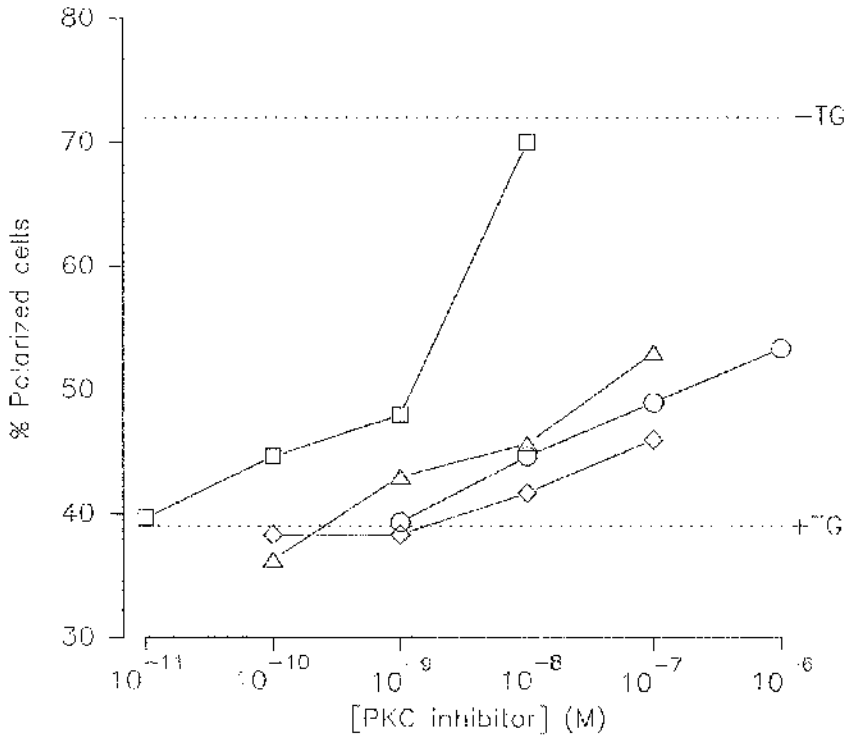
Perhaps PHA stimulates an isoenzyme unaffected by PMA but affected by the specific inhibitors. On the other hand, a prolonged PMA exposure may activate an alternative pathway exploited by the non-specific mitogen PHA.

Rounding up of repolarized Jurkat cells stimulated through the PGE_2 receptor were not affected by the PKC inhibitors (results not shown). Thus, the increase in $[cAMP]_i$ induced by PGE_2 most likely affects the locomotory machinery by a mechanism independent of PKC.

11.2.3.4. Effect of PKC inhibitors on thapsigargin- and Ca^{2+} ionophore-stimulated response

Thapsigargin induces rounding up similar to that seen with αCD3. Thapsigargin releases Ca^{2+} from intracellular stores and the extracellular Ca^{2+} must be present for the transient response to take place. Although, thapsigargin is not known to stimulate the production of DAG or to stimulate PKC directly, it was speculated that the intracellular Ca^{2+} concentration could temporarily rise to a level sufficient to activate PKC in the absence of the physiological activator DAG. Alternatively, Ca^{2+} may stimulate a biochemical pathway which results in products capable of stimulation of certain PKC isoforms. Indeed, other components of glycerolipid metabolism, such as cardiolipin, arachidonic acid, and lipoxin A were shown to be such stimulators (Hug and Sarre, 1993). If PKC is involved in Ca^{2+}-mediated changes in cell morphology, the thapsigargin-stimulated rounding up should be susceptible to the inhibition by PKC inhibitors. To test this hypothesis, repolarized Jurkat cells were preincubated with the PKC inhibitors and then stimulated with thapsigargin. Figure 63 shows that the PKC inhibitors decreased the extent of thapsigargin-induced rounding up in a dose-dependent manner. However, the PKC inhibitors were more effective on αCD3-induced rounding up, when compared to thapsigargin, as suggested by a steeper dose-response curve (figure 61). The minimum effective doses of PKC

Figure 63: Dose-dependent inhibition of thapsigargin-stimulated morphology change in repolarized dbcAMP-cultured Jurkat cells preincubated with PKC inhibitors. The cells were cultured with 1 mM dbcAMP for 4 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min in the presence of various concentrations of staurosporine, Ro31-8220, GF109203X, or CGP041521. Thapsigargin was added to final concentration of 500 nM and the incubation was continued for 5 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Staurosporine (square), Ro31-8220 (diamond), GF109203X (circle) and CGP041521 (triangle).



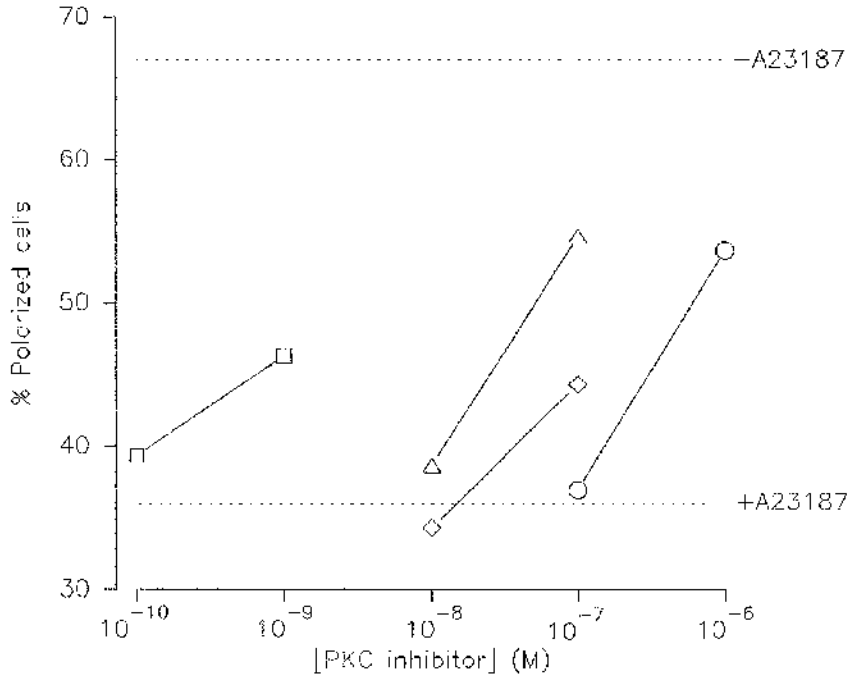
inhibitors measured with thapsigargin compared approximately with that observed with αCD3. GF109203X ($ED_{50} > 10^{-7}M$) was only slightly more effective than Ro31-8220 ($ED_{50} > 10^{-7}M$). CGP041251 (ED_{50} $10^{-6}M$) was about 10-fold more effective than either of the above drugs. Staurosporine (ED_{50} $2x10^{-9}M$) again proved to be the most active, being 10-fold more effective than CGP041251 and about 100-fold better than GF109203X or Ro31-8220. Although staurosporine apparently nearly abolished the thapsigargin effect, the result may be difficult to interpret because staurosporine at $10^{-8}M$ tends to induce unusual morphologies. The exaggerated morphological change suggests a toxic effect of staurosporine.

The stimulation of dbcAMP-cultured Jurkat cells with thapsigargin triggers a release of intracellular Ca^{2+} stores and the subsequent influx of extracellular Ca^{2+}. This increase can be also induced using Ca^{2+} ionophores and it was shown that such a treatment results in a rounding up of the cells. The pretreatment of the repolarized cells with PKC inhibitors affected dose-dependently the ability of A23187 to change the morphology of the repolarized Jurkat cells (figure 64). Thus, the effects induced by Ca^{2+} mobilizing agents are also mediated by the PKC.

11.2.4. Inhibition of protein phosphatase and the effect on polarized morphology

The above experiments suggest that PKC plays a key role in regulating the shape change of locomotory cells. The equilibrium established by the phosphorylation activity of kinases and the dephosphorylation activity of phosphatases would determine the phosphorylation state of a specific intracellular protein which in turn would determine the morphology and locomotory capability of the cell. The data so far suggests that the polarized morphology is associated with a low degree of protein phosphorylation. Conversely, a state of high protein phosphorylation results in rounding up of the cells. Based on the proposed reversible biochemical reaction, rounding up should be achieved either by

Figure 64: Dose-dependent inhibition of A23187-stimulated morphology change in repolarized dbcAMP-cultured Jurkat cells preincubated with PKC inhibitors. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min in the presence of various concentrations of staurosporine, Ro31-8220, GF109203X or CGP041521. A23187 to final concentration of 10^{-6}M was added and the incubation was continued for 5 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Staurosporine (square), Ro31-8220 (diamond) and GF109203X (circle).



activation of PKC, as was shown above, or by inhibition of the phosphatase.

11.2.4.1. Effect of calyculin A on polarized Jurkat cells

Calyculin A is a specific inhibitor of protein phosphatases. As predicted, calyculin A was shown to round up repolarized Jurkat cells. The speed of changes in morphology (figure 65) resembles changes observed in the presence of PMA (figure 55). The rounding up of the repolarized cells was complete by 5 min after the application of either calyculin A or PMA. The dose-response experiments with calyculin A revealed that the minimum effective dose is 10^{-11}M and the estimated ED_{50} dose for polarization inhibition in Jurkat cells was $2x10^{-9}$M (figure 66).

11.2.4.2. Effect of PKC inhibitors on the morphological changes induced by calyculin A

The data showed that preincubation with PKC inhibitors notably inhibits the rounding up in response to calyculin A (figure 67). The activity of PKC inhibitors was similar to that seen using αCD3. Staurosporine (ED_{50} $2x10^{-9}$M) was about 1000-fold more potent than GF109203X (ED_{50} 10^{-6}M) or Ro31-8220 ($ED_{50} > 10^{-7}$M) and about 100-fold more effective than CGP041521 (ED_{50} $2x10^{-7}$M).

11.2.5. Translocation of PKC to the membrane of activated Jurkat cells

PKC activity translocated to the plasma membrane of dbcAMP-cultured Jurkat cells stimulated with αCD3, PHA, thapsigargin and PMA was measured (figure 68). The activity was determined at crucial time-points identified in experiments dealing with the changes in cell morphology. The time-course of the αCD3- and thapsigargin-stimulated effect was shown to be transient with maximum rounding up at 15 and 5 min respectively. In both cases, the cells experienced substantial recovery

Figure 65: Time-course of the action of calyculin A on repolarized dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min. Calyculin A was added to final concentration of $2x10^{-8}$M and the incubation was continued for various time intervals. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

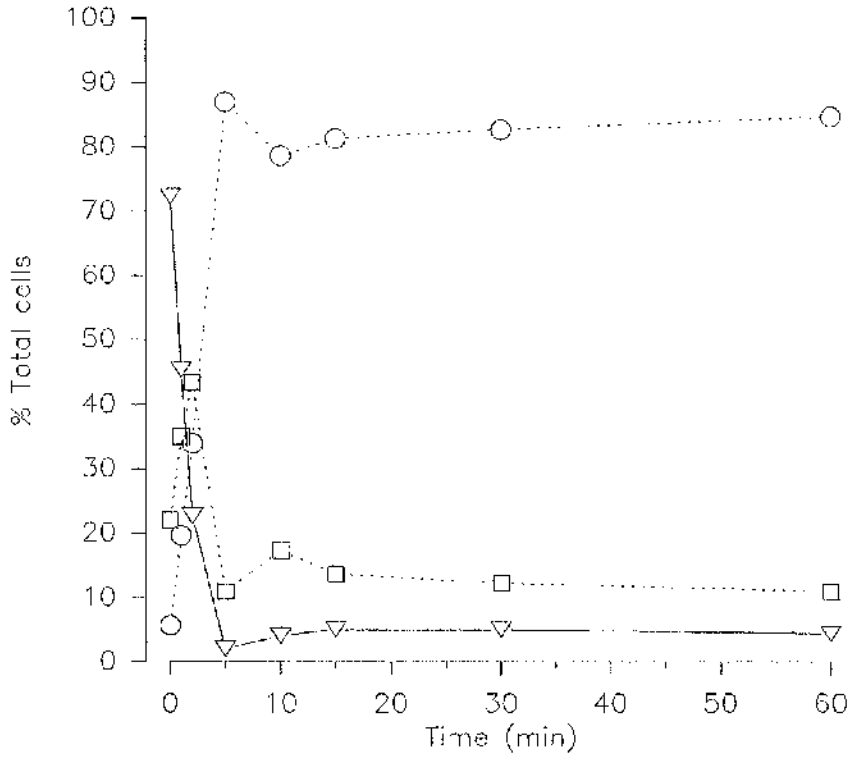


Figure 66: Dose-response of repolarized dbcAMP-cultured Jurkat cells to calyculin A. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min. Various concentrations of calyculin A were added and the incubation was continued for 5 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).

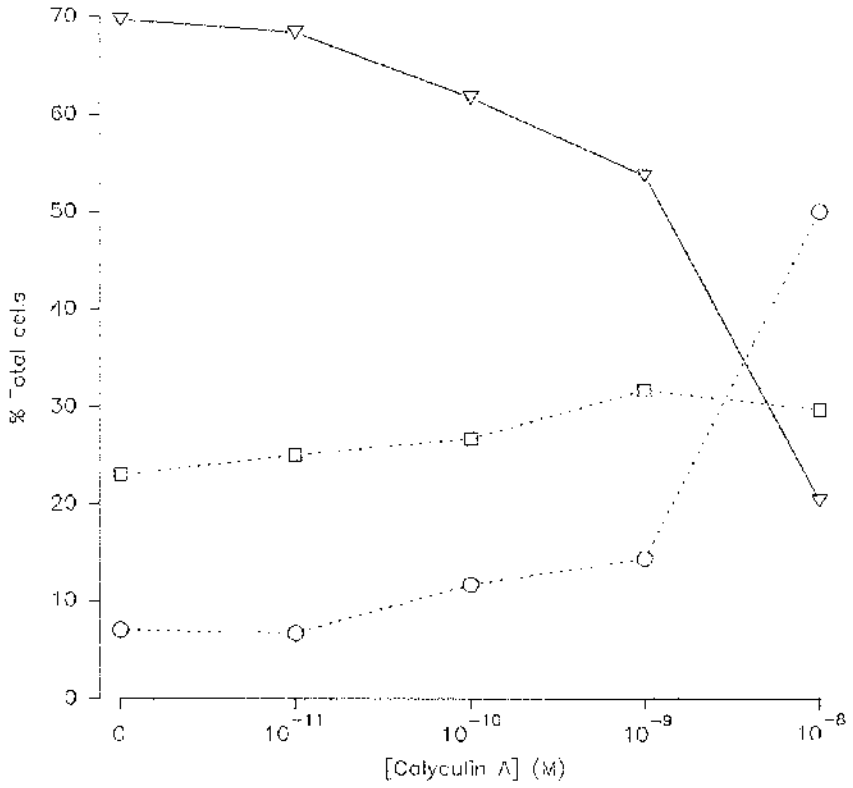


Figure 67: Dose-dependent inhibition of calyculin A-stimulated morphology change in repolarized dbcAMP-cultured Jurkat cells preincubated with PKC inhibitors. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min in the presence of various concentrations of staurosporine, Ro31-8220, GF109203X, or CGP041521. 10^{-8}M calyculin A was added and the incubation was continued for 5 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Staurosporine (square), Ro31-8220 (diamond), GF109203X (circle) and CGP041521 (triangle).

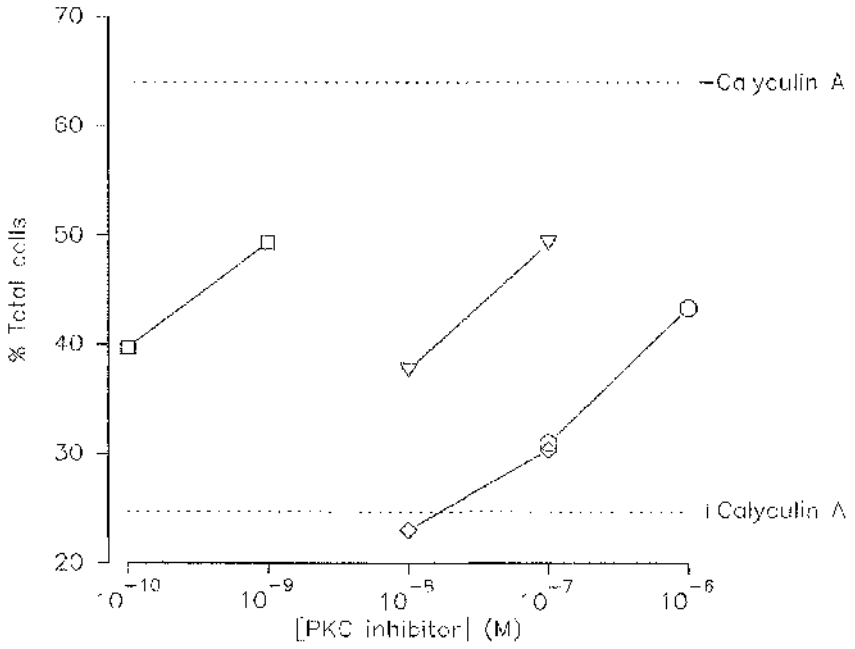
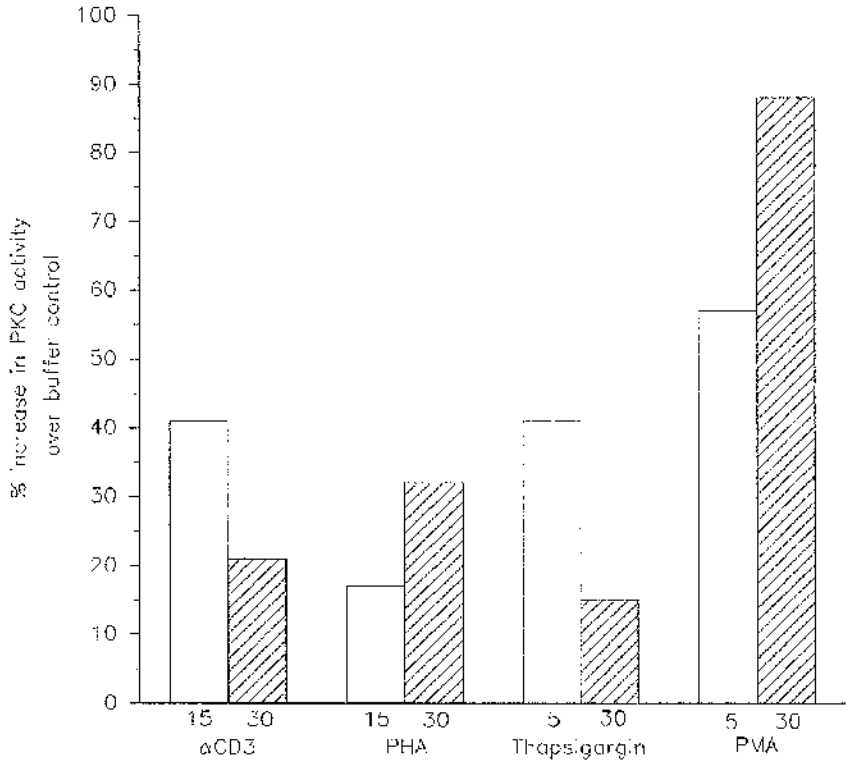


Figure 68: PKC activity translocated to the membrane of dbcAMP-cultured Jurkat cells stimulated with αCD3, PHA, thapsigargin, and PMA. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min. Aliquots of the stimulators were added to the final concentration of 50 ng/ml, 1 μg/ml, 1 μM, and 10^{-8}M respectively. The incubation was continued for various time intervals as shown below each bar. The cell extract representing the cytoplasmic and membrane fraction was then prepared and assayed for PKC activity using a commercial kit. Results were expressed as percent increase relative to the corresponding buffer control. Figure represents similar results obtained in a duplicate experiment and in several preliminary experiments.



of polarized morphology within 30 min after the introduction of a stimulus. PKC was proposed to play an important role in the mechanism leading to the loss of polarized morphology of dbcAMP-cultured Jurkat cells. The dynamics of the increase in PKC activity translocated to the plasma membrane supported this view. The membrane-associated PKC activity in cells stimulated with αCD3 or thapsigargin, for 15 and 5 min respectively, increased by 40% when compared to the buffer control. As predicted by the shape change observations, the PKC activity in cells stimulated with either agent declined to about 20% by 30 min after stimulation. In contrast, PHA and PMA, which induce permanent rounding up of dbcAMP-cultured Jurkat cells, induced an increased membrane-bound PKC activity at 15 and 5 min respectively and still higher activity after 30 min of stimulation. An important limitation of the assay used should be noted. Although the method accurately estimates the amount of PKC in the cell fractions examined, the activation status of these enzymes *in vivo* is uncertain. Indeed, the method relies on the assumption that membrane-associated PKC is activated. In addition, to stimulate PKC, in cell fractions examined, to phosphorylate a specific substrate PMA is used together with other activation co-factors such as phosphatidylserine and Ca^{2+}. Thus, PKC isoforms that are PMA insensitive or that may require other than these classical PKC activators would not be detected. However, the PMA-depletion experiments suggested that such PKC isoforms may not be involved in the lymphocyte morphology regulation or that their role is limited.

11.2.6. Expression of PKC isoforms in Jurkat cells

The overwhelming evidence of PKC involvement in the regulation of lymphocyte motility prompted the inquiry into the identity of the PKC isoform responsible. The expression of PKC isoforms in Jurkat from routine culture, culture containing 1 mM dbcAMP, and from culture containing dbcAMP and 10^{-7}M PMA was examined. For this purpose,

Western blot of the whole cell protein extract, separated by SDS-PAGE was performed and probed with a panel of monoclonal antibodies (figure 69 and 70). PKCα, PKCγ, PKCϵ, PKCΘ, PKCι, PKCλ, PKCμ, and PKCζ were detected. The first four isoforms are known to be PMA-sensitive and in agreement with this fact, the PKC expression was significantly lowered as indicated by visibly lighter or missing bands in lane 3. Thes isoforms may play a crucial role in the αCD3-stimulation rounding up of Jurkat cells as indicated by the PKC-depletion experiments.

Interestingly, the optical density of bands obtained with PKCα, PKCγ, and PKCϵ appeared to be higher in dbcAMP-cultured Jurkat (lane 2) cells compared to the cells from routine culture (lane 1). The amount of protein examined in the three lanes was relatively similar as indicated by similar density of other PKC bands and of some non-specific bands. Thus, the difference in optical density of the bands noted above reflects the actual differences in PKC expression. This apparent contradiction between the increased PKC expression and acquisition of polarization in dbcAMP-cultured cells may be explained in three ways: (1) dbcAMP acts downstream of PKC thus counteracting the increased phosphorylation of proteins regulating the motility; (2) dbcAMP culture results in massive induction of protein phosphatases which would dephosphorylate the regulatory proteins; or (3) dbcAMP-cultured Jurkat cells are depleted of endogenous activators of the crucial PKC isoforms. Indeed, the PKC expression may not be a reliable measure of PKC activity *in vivo* as a constitutively motile variant of MOLT-4 cell line did not appear different form the non-motile MOLT-4 when PKC expression was examined (Dr. Matthews personal communication). Yet the motility in these cell lines was subject to regulation by PKC activators and inhibitors.

The differences in optical density of bands obtained with different monoclonal antibodies (such as PKCα and PKCγ) cannot be translated into conclusions with respect to the quantitative differences in the expression of these two PKC isoforms. The difficulty lies in accounting for the different affinities of the monoclonal antibodies used.

Figure 69: Protein kinase C expression in Jurkat cells from routine culture (lane 1), from culture containing 1 mM dbcAMP (lane 2) and from culture containing 1 mM dbcAMP with 10^{-7}M PMA (lane 3). Western blots of whole cell protein extracts were performed using monoclonal antibodies specific for various PKC isoforms. Molecular weight markers are shown in kDa. Molecular weight of expected band is represented by arrow (>). Frame A PKCα (82 kDa), frame B PKCß (80 kDa), frame C PKCγ (80 kDa), frame D PKCδ (78 kDa), frame E PKCε (90 kDa), and frame F PKCθ (79 kDa).

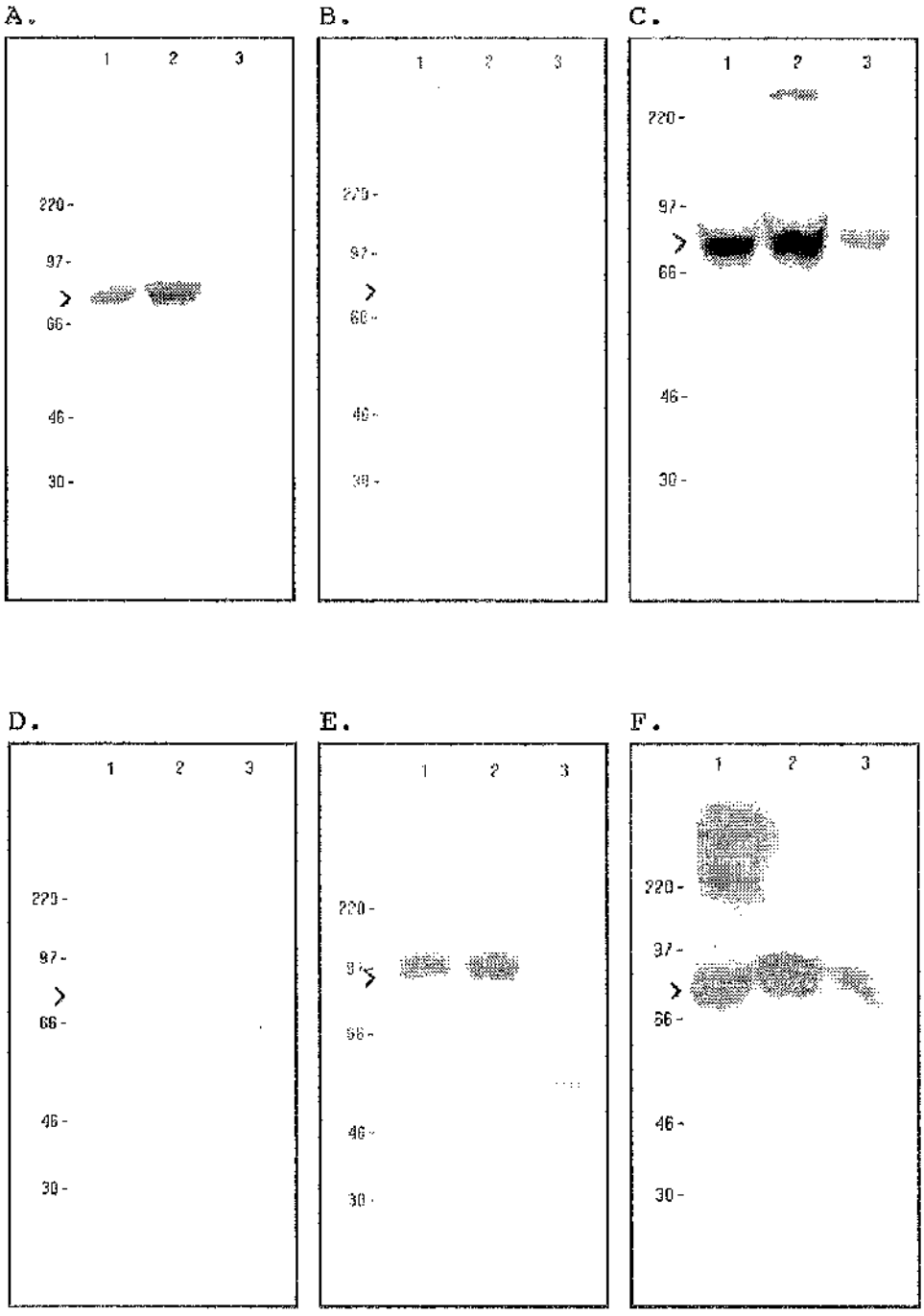
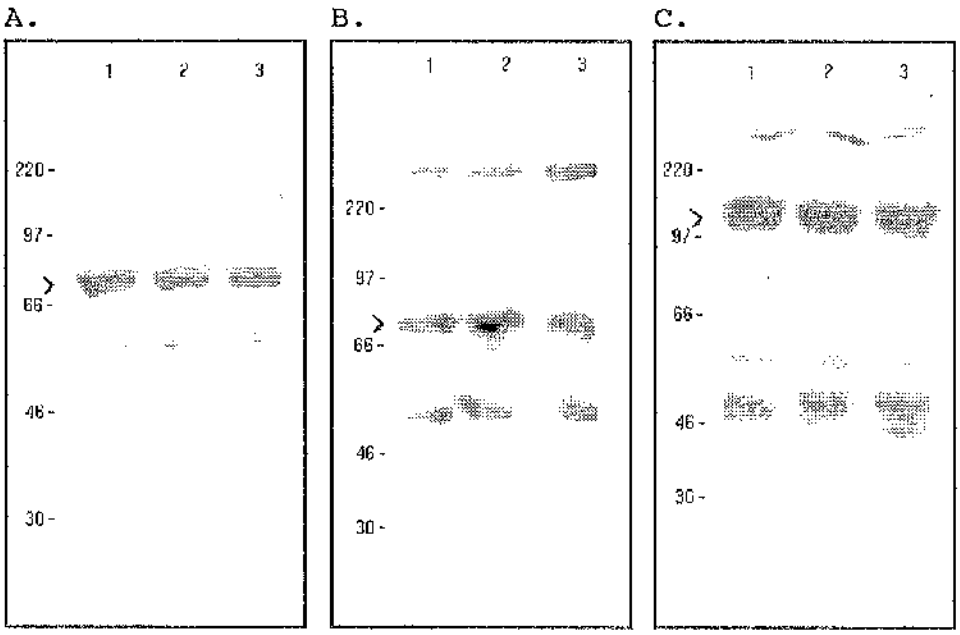
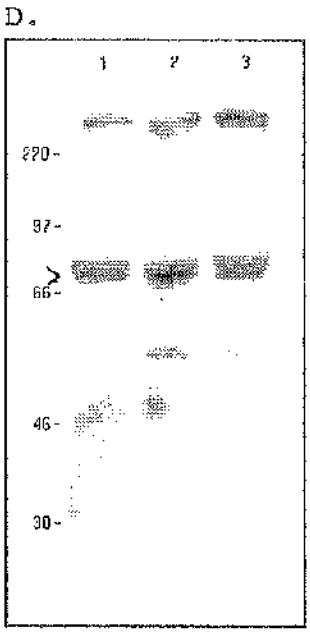


Figure 70: Protein kinase C expression in Jurkat cells from routine culture (lane 1), from culture containing 1 mM dbcAMP (lane 2) and from culture containing 1 mM dbcAMP with 10^{-7}M PMA (lane 3). Western blots of whole cell protein extracts were performed using monoclonal antibodies specific for various PKC isoforms. Molecular weight markers are shown in kDa. Molecular weight of expected band is represented by arrow (>). Frame A PKCι (74 kDa), frame B PKCλ (74 kDa), frame C PKCμ (115 kDa), and frame D PKCξ (72 kDa).





11.3. SUMMARY

In conclusion, the above experiments showed that incubation of dbcAMP-cultured Jurkat cells with PMA caused a rapid, dose-dependent reversal of polarization. Depletion and inhibition experiments showed that this effect of PMA is due the activation of PKC. In addition, the involvement of PKC in the effect of αCD3, PHA, A23187 and thapsigargin was suggested. Direct measurements of PKC activity in dbcAMP-cultured Jurkat cells stimulated with αCD3, PHA, thapsigargin and PMA revealed dynamics that link the PKC activation to the changes in cell morphology observed. In addition, Western blots revealed that the PMA-sensitive PKC isoforms that may be responsible for this regulatory activity are PKCα, PKCγ, PKCε, or PKCΘ. Changes in phosphorylation equilibrium caused by incubation of dbcAMP-cultured Jurkat cells with calyculin A caused changes in morphology similar to those observed with PMA.

12. INVOLVEMENT OF TYROSINE PHOSPHORYLATION AND G PROTEIN IN SURFACE RECEPTOR COUPLING TO INTRACELLULAR SIGNALLING PATHWAYS

12.1. BACKGROUND

Lymphocytes monitor the status of their environment by receiving messages in the form of soluble factors released by tissues, as well as by a physical contact with neighbouring cells. Such events, taking place on the extracellular face of cell membrane, are transmitted into the cytoplasm of a cell via receptors. Most such receptors fall into one of three known classes: (1) channel-linked receptors; (2) catalytic receptors whose cytoplasmic domain constitutes a PTK or is associated with a non-receptor TPK; and (3) G protein-linked receptors. G proteins are well established to perform various functions in activation and inactivation of intracellular enzymes which are ultimately responsible for the generation intracellular messenger molecules. Similarly, protein-tyrosine phosphorylation regulates the activity of ever-expanding range of intracellular enzymes and regulatory proteins.

The role of the TcR/CD3 complex in the acquisition of locomotory capacity in PBL has been documented (Wilkinson and Higgins, 1987a). In addition, the locomotory capacity of dbcAMP-cultured Jurkat cells is regulated by signals generated upon stimulation of TcR/CD3 complex. Both, the increase in $[Ca^{2+}]_i$ and the activation of PKC were demonstrated to reverse the polarization in these cells. The increase in $[Ca^{2+}]_i$ and the activation of PKC via the generation of DAG, are the result of activation of intracellular PLC. The activity of PLC was shown to be regulated by G proteins associated with surface molecules in numerous cell types. In addition, an increasing evidence links tyrosine phosphorylation as a regulatory linkage between the various surface structures and PLC-generated second messengers in T cells. The purpose of the following experiments was to determine the involvement of PTK and G proteins in the pathways that link TcR/CD3 complex with the locomotory machinery.

12.1.1. Role of protein-tyrosine phosporylation signal transduction

12.1.1.1. Lymphocyte surface molecules involved in antigen-mediated activation

T lymphocyte activation normally requires the interaction of clonotypic TcR with a peptide antigen presented on the MHC molecule of an antigen-presenting cell. The signal transduction is mediated by the associated CD3 complex and the ζ/η chains, described in a previous chapter. In addition, CD4/CD8 co-receptor molecules participate in the antigen recognition event and function to increase the antigen-receptor binding affinity and constitute an essential element in the signal transduction pathway. The CD4 and CD8 molecules are expressed on mutually exclusive subsets of T lymphocytes and interact with distinct invariant determinants on class II and class I MHC molecules respectively. CD4 and CD8 are transmembrane molecules that belong to the immunoglobulin superfamily and share only limited sequence homology. CD4 is a 55-60 kDa glycoprotein that is expressed in a monomeric form, while CD8 is expressed as a homodimer of two 32-34 kDa α chains or a heterodimer of an α and a 25-26 kDa ß chain.

12.1.1.2. Non-receptor PTK

One of the earliest events that follows T cell activation via the TcR is rapid increased tyrosine phosphorylation of several endogenous substrates (Samelson *et al.*, 1986; Baniyash *et al.*, 1988b; Hsi *et al.*, 1989; June *et al.*, 1990a) including TcR ζ chain and the proto-oncogene *vav* (Margolis *et al.*, 1992). Of particular interest is the tyrosine phosphorylation of $PLC\gamma_1$ in response to TcR/CD3-mediated PTK activation (Park *et al.*, 1991; Secrist *et al.*, 1991; Weiss *et al.*, 1991).TPK generally belong to either the receptor class or the non-receptor class of enzymes. The receptor TPK bind polypeptide growth factors, such as PDGF, EGF, FGF,

CSF-1 and insulin, by their extracellular sequences forming the large glycosylated extracellular ligand-binding domain. The transmission of intracellular signals is initiated by activation of their intrinsic PTK activity mediated by cytoplasmic catalytic domains. The members of the non-receptor TPK class associate with other surface proteins which generally lack endogenous enzyme activity. None of the surface molecules involved in antigen-induced lymphocyte activation posses a PTK domain in their cytoplasmic portion (Ashwell and Klausner, 1990). However, several of the surface molecules engaged during such activation associate with at least three non-receptor PTK that may function as their signal transducing element.

12.1.1.3. Lymphocyte activation-associated PTK

Several non-receptor PTK have been proposed to mediate signalling functions in lymphocytes (Perlmutter *et al.*, 1988; Bolen, 1991). However, recent studies have identified the involvement of three cytoplasmic PTK in proximal TcR signalling. These PTK are members of two distinct families: $p59^{fyn}$ and $p56^{lck}$ are *src*-related PTK while ZAP-70 (zeta chain-associated protein) is a *syk*-related PTK.

The $p56^{lck}$ (Casnellie *et al.*, 1982; Voronova and Sefton, 1986; Adler *et al.*, 1988; Perlmutter *et al.*, 1988) is expressed almost exclusively in lymphocytes, including all mature T cells and thymocytes (Marth *et al.*, 1985; Veillette *et al.*, 1987; Reynolds *et al.*, 1990). Initially, its participation in antigen-induced T cell activation was based on finding that $p56^{lck}$ is directly associated with the cytoplasmic domains of CD4 and CD8α co-receptor molecules (Rudd *et al.*, 1988; Veillette *et al.*, 1988b; Barber *et al.*, 1989) and later with IL-2rß (Hatakeyama *et al.*, 1991). The $p56^{lck}$ interacts with CD4/CD8 through a region in its amino-terminal which is unique among the *src*-related PTK (Shaw *et al.*, 1989). A specific $p56^{lck}$-binding consensus region of 13 amino acids was identified in the cytoplasmic tail of CD4 and CD8α (Barber *et al.*, 1989; Zamoyska

et al., 1989) where six residues were found to be essential in the cysteine-dependent association (Vega *et al.*, 1990; Turner *et al.*, 1990; Shaw *et al.*, 1990). Indeed, T cell hybridomas with mutations in CD4 $p56^{lck}$-binding sequence were unable to respond to antigen (Gleichenhous *et al.*, 1991). In addition, antibody-mediated cross-linking of CD4 or CD8 substantially increases *in vitro* PTK activity of $p56^{lck}$ (Veillette *et al.*, 1989ab; Luo and Sefton, 1990). The functional involvement of $p56^{lck}$ in TcR-mediated signalling was also confirmed. Insulin-specific T cell hybridomas transfected with activated forms of $p56^{lck}$ showed increased sensitivity to the antigen as measured by IL-2 production (Abraham *et al.*, 1991). In addition, the *lck*$^-$ mutant of Jurkat could not mobilize intracellular Ca^{2+} in response to αCD3 stimulation despite normal expression of TcR/CD3 and $p59^{fyn}$ (Straus and Weiss, 1992). Similarly, a mouse CTLL T cell line lacking $p56^{lck}$ expression showed a defective TcR-mediated cytotoxic activity (Karnitz *et al.*, 1992). All four domains of the PTK seem to be required for coupling of TcR-stimulated signals (Caron *et al.*, 1992).

There are two isoforms of $p59^{fyn}$, $p59^{fyn(B)}$ (neuronal type) and $p59^{fyn(T)}$ (haematopoietic cell type), which result from mutually exclusive splicing of alternative seventh exons (Cooke and Perlmutter, 1989). The association of $p59^{fyn}$ with TcR/CD3 and its involvement in propagating signals through this complex was suggested by five lines of evidence: (1) the expression of $p59^{fyn}$ by thymocytes correlates with the acquisition of TcR responsiveness during their differentiation into mature T cells (Cooke *et al.*, 1991); (2) co-immunoprecipitation with the CD3ε component (Samelson *et al.*, 1990); (3) co-capping of the TPK with the TcR complex (Gassman *et al.*, 1992); (4) enhancement of TcR responsiveness by overexpression of $p59^{fyn}$ (Cooke *et al.*, 1991); and (5) mice with elevated $p59^{fyn}$ activity (Katagiri *et al.*, 1989) show constitutive tyrosine phosphorylation of the TcR ζ chain (Samelson *et al.*, 1986). Thus, it is suggested that $p59^{fyn}$ linked with CD3ε is essential for the TcR-linked signal transduction by mediating the phosphorylation of the TcR ζ chain.

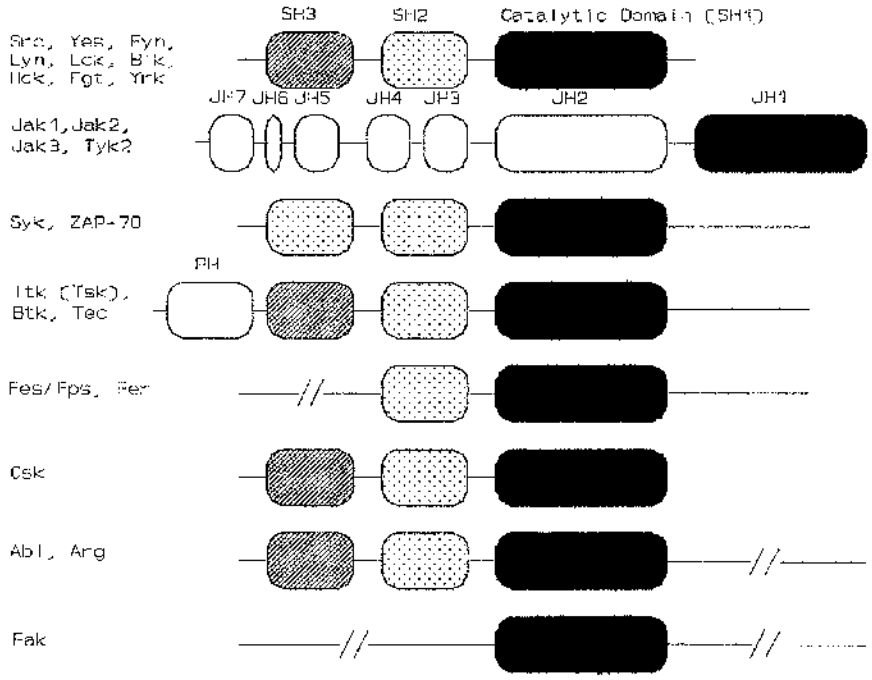
Expression of the $p60^{syk}$ and ZAP-70 TPK appears to be limited to cells of haemopoietic origin, suggesting that their function is linked to cell type-specific signal transduction. ZAP-70 (Chan *et al.*, 1992b) is expressed exclusively in T cells and natural killer cells while $p60^{syk}$ occurs predominantly in B cells, thymocytes and myeloid cells. In contrast to $p59^{fyn}$ and $p56^{lck}$, ZAP-70 associates with the TcR ζ chain (Chan *et al.*, 1991) and CD3ε (Wange *et al.*, 1992; Straus and Weiss, 1993) only following TcR stimulation. This association requires the presence of $p59^{fyn}$ and $p56^{lck}$. These PTK efficiently phosphorylate the CD3ε and the TcR ζ chain (Straus and Weiss, 1993; Qian *et al.*, 1993) which in turn recruit ZAP-70 to the activated receptor complex (Chan *et al.*, 1994).

A tyrosine-based activation motif, present in a number of other signal transducer elements, was identified in the TcR ζ chain (3 copies) as well as in CD3ε (Samelson and Klausner, 1992). This so called ARAM consists of two tyrosine-containing sequences (YXXL) separated by six to eight amino acids (Reth, 1989). These ARAM are potential sites for phosphorylation upon activation through the TcR and are able to function in coupling of the receptor complex to cytoplasmic signalling machinery (Romeo *et al.*, 1992; Irving *et al.*, 1993). Indeed, CD3ε chain is phosphorylated at these two tyrosine residues upon T cell activation *in vivo* (Sancho *et al.*, 1993). Efficient association is mediated by both SH2 of ZAP-70 and phosphotyrosine in ARAM of the ζ chain and CD3ε of activated TcR (Wange *et al.*, 1993). The proximity of ZAP-70 and TcR complex results in tyrosine phosphorylation of ZAP-70 (Iwashima *et al.*, 1993) which in turn acquires the capability to recruit SH2-containing mediators of intracellular signalling.

12.1.1.4. Structure of non-receptor PTK

The family of non-receptor PTK includes at least 24 members. The common domain relationships between the different families are shown in figure 71. The major area of sequence homology between the non-receptor

Figure 71: Diagramatic representation of structures of known non-receptor PTK members. The amino-terminus is on the right side of the figure. The following abbreviations are used: SH, Src homology domain; JH, Jak homology domain; PH, pleckstrin homology domain. JH1 and JH2 constitute PTK and PTK-like domain respectively. Size of and spacing between various domains is not shown to scale.



PTK occurs in the catalytic domain which includes ATP binding site and tyrosine autophosphorylation site. The catalytic domain is responsible for the PTK activity.

The members of the *src* family, along with a single isoform of the *abl/arg* family have an exclusive mechanism for promoting their association with the inner surface of the plasma membrane. This mechanism is based on the post-translational myristylation of a common glycine residue at position two (Marchildon *et al.*, 1984; Voronova *et al.*, 1984).

The SH3 domains (Stahl *et al.*, 1988; Mayer *et al.*, 1988) are thought to recognize proline-rich peptide ligands which are present in some guanine nucleotide exchange factors and GTPase activating proteins (Ren *et al.*, 1993). In adition, SH3 domains are found in actin-binding proteins including myosin, spectrin, and a yeast cytoskeletal protein (Drubin *et al.*, 1990) and function as possible mediators of interactions of such molecules with the components of the cytoskeleton.

The SH2 domains found in non-receptor PTK are capable of high affinity binding to selected phosphotyrosine proteins (Pawson, 1989; Matsuda *et al.*, 1991. The highly conserved sequence FLVRES of SH2 is thought to be required to bind to amino acids that flank the phosphotyrosines fitting into a specific binding pocket of SH2 (Pazin and Williams, 1992). The SH2 domain may interact with a phosphotyrosine residue on an adjacent domain in the same molecule (such as tyrosine 528,505 or 527 of $p59^{fyn}$, $p56^{lck}$ or $p60^{src}$, respectively) thus downregulating the PTK activity (Matsuda *et al.*, 1990). Indeed, mutation or deletion of the SH2 domain produces a transforming $p59^{fyn}$ protein (Hirai and Varmus, 1990; O'Brien *et al.*, 1990; Kawakami *et al.*, 1988; Semba *et al.*, 1990). Similarly, replacement of the regulatory tyrosine residue with phenylalanine or serine induces a constitutive activation of the kinase (Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Marth *et al.*, 1988). The negative regulatory site at the carboxy terminus of *src* family PTK is tyrosine phosphorylated by $p50^{csk}$ (Perlmutter *et al.*, 1988).

In addition, SH2 regions were identified also in cytoplasmic proteins involved in mitogenic pathways such as PLCγ (Stahl *at al.*, 1988; Suh *et al.*, 1988), the regulatory subunit (p85) of PI 3 kinase (Escobeno *et al.*, 1991; Skolnik *et al.*, 1991; Otsu *et al.*, 1991), the ras-GTPase activating protein (McCormick, 1989) that regulates *ras* activity (Downward *et al.*, 1990), and the *crk*, *abl*, and *vav* oncogene products (Mayer *et al.*, 1988). Thus, SH2 domains mediate the recognition and association of such cytoplasmic proteins with PTK-linked receptors containing phosphotyrosine residues (Koch *et al.*, 1991).

All PTK studied autophosphorylate on tyrosine residues in response to ligand binding (Pazin and Williams, 1992). The phosphotyrosine residues then serve as highly selective sites for binding of SH2-containing cytoplasmic signalling components. Such components thus bind to activated, autophosphorylated receptors and themselves become substrates for serine/tyrosine phosphorylation (Margolis *et al.*, 1991; Anderson *et al.*, 1990). For example, PDGFß receptor binds upon activation, among others, PI 3-kinase on tyrosine 708 and 719 (Coughlin *et al.*, 1989; Escobeno *et al.*, 1991; Kazlauskas *et al.*, 1992), GTPase-activating protein on tyrosine 739 (Kazlauskas *et al.*, 1992; Fantl *et al.*, 1992) and PLCγ binds on tyrosine 977 and 989 (Pazin and Williams, 1992). The tight interaction of the SH2 of $PLC\gamma_1$ is followed by the tyrosine phosphorylation of its tyrosine 771, 783 and 1254; the resulting conformational change in $PLC\gamma_1$ allows its SH3 domain to interact with the membrane cytoskeleton causing the translocation of $PLC\gamma_1$ from cytosol to the cellular membrane (Rhee, 1991). Interestingly, coexpression of $p59^{fyn}$, but not $p56^{lck}$, with TcR ζ chain produced tyrosine phosphorylation of the ζ chain and $PLC\gamma_1$ and calcium mobilization in response to receptor cross-linking (Hall *et al.*, 1993). The coexpression of the phosphotyrosine phosphatase CD45 enhanced these effects presumably by activating the $p59^{fyn}$ kinase (Pingel and Thomas, 1989; Koretsky *et al.*, 1990, 1991).

12.1.1.5. PLC is activated by PTK

Contrary to some reports (Kim *et al.*, 1990) several lines of evidence implicate a regulatory role for tyrosine phosphorylation in activation of PLCγ. The tyrosine phosphorylation of several intracellular substrates, stimulated by activation through TcR/CD3, precedes measurable hydrolysis of $PI(4,5)P_2$ and $[Ca^{2+}]_i$ increase (June *et al.*, 1990a). In addition, PLCγ is catalytically activated by tyrosine phosphorylation by growth factor receptors (Nishibe *et al.*, 1990; Goldschmidt-Clermont *et al.*, 1991; Kim *et al.*, 1991). PDGF- and EGF-induced activation of PLC is independent of G proteins (Hasegawa-Sasaki *et al.*, 1988; Cattaneo and Vincentini, 1989; Hepler *et al.*, 1990; Johnson and Garrison, 1987; Huang and Ives, 1989). Treatment of cells with EGF or PDGF promoted the association of $PLC\gamma_1$ with the receptors as detected by coimmunoprecipitation (Wahl *et al.*, 1989a; Huckle *et al.*, 1990) and this association was dependent on the receptor PTK activity (Mollenaar *et al.*, 1988; Escobeno *et al.*, 1988; Margolis *et al.*, 1990; Morrison *et al.*, 1990; Kumjian *et al.*, 1991). The tyrosine phosphorylation seems to be isoenzyme specific as the treatment with PDGF or EGF results in the increase of phosphorylation of $PLC\gamma_1$, but not $PLC\beta_1$ or $PLC\delta_1$ (Wahl *et al.*, 1989ab; Meisenhelder *et al.*, 1989; Margolis *et al.*, 1989; Kumjian *et al.*, 1989; Nishibe *et al.*, 1989). The phosphorylation is independent of receptor internalization (Wahl *et al.*, 1989a; Meisenhelder *et al.*, 1989). Similarly, stimulation of Jurkat cells with αCD3 resulted rapidly and transiently in phosphorylation on serine and tyrosine residues of $PLC\gamma_1$ while $PLC\beta_1$ was not affected (Park *et al.*, 1991).

12.1.1.6. PTK inhibitors interfere with TcR/CD3-mediated lymphocyte activation

Further evidence for a requirement for a regulatory role of tyrosine phosphorylation in activation of PLCγ by a TcR/CD3 was derived from

studies of the effects of inhibitors of protein tyrosine phosphorylation (June *et al.*, 1990b; Mustelin *et al.*, 1990; Stanley *et al.*, 1990; Trevillyan *et al.*, 1990; Augustine *et al.*, 1991; Norton *et al.*, 1991). The best known of these inhibitors are the benzoquininoid ansamycin antibiotic herbimycin A (June *et al.*, 1990b) and the isoflavone genistein (Mustelin *et al.*, 1990; Trevillyan *et al.*, 1990; Norton *et al.*, 1991). Herbimycin A is an *in vivo* inhibitor of *src*-related PTK which acts by depleting the intracellular level of the enzymes (Uehara *et al.*, 1989ab). In T cells, herbimycin A binds to the carboxy-terminus of *src*-related PTK via sulphydryl groups and increases their rate of degradation. As a result, the steady-state levels of *src*-related PTK $p56^{lck}$ and $p59^{fyn}$ are reversibly depleted by 90-97% (June *et al.*, 1990b). In contrast, genistein is an *in vitro* PTK inhibitor acting by non-competitive inhibition of ATP hydrolysis (Akiyama *et al.*, 1987).

Previous studies have shown that herbimycin A reduces the immunoprecipitated activity of $p56^{lck}$ and $p59^{fyn}$ while the activity of serine-threonine kinase c-*raf*, and aluminum fluoride-induced activation of G protein-associated PLC remain unaffected (June *et al.*, 1990b). In addition, the expression of IL-2r and IL-2 secretion, induced by the combination of calcium ionophore and a phorbol ester, are not inhibited by herbimycin A (June *et al.*, 1990b). Thus, the target for herbimycin A is likely to be a *src* family member proximal to PKC.

Genistein has been shown to inhibit the TcR/CD3-induced tyrosine phosphorylation of CD3ζ in human PBL (Mustelin *et al.*, 1990). In addition, genistein inhibits the appearance of a number of tyrosine phosphoproteins normally induced by the activation of PBL by antigen (Norton *et al.*, 1991). Genistein inhibits the production of IL-2 in response to stimulation via TcR/CD3, as well as by the combination of calcium ionophore and a phorbol ester (Mustelin *et al.*, 1990; Trevillyan *et al.*, 1990). Thus, the target for genistein activity is most likely distal to the activation of PLC.

In Jurkat cells transfected with the human muscarinic receptor type 1 (hMR1), different isoenzymes of PLC can be activated in response to

carbachol as well as to αTcR (Goldsmith *et al.*, 1989). However, the pathways involved in signalling through these two receptors are different where the seven transmembrane-domain hMR1 activates PLCß in a PTK-independent manner via a G protein (Nathanson, 1987; Dohlman *et al.*, 1987; Peralta *et al.*, 1987; Matesic *et al.*, 1989). In such a system, herbimycin A markedly inhibited the resting and induced levels of phosphotyrosine-containing proteins, including PLCγ_1 and TcR ζ chain, and prevented activation of PLC by αTcR but not by carbachol (Graber *et al.*, 1992). However, genistein inhibited activation of PLC by both TcR/CD3 and hMR1 but the inhibition was only partial. In contrast to herbimycin A, genistein was cytotoxic and inhibited protein synthesis in both Jurkat cells and PBL (Graber *et al.*, 1992).

12.1.2. Role of G protein in signal transduction

12.1.2.1. G proteins and leukocyte locomotion

A pertussis toxin-sensitive G protein is known to be involved in chemotactic-factor induced activation of neutrophils (Becker *et al.*, 1985; Snyderman *et al.*, 1986). The migration of lymphocytes into filters was also inhibited by pertussis toxin (Spangrude *et al.*, 1985a), as was inhibited the capacity of lymphocytes to cross HEV and enter lymph nodes *in vivo* (Spangrude *et al.*, 1984; Braaten *et al.*, 1984). However, T lymphocytes that have been rendered incapable of normal recirculation by pertussis toxin exhibit normal blastogenesis after stimulation with T cell mitogens (Spangrude *et al.*, 1984; 1985b). Similarly, pertussis toxin had little inhibitory effect on growth-induced locomotor capacity stimulated by αCD3 (Wilkinson and Watson, 1990). On the other hand, pertussis toxin inhibited immediate polarization in response to FCS or IL-8 in lymphocytes direct from blood or after overnight culture with FCS; an overnight exposure to pertussis toxin rendered lymphocyte locomotor responses to chemoattractants insensitive to this toxin (Wilkinson and Watson, 1990).

In a different system, cultured high endothelial cells stimulated rat lymph node lymphocyte motility by two mechanisms: one which was rapid and pertussis toxin-sensitive and one which was slower, pertussis toxin insensitive and dependent on lymphocyte adhesion to the high endothelial cells (Harris, 1991).

12.1.2.2. G proteins couple receptors to PLCß, activation

The involvement of G proteins in PLC activation has been suggested as a result of numerous studies which have shown that GTP and its nonhydrolyzable analogues stimulate phosphatidylinositol metabolism in permeabilized cells, crude membrane fractions, or partially purified enzyme preparations (Fain *et al.*, 1988). Also, direct stimulation of G proteins with AlF_4^- or GTPγS was shown to activate PLC in intact or permeabilized T cell populations (Cogeshall and Altman, 1989). In addition, type 1 muscarinic receptor, known to be coupled to G proteins in other systems, was transfected to Jurkat resulting in ligand-induced PLC activation (Goldsmith *et al.*, 1989). The search for a G protein involved in the PLC activation uncovered a new family of pertussis toxin-insensitive G proteins designated G_q; these G proteins specifically activate $PLC\beta_1$ but not $PLC\gamma_1$ or $PLC\delta_1$ (Taylor *et al.*, 1991; Taylor and Exton, 1991).

12.1.2.3. Structure of α_q

Members of the α_q family lack the cysteine residue four amino acids from the carboxy terminus constituting the site for ADP-ribosylation catalyzed by pertussis toxin (Strathman and Simon, 1990; Wilkie *et al.*, 1991). α_q has a threonine for alanine substitution in the sequence GAGES known to be important for GTPase activity in α_s, α_i and α_o. These G_α bind GTPγS only with slow rates and stoichiometry probably due to high-affinity GDP binding.

12.1.2.4. Mechanism of PLC activation by α_q

As suggested earlier (Smith *et al.*, 1986), α_q stimulates PLC and this stimulation occurs over a wide range of Ca^{2+} concentrations. The binding of α_q results, in addition to increasing the maximal activity of PLC, in a changed apparent affinity of the enzyme for Ca^{2+} from 1 μM to 0.1 μM (Smrcka *et al.*, 1990).

12.1.2.5. Pertussis toxin-sensitive coupling of PLC

Pertussis toxin-sensitive activation has been demonstrated when this toxin blocked phosphatidylinositol metabolism, induced by chemoattractant in leukocytes or in HL-60 (Kikuchi *et al.*, 1986; Nakamura and Ui, 1985; Varghese *et al.*, 1984); α_i and α_o reconstituted the PLC activity in pertussis toxin-treated HL-60 cells (Kikuchi, 1986). Indeed, the formyl-peptide receptors were shown to couple to both α_{i2} and α_{i3} (Gierschik and Jakobs, 1987) and to co-purify with α_{i2} (Polakis *et al.*, 1988). Similarly, pertussis toxin blocked PLC activation in *Xenopus* oocytes stimulated by muscarinic receptors (Moriarty *et al.*, 1990). The responsiveness could be restored by microinjection of G_o but not G_i and GTPγS-preactivated α_o mimicked the receptor stimulation.

Effects of pertussis toxin on receptor-regulated PLC activation have been observed with receptors previously thought to be pertussis toxin-insensitive (Pfeilschifter and Bauer, 1986; Shayman *et al.*, 1987). There is evidence that different receptors can be coupled to PLC, in a single cell, by G proteins of different pertussis toxin sensitivity (Voyno-Yasenetskaya *et al.*, 1989; Perney and Miller, 1989; Ashkenazi *et al.*, 1989). In contrast, a receptor may be coupled to PLC by pertussis toxin-sensitive G protein in one cell type and by an insensitive one in another (Moriarty *et al.*, 1989).

12.2. EXPERIMENTAL

12.2.1. Involvement of PTK in the acquisition of polarized morphology by PBL

The majority of PBL activated by a prolonged culture with PPD (Wilkinson, 1986) or with αCD3 (Wilkinson and Higgins, 1987a) acquire the locomotory capacity. In addition to the two PTK inhibitors described above, several additional reagents (Gibco #3169SA) were used in the preliminary study. These PTK inhibitors included: Methyl 2,5-dihydroxycinnamate (MDC), lavendustin A, RCAM-lysozyme, tyrphostin, and 2-hydroxy-5-(2,5-dihydroxybenzyl) aminobenzoic acid (HDAA). While lavendustin A and HDAA seem to inhibit the PTK similarly to genistein, by inhibition of ATP hydrolysis, MDC and tyrphostin were shown to block the substrate binding to the EGF receptor-associated PTK and also inhibit EGF receptor autophosphorylation. RCAM-lysozyme is an inhibitor of insulin receptor kinase autophosphorylation.

Genistein and tyrphostin, with minimum effective dose of 10 ng/ml (data not shown), resulted in a slight but noticeable decrease in polarization in cultures activated with PPD. None of the inhibitors, with the exception of herbimycin A (Prof. P.C. Wilkinson, personal communication), affected the polarization observed following a culture with αCD3. Thus, it appears that the target PTK, affected by genistein, is engaged upon activation by antigen only. Indeed, the CD4 molecule and the associated $p56^{lck}$ PTK plays an important accessory role during such an activation process. On the other hand, herbimycin A affects a broad range of *src* family PTK including TcR/CD3-associated $p59^{fyn}$. It can therefore be concluded, that the acquisition of the locomotory capacity is a consequence of the same biochemical processes that ultimately result in the full activation of the lymphocytes.

12.2.2. Involvement of PTK in the maintenance of the polarized morphology by Jurkat cells

The same set of PTK inhibitors was applied to dbcAMP-cultured Jurkat cells prior to the treatment of these motile cells with αCD3. Only a pretreatment with herbimycin A, at 50 ng/ml for 30 min, resulted in a 50% inhibition of the αCD3-induced rounding up of Jurkat cells (figure 72). This result agrees with the results obtained with αCD3-cultured PBL as well as with the studies of PLC activation in Jurkat cells in response to the αCD3 activation (Graber *et al.*, 1992).

In the next series of experiments, herbimycin A was used in an attempt to disrupt the response of Jurkat cells to PMA and PHA. Both of these agents were found to induce rounding up of polarized Jurkat cells. In contrast to αCD3, whose effect is transient, PMA and PHA effect is permanent. Figure 73 shows the results obtained in a typical experiment. Rounding up of the cells in response to PMA was not affected at either 10^{-9}M or 10^{-8}M (figure 73-A) and PHA was equally effective in the presence and the absence of the PTK inhibitor (figure 73-C). Again, rounding up of cells in αCD3 was consistently eliminated at all doses, following the preincubation with herbimycin A (figure 73-B).

These results clearly show that both PMA and PHA bypass the PTK necessary for signal delivery through the TcR/CD3. Indeed, PMA binds to and directly activates the PKC, a signal element down-stream of PTK-regulated PLC. PHA binds to a variety of surface structures and thus has the potential to induce a number of signalling pathways. Clearly, PHA is able to bypass the PTK-dependent mechanisms and activate the pertinent biochemical processes in PTK-depleted Jurkat cells.

12.2.3. Effect of herbimycin A pretreatment on tyrosine phosphorylation of αCD3-stimulated Jurkat cells

The effect of herbimycin A on the αCD3-stimulated changes in

Figure 72: Effect of various PTK inhibitors on αCD3-induced morphology change of dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min in the presence of 50 ng/ml herbimycin A or 1 μg/ml of lavendustin A, RCAM-lysozyme, tyrphostin, genistein, methyl[2,5-diOH] cinnamate, or 2-(OH)-[2,5-diOH]benzyl] aminobenzoic acid. OKT3 was added to final concentration of 20 ng/ml and the incubation was continued for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast.

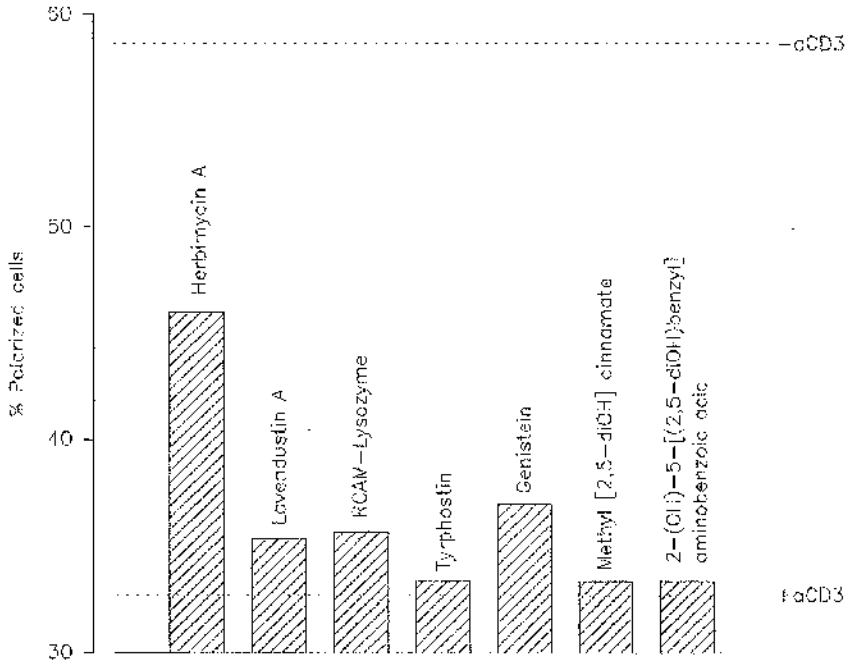
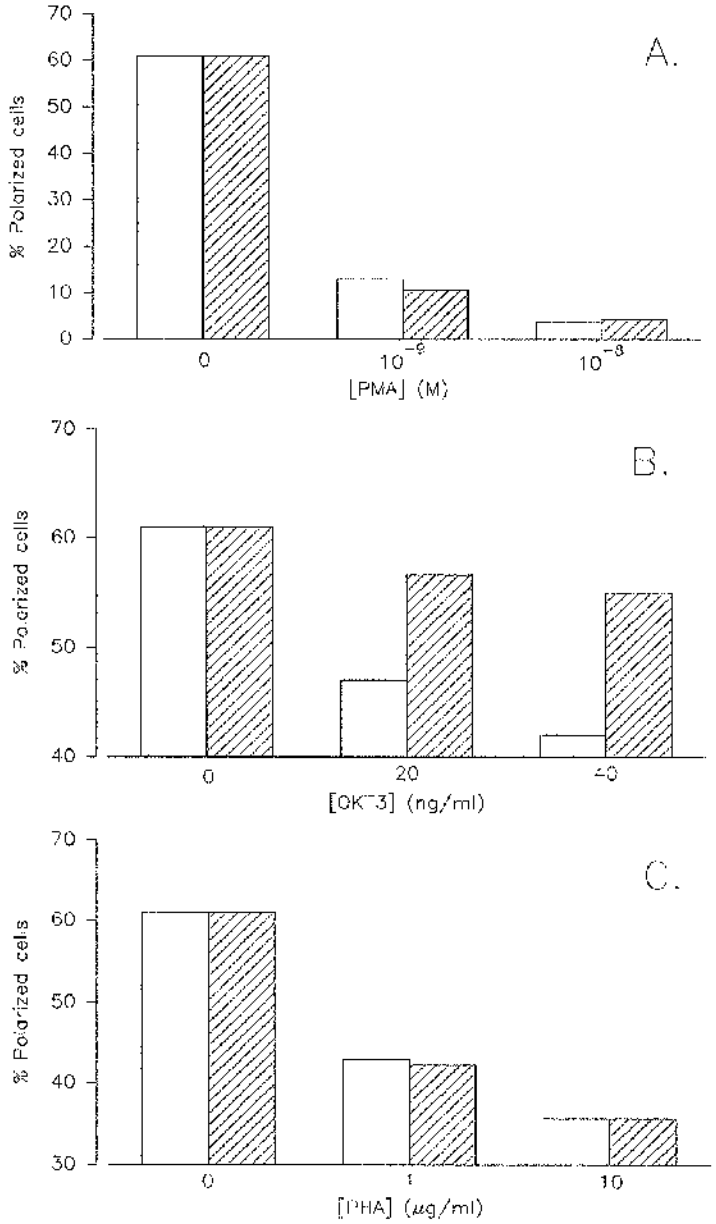


Figure 73: Effect of herbimycin A on (A) PMA-, (B) αCD3-and (C) PHA-induced morphology change of dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min in the presence of 50 ng/ml herbimycin A. Different concentrations of PMA, OKT3, or PHA were then added and the incubation was continued for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Polarized cells preincubated in the absence (open bar) and presence (hatched bar) of herbimycin A.



dbcAMP-cultured Jurkat cells was shown. The effect of this PTK inhibitor on the intracellular protein-tyrosine phosphorylation was examined using Western blotting of whole-cell protein extracts separated by SDS-PAGE (figure 74). Comparison of lane 1 and 3 reveals that preincubation for 30 min with 50 ng/ml herbimycin A (lane 3) does not affect the basal level of proteins containing phosphotyrosine residues. The activation with αCD3, of dbcAMP-cultured Jurkat cells repolarized for 30 min in buffer (lane 2), resulted in an increase of protein-tyrosine phosphorylation of several substrates detected as bands whose optical density increased when compared to the control (lane 1). In contrast, preincubation with herbimycin A in most cases disabled the ability of Jurkat cells to increase protein-tyrosine phosphorylation in response to αCD3 (lane 4). Thus, many bands remained unchanged when compared to the control (lane 3) and in some case even decreased in density (*i.e.* major band at about 115 kDa). Such observation suggests that specific phosphatases are also activated by αCD3 and for certain substrates may dominate after the equilibrium is disturbed by the PTK inhibitor.

12.2.4. Effect of pertussis toxin on repolarized dbcAMP-cultured Jurkat cells

The locomotory machinery of dbcAMP-cultured Jurkat cells was shown to be dose-dependently blocked by pertussis toxin (figure 75). The repolarization of these cells was noticeably reversed by a 10 ng/ml dose, where the decrease in polarized cells was reflected in a corresponding increase in the number of cells with an irregular outline. A dose of 100 ng/ml was necessary to bring about an increase in the round cells.

The sensitivity of the TcR/CD3 signal to pertussis toxin suggests the involvement of a G protein at some point in the signalling pathway. However, the connection of this G protein with the activation of PLCß is unclear. In addition, the location of the G protein with respect to TcR/CD3, PKC or Ca^{2+} mobilization could not be determined and will

Figure 74: Effect of Herbimycin A on tyrosine phosphorylation induced in dbcAMP-cultured Jurkat cells stimulated with αCD3. Jurkat cells were cultured for 3 days in the presence of 1 mM dbcAMP. Cells were then washed and preincubated with (lane 3 and 4) or without (lane 1 and 2) 50 ng/ml Herbimycin A for 30 min at 37°C. αCD3 was added to the final concentration of 10 ng/ml (lane 2 and 4) and the incubation was continued for 15 min. Finally, cells were washed with ice-cold TBS/vanadate and Western blots of whole cell extracts were performed. Bands containing phosphotyrosine residues were visualized using a specific HRPO-conjugated monoclonal antibody. Molecular weight markers of 97, 69, and 46 kDa are shown in the left margin.

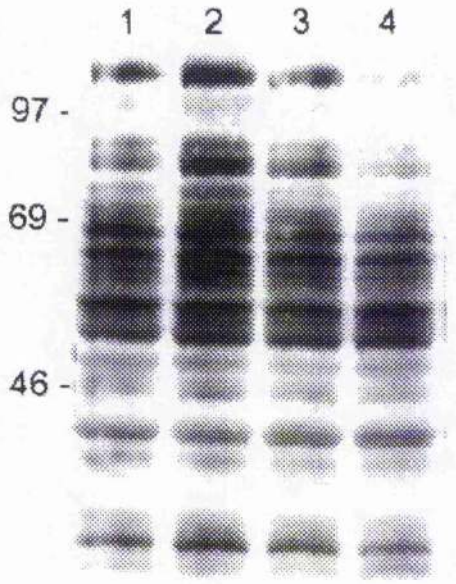
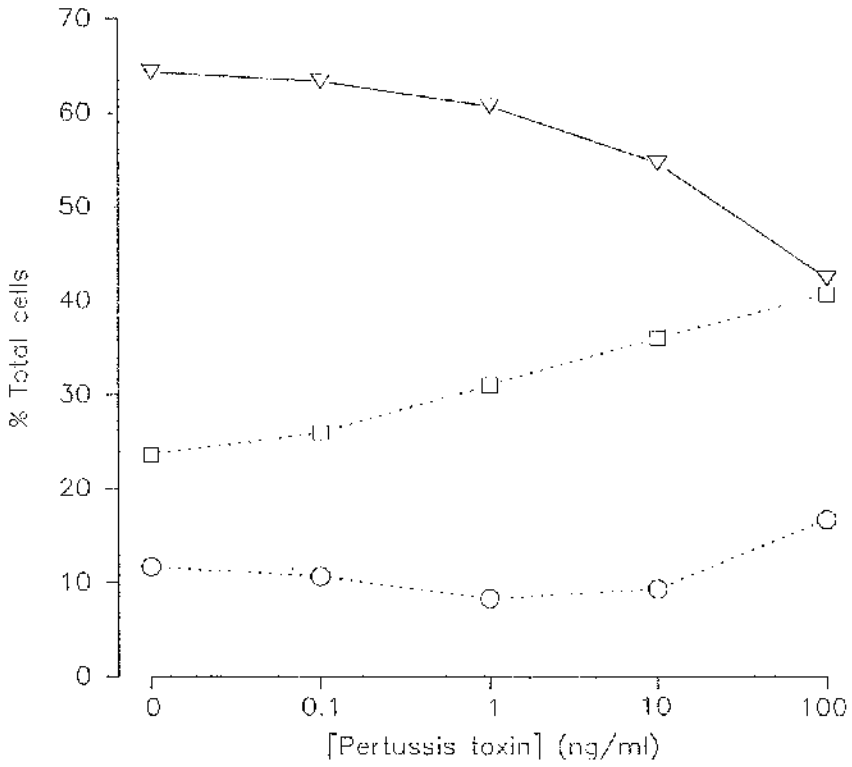


Figure 75: Dose-response of repolarized dbcAMP-cultured Jurkat cells to treatment with pertussis toxin. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min. Pertussis toxin was then added and the incubation was continued for 30 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Round cells (circle), irregular cells (square) and polarized cells (triangles).



require further study.

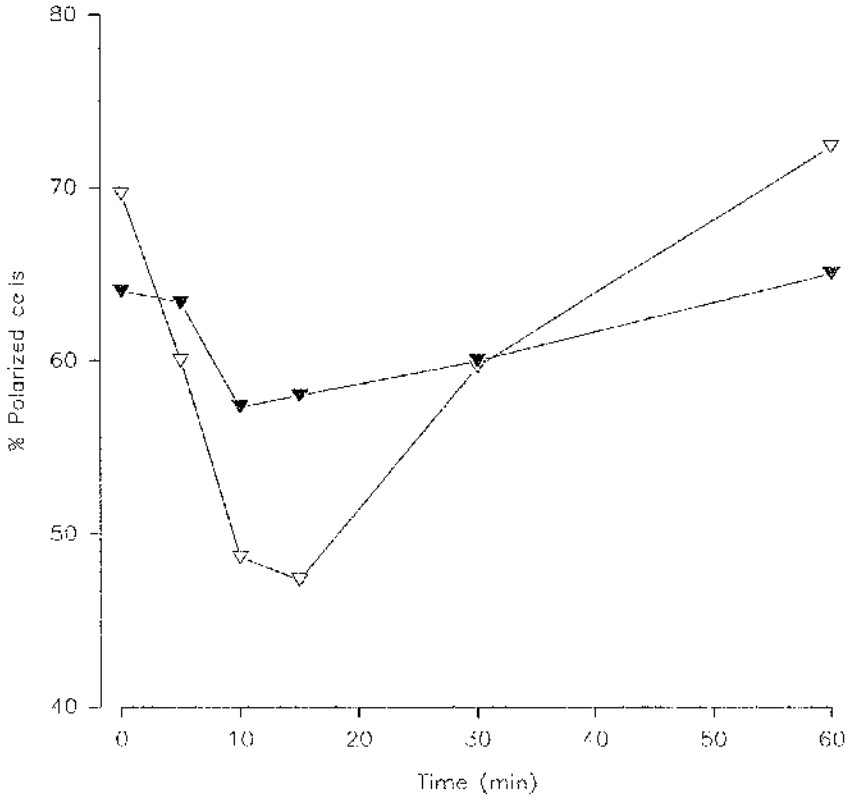
12.2.5. Effect of pertussis toxin on the TcR/CD3 signal

In an attempt to determine if a pertussis toxin-sensitive G protein couples the TcR/CD3 signal, the dbcAMP-cultured Jurkat cells were preincubated with 1 ng/ml dose of the toxin. At this dose, pertussis toxin was found to have a slight effect on the repolarization of the cells. A time-course of αCD3-stimulated shape change of the repolarized cells was then observed (figure 76). It can be seen that although the αCD3 effect was not suppressed, a significant inhibition was observed.

12.3. SUMMARY

In conclusion, the above experiments showed that incubation of PBMC with herbimycin A uncoupled pathways responsible for αCD3-mediated lymphocyte activation while genistein and tyrphostin affected the PPD-stimulated pathways. In line with these results, the preincubation of dbcAMP-cultured Jurkat cells with herbimycin A, but not with any other PTK inhibitors tested, uncoupled TcR/CD3 from signalling pathway which ultimately leads to the reversal of polarization. Several intracellular protein substrates for PTK were shown to be affected by the PTK inhibitor. The results further suggested that pertussis toxin-sensitive G protein is involved in the maintenance of the locomotory capacity in dbcAMP-cultured Jurkat cells. In addition, such G protein seems to be involved in the αCD3-stimulated pathway that ultimately leads to the reversal of cell polarization.

Figure 76: Effect of pertussis toxin on αCD3-induced morphology change of dbcAMP-cultured Jurkat cells. The cells were cultured with 1 mM dbcAMP for 3 days, washed twice with HBSS/MOPS and repolarized at 37°C for 30 min in the presence of 1 ng/ml pertussis toxin. OKT3 was added to final concentration of 20 ng/ml and the incubation was continued for 15 min. The cells were finally fixed with glutaraldehyde and the morphology was examined and quantified under phase contrast. Polarized cells preincubated in the absence (open triangles) or presence (full triangles) of pertussis toxin.



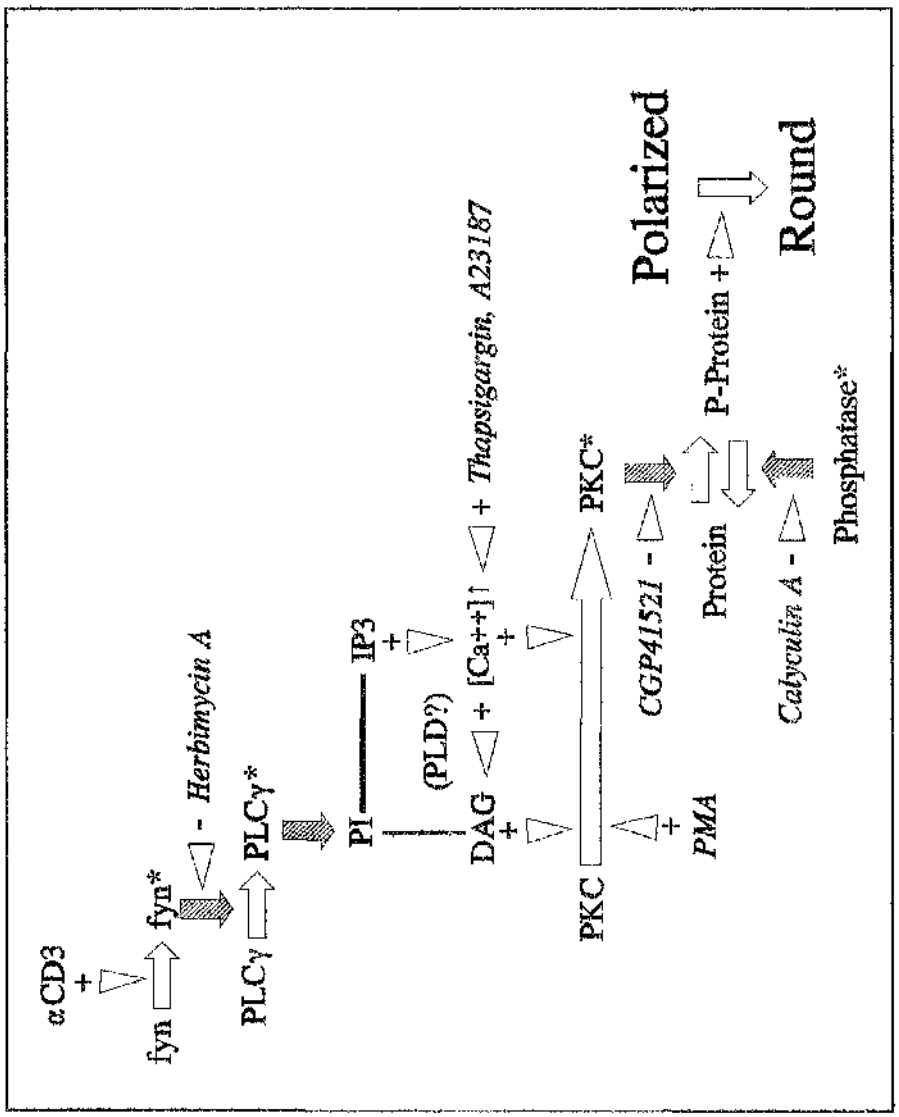
13. GENERAL DISCUSSION AND CONCLUSIONS

Resting lymphocytes display a round shape and are non-motile. Although the normal Jurkat cells are actively dividing, their shape resembles that of resting lymphocytes. Such a dividing Jurkat cell redirects most of its intracellular resources to the synthesis of new proteins and nucleic acids, ultimately resulting in a generation of a daughter cell. From a physiological point of view, the lack of motility is not surprising. The maintenance of a locomotory activity places demands on the intracellular energy resources and, in addition, engages intracellular machinery instrumental in the events of cell division. Indeed, the DNA-labelling experiments showed that the majority of Jurkat cells are in the S and G_2/M phase of the cell cycle. This indicates that the progress through G_0/G_1 phase of the Jurkat cell cycle is very rapid. In contrast, the majority of PBL are resting lymphocytes and by definition at G_0 phase. The acquisition of locomotory capacity in lymphocytes was shown to be associated with the transcription of new genes and active protein synthesis in lymphocytes transiting through G_1 phase (Wilkinson, 1986). To induce locomotion in G_0 lymphocytes, a progress into G_1 phase of the cell cycle must be initiated, usually by activation with antigen, mitogens or αCD3. Thus, the manipulation required to induce the locomotory capacity in the PBL and Jurkat cells is quite opposite. While a positive signal is required to remove the block which maintains PBL at G_0, a block must be applied in the Jurkat cell cycle which slows down or arrests their progress through G_1. A prolonged culture in the presence of dbcAMP was successfully used to retain the majority of the Jurkat cells in G_1. This was confirmed (1) by DNA-labelling experiments, (2) by lack of cell division in these cells and (3) by the decrease in cell size to one more resembling the size of resting lymphocytes. The microscopic observations revealed a concominant acquisition of locomotory capability in such dbcAMP-cultured Jurkat cells. It is not clear if the increase in cell motility is coincidental with the arrest

in G_1 phase or if the cell motility is a property of cells in the G_1 phase of cell cycle. Although far from definite proof, the data indicate that the first suggestion is most likely. There is no question that a low rate of cell division (presumably with the accumulation of cells in the G_0/G_1 phase) is required for the subsequent acquisition of locomotory capacity. The low rate of division was achieved either by a prolonged culture with a relatively high dose of dbcAMP (0.75 to 1 mM) or temporarily, using the lag phase Jurkat cells. The increase in the numbers of motile cells was self-evident at the high dbcAMP concentrations. However, in the second case, the relatively low concentration of dbcAMP (0.25 to 0.50 mM) was able to induce cell locomotion for the duration of the low-rate lag phase cell division. When the cells returned to near-log phase growth, these low dbcAMP doses were no longer able to induce cell motility. Thus, the cell growth seems to be affected by culture in the presence of high doses of dbcAMP, while locomotory capability can be induced through a separate mechanism by a lower dose of dbcAMP in cells present in the G_1 phase of cell cycle.

The regulation of locomotory machinery was shown to involve protein phosphorylation catalyzed by at least one of the PMA-sensitive PKC isoforms. The evidence was accumulated in experiments where (1) PKC was specifically activated by PMA, (2) PKC was depleted from the cells by prolonged incubation with PMA, (3) the PKC activity was inhibited by agents relatively specific for PKC, and (4) protein phosphorylation was altered by inhibition of protein phosphatase. The Western blot analysis has confirmed four such PKC isoform candidates: PKCα, PKCγ, PKCϵ, or PKCΘ. The results consistently indicated that an increase in PKC-catalyzed protein phosphorylation leads to an inhibition of locomotory capacity. Thus, the scheme depicted in figure 77 can is suggested for a mechanism that regulates the motility of dbcAMP-cultured Jurkat cells. In this scheme, 'Protein' and 'Protein-P' are dephosphorylated and phosphorylated forms of the putative regulatory protein involved in the mechanism responsible for the change in cell

Figure 77: Proposed signalling mechanisms involved in the regulation of morphological changes associated with a locomotory response in T cell line Jurkat. Italics indicate regents used to activate or inhibit various stages of the pathway. Asterisks (*) indicate activated species of various biochemically active molecules. Triangles signify an effect, positive or negative if preceded by a '+' or a '-' respectively. Hatched arrows show reaction where an enzymatic activity of an activated species is utilized. Blank arrows indicate conversion brought about by intracellular biochemical or biological events.



morphology. The equilibrium in dbcAMP-cultured Jurkat cells is biased so that the regulatory protein is constitutively dephosphorylated. By pharmacological intervention, this bias can be changed so that the round cell morphology is induced. This situation is analogous in fresh resting PBL (Prof. P.C. Wilkinson, personal communication). In these cells, the equilibrium is biased towards the phosphorylated form of the regulatory protein resulting in a round morphology. By interfering with the phosphorylation process using one of the specific PKC inhibitors, a majority of the cells are induced to polarize within 30 min. The involvement of phosphatase in this process was ascertained when the preincubation of the fresh PBL with calyculin A eliminated the polarizing effect of the PKC inhibitors.

In support of the scheme described above, Thorp *et al.* (1994) showed that a specific PKC inhibitor GF109203X caused, within an hour, an increase in shape-changing in freshly isolated PBL. Similarly, Zimmermann and Keller (1992) showed that another specific PKC inhibitor, CGP41251, caused a dose-dependent increase in the polarization of Walker carcinoma cells. In addition, Keller *et al.* (1990) showed that neutrophils can be stimulated to change shape upon addition of H-7. In contrast, less specific PKC inhibitors such as staurosporine and H-7 were found to inhibit motility not only in a spontaneously shape-changing variant of U937 monocytoid cells and αCD3-activated PBL (Thorp *et al.*, 1994) but also in spontaneously and colchicine-stimulated Walker carcinoma cells (Zimmermann and Keller, 1992). Surprisingly, GF109203X also inhibited motility in U937 cells (Thorp *et al.*, 1994). A presence of multiple PKC isotypes involved in the induction and in the maintenance of the locomotory morphology was suggested to explain the contradicting experimental results (Thorp *et al.*, 1994). Perhaps these contradicting results reflect the differences in susceptibility of these isoenzymes to the various PKC inhibitors. Indeed, the differential tissue expression, and differences in activation requirements of PKC isoforms are very likely building blocks for numerous complex regulatory systems (Kikkawa *et al.*, 1989). In addition

to the mentioned classical PMA-sensitive PKC isoforms, PMA-insensitive isoforms PKCι, PKCλ, PKCμ, and PKCζ were detected. Their involvement in the acquisition and maintenance of lymphocyte locomotion, downstream of the classical PKC, is conceivable.

Surprising but consistent observations revealed that the increase in $[Ca^{2+}]_i$ also results in loss of locomotory morphology. This evidence was obtained in experiments where $[Ca^{2+}]_i$ was elevated (1) directly using a Ca^{2+} ionophore A23187, (2) via a TcR/CD3-medaited intracellular Ca^{2+} release, and (3) directly using thapsigargin to pharmacologically induce the release of intracellular Ca^{2+} stores. The presence of extracellular Ca^{2+} is vital for locomotion inhibition. Thus, the role of an intracellular Ca^{2+} release serves to trigger a massive influx of extracellular Ca^{2+}. The large increase in $[Ca^{2+}]_i$ then leads to the changes in the cell morphology.

The inhibition or 'rounding up' caused by the activation of PKC and by the elevation of $[Ca^{2+}]_i$ were found to use the same intracellular mechanism, namely increase in phosphorylation of regulatory proteins. Indeed, the preincubation of Jurkat cells with PKC inhibitors dose-dependently decreased the effect of $[Ca^{2+}]_i$ induced by the agents mentioned above. In addition, the depletion of PKC virtually disabled the TcR/CD3-mediated locomotion inhibition. How is Ca^{2+} capable of affecting the phosphorylation of intracellular proteins?

Mammalian cells express several Ca^{2+}/calmodulin-dependent protein kinases (Stull *et al.*, 1986). Receptor-mediated increases in $[Ca^{2+}]_i$ were suggested to activate protein kinases and lead to the appearance of new phosphoproteins (Tsien *et al.*, 1982b). Indeed, a 5 min treatment of Jurkat cells with ionomycin or αTcR resulted in an appearance of the same set of two cytoplasmic acidic phosphoproteins suggesting that these reagents activate the same protein kinases (Imboden *et al.*, 1985). Ionomycin treatment also caused increased phosphorylation in human peripheral blood T cells (Chaplin *et al.*, 1980), including phosphorylation of the CD3γ, CD4 and CD8 in quiescent blood T cells (Cantrell *et al.*, 1989; Breitmeyer *et al.*, 1987). Unlike PMA, which induced phosphorylation of

serine 126 of CD3γ, ionomycin induced phosphorylation of serine 123 and 126 (Cantrell *et al.*, 1989). Thus, the relative similarity and overlap in the phosphorylation patterns of Ca^{2+}/calmodulin-dependent protein kinases and PKC could result in similar physiological changes in the cells. However, the relative specificity of the PKC inhibitors used, as well as that of PMA-mediated PKC depletion, provide evidence contradicting the involvement of a Ca^{2+}/calmodulin-dependent protein kinase. Thus, some PKC isoform must be directly or indirectly activated by an elevated $[Ca^{2+}]_i$.

The activation of PKC by increasing $[Ca^{2+}]_i$ has been documented. Although ionomycin alone, at a concentration of 200-300 nM, does not cause PKC translocation to the T cell membrane (Isakov and Altman, 1987), increasing the Ca^{2+} concentration from 100 nM to 500 nM was shown to promote the association of PKC with membranes (Wolff *et al.*, 1985). Such a Ca^{2+}-mediated binding is reversible and occurs within the range of receptor-mediated $[Ca^{2+}]_i$ increases but below the 5 to 50 μM required for Ca^{2+} to activate PKC in the absence of DAG. An alternative mechanism may activate PKC, whereby a $[Ca^{2+}]_i$ increase to 1-10 μM range enables calpain to cleave the native PKC thus liberating the active kinase domain (Kishimoto *et al.*, 1983). Indeed, several PKC substrates have been shown to be phosphorylated in response to Ca^{2+} ionophore alone (Chatila *et al.*, 1989). However, the quantitative measurements of the increases in $[Ca^{2+}]_i$ indicated that such high concentrations were never attained with any of the activators used; the peak concentrations detected were about 500 nM, 300 nM, and 600 nM for αCD3, PMA and thapsigargin respectively. Thus, there must be a mechanism whereby an increase in the $[Ca^{2+}]_i$ results in the generation of PKC activators such as DAG, free fatty acid and lysophospholipids.

The likely link between $[Ca^{2+}]_i$ increases and the activation of PMA-sensitive PKC is therefore formed by a Ca^{2+}-sensitive regulatory component. Upon its activation, a second messenger is produced which in turn activates the PKC; additional complexities involved in the activation of PKC have been described (Asaoka *et al.*, 1992; Nishizuka, 1992). An

alternative source of DAG, the PKC coactivator, is PCh. PCh is hydrolyzed by activated PLD, resulting in the production of phosphatidic acid which is then converted to DAG by the removal of its phosphate. Mammalian PLD is associated mainly with the particulate fraction and its kinetic properties vary greatly from tissue to tissue. Rat brain PLD shows a considerable activity at submicromollar Ca^{2+} concentrations. PKC activity stimulated by PCh-derived DAG may be greatly enhanced by *cis*-unsaturated fatty acids, including oleic, linoleic, linolenic, arachidonic and docosahexaenoic acids. Indeed, PLA_2 hydrolyzes phospholipids to liberate such free fatty acids together with lysophospholipids. Arachidonic acid-selective and -non-selective cytosolic PLA_2 are both present within cells. The enzymes are active in the presence of Ca^{2+} at concentration below 1 μM. They appear to translocate to membrane in response to agonists that mobilize intracellular Ca^{2+}. Thus it is conceivable that a rise in $[Ca^{2+}]_i$ may induce an increase in the activity of certain PKC isoforms.

The signal coupling of TcR/CD3 to the intracellular machinery that affects Jurkat cell motility was also briefly examined. The involvement of PLC in the regulation of motility in dbcAMP-cultured Jurkat cells is very likely. Antigen receptors on B cells and T cells associate with PLC and upon stimulation induce generation of Ca^{2+} and DAG as second messengers; the increase of $[Ca^{2+}]_i$ and the activation of PKC were both found to play a role in the regulation of Jurkat cell motility. The involvement of G proteins in the signalling pathways of neutrophils and mast cells leading to PLC activation has been accepted (Harnett and Klaus, 1988). Mechanisms coupling these surface receptors with PLC in B and T cells have been discussed by Harnett and Rigley (1992). In B cells it has been shown that surface IgM and IgD receptors are coupled to the PLCß by pertussis toxin-sensitive forms of G_p. Although in T cells, a membrane-bound PLC isoform has been shown to be under G protein control, such a G protein-coupled PLC has not been found associated with TcR.

The activation of PLCγ1 in Jurkat cells, stimaulated through TcR/CD3, involves tyrosine phosphorylation by nonreceptor PTK $p56^{lck}$

amd $p59^{fyn}$ (Rhee and Choi, 1992). PTK inhibition data reported in this document indicate that Jurkat cell rounding up that was induced by αCD3, but not by PHA or PMA, is coupled by a non-*lck* PTK. Western blot analysis confirmed that 30-min preincubation with herbimycin A resulted in a significant decrease in tyrosine phosphorylation of numerous intracellular substrates; PTK $p59^{fyn}$ known to be associated with the CD3 molecule, and PLCγ are possible candidates. However, the identification of these substrates would require further investigation using methods such as immunoprecipitation with specific monoclonal antibodies.

Results presented in this document have further suggested an involvement of a pertussis toxin-sensitive G protein coupling the pathway involved in the maintenance of motility of the dbcAMP-cultured Jurkat cells. Interestingly, the TcR/CD3-mediated signal was also significantly inhibited in pertussis toxin-pretreated Jurkat cells. This latter effect was observed at pertussis toxin concentrations 10- to 100-fold lower than those necessary to partially reverse the Jurkat cell polarization on its own. This difference in pertussis toxin sensitivity may indicate the presence of multiple G proteins downstream of PLCγ.

The presence of both the pertussis toxin-sensitive G protein and of a non-receptor PTK in a single regulatory pathway is interesting. Harnett and Rigley (1992) suggested the possibility of coupling by a nonclassical/PTK-activated G protein. Two members of the *ras* superfamily of small G proteins, Rho and Rac, were suggested to be involved in various aspects of cytoskeleton regulation. These include the control of focal adhesion, stress fibers, and of actin components which are involved in membrane ruffling (Chavrier *et al.*, 1993). Clearly, the components of the signal transduction pathway responsible for the regulation of motility in the Jurkat cells and in lymphocytes in general need to be identified and further characterized.

The existence of a regulatory protein, reversibly phosphorylated as a means in regulation of cell motility, reflects the reversible character of this physiological behaviour in leukocytes. The components of cellular

locomotory machinery and their regulation have been recently reviewed (Stossel, 1993). Indeed, the binding of Ca^{2+}-calmodulin to MARCKS or the phosphorylation of MARCKS by PKC suggest a possible mechanism for the control of locomotion. In addition, many of the proteins in focal contacts are substrates for protein kinases, have intrinsic protein kinase activity, or both. It is likely that phosphorylation reactions govern binding activities of these proteins to each other and to actin. The identification of the regulatory proteins is in progress. ^{32}P-labelling has shown that the PMA-induced switch from motile to non-motile MOLT-4 is associated with increased phosphorylation of a number of proteins separated by SDS-PAGE (Southern *et al.*, 1995). However, the identification and localization of these proteins will require further study.

14. ABBREVIATIONS

αCD3	monoclonal antibody specific for CD3
αIg	monoclonal antibody specific for immunoglobulin
αTcR	monoclonal antibody specific for T cell receptor chain
AC	accessory or antigen presenting cell(s)
ATP	adenosine trisphosphate
°C	degrees Celsius
Ca^{2+}	calcium ion
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
cAMP	adenosine-3',5'-cyclic monophosphate
$[cAMP]_i$	intracellular cAMP concentration
cAMPS	adenosine-3',5'-cyclic monophosphothioate (Rp-isomer)
CD	cluster of differentiation
Con A	concanavalin A
CTL	cytotoxic T lymphocytes
DAG	diacylglycerol
dbcAMP	dibutyryl cAMP
dH_2O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxy nucleic acid
ED_{50}	50% effective dose
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol-bis(ß-aminoethylether)N,N,N',N', tetraacetic acid
FACS	fluorescence-activated cell sorting
Fc	crystallizable fragment of immunoglobulin
FCS	foetal calf serum
FSC	forward scatter measured by FACS instrimentation
G_1 phase	first gap phase of cell cycle
G_2 phase	second gap phase of cell cycle
G_α	alpha subunit of GTP-binding protein

G_β	beta subunit of GTP-binding protein
G_γ	gamma subunit of GTP-binding protein
G_i	inhibitory GTP-binding protein
G_s	stimulatory GTP-binding protein
GTP	guanosine trisphosphate
HBSS	Hanks' balanced salt solution
HEV	high endothelial venules
hr(s)	hour(s)
hrIL-8	human recombinant IL-8
HSA	human serum albumin
IBMX	isobutylxanthine (phosphodiesterase inhibitor)
IFN-γ	interferon gamma
IgG	immunoglobulin gamma
IL-1	interleukin 1
IL-2	interleukin 2
IL-2r(ß)	IL-2 receptor (beta chain)
IL-4	interleukin 4
IL-8	interleukin 8
IP	inositol phosphate
IP_2	inositol bisphospahte
IP_3	inositol trisphosphate
l	litre(s)
M	molar
Mg^{2+}	magnesium ion
mg	milligram(s)
μg	microgram(s)
min	minute(s)
ml	milliliter(s)
μl	microliter(s)
mM	millimolar
μM	micromolar
M phase	mitosis phase of cell cycle

MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein 1α
MLC	mixed lymphocyte culture
mRNA	messenger RNA
NAP-1	neutrophil activating protein 1
ng	nanogram
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffred saline
PCh	phosphatidyl choline
PCV	postcapillary venule(s)
PDB	phorbol 12,13 dibutyrate
PE	phycoerythrin
PGE_2	prostaglandin E_2
PHA	phytohaemagglutinin
PI	phosphatidylinositol
PI(4)P	phosphatidylinositol 4-phosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein serine/threonine kinase
PKC	Ca^{2+}-dependent protein serine/threonine kinase
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PPD	purified protein derivative of *Mycobacterium tuberculosis*
PTK	protein tyrosine kinase
S phase	synthetic phase of cell cycle
sec	second(s)
SSC	side scatter measured by FACS instrumentation
Seph-	Sepharose bound-
RNA	ribonucleic acid
TBS	Tris-buffered saline

TcR	T cell antigen receptor
Tγ	suppressor T lymphocyte
Tμ	helper T lymphocyte
W-7	N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide

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