Investigation of the signal transduction pathways involved in the induction of T-lymphocyte motility

Thesis

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INVESTIGATION OF THE SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN THE INDUCTION OF T-LYMPHOCYTE MOTILITY

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September 1996
"This is as strange a maze as e'er men trod,
And there is in this business more than nature
Was ever conduct of"

William Shakespeare - The Tempest, Act V. Scene 1
ABSTRACT

Induction of lymphocyte motility is an essential early step in extravasation of lymphocytes into inflammatory sites and also into lymphoid tissues in the process of lymphocyte recirculation. Lymphocyte motility requires a change from a spherical morphology to a constantly changing irregular shape. In this study, a variety of agents have been investigated for induction of this shape changing morphology in freshly isolated human peripheral blood T-lymphocytes (PBTLs) and a non-motile variant of the MOLT-4 human lymphoid cell line. The MOLT-4 cells proved to be non-responsive to most of the agents tested, however, 5 agents were found to cause significant polarisation in PBTLs. IL-2, IL-15, fetal calf serum (FCS) and nocadazole induce shape change in 20-40% of PBTLs. However, the most potent inducer of shape change found were the PKC inhibitors of the bisindolylmaleimide (Bis) type, which show effects on over 60% of PBTLs, as reported recently\(^{(131)}\). Do these diverse inducers of shape changing in PBTLs act by a common signal transduction pathway? With IL-2, IL-15, FCS, nocadazole and Bis., no common changes in intracellular calcium flux, intracellular pH, inositol triphosphate levels, renaturable kinase activity and tyrosine phosphorylation have been found. So if a final common signal transduction pathway exists, it must involve other second messenger systems.

However, a number of pharmacological agents were found to prevent the induction of shape change in PBTLs, indicating that they could be targeting a common second messenger element involved in motility signal transduction. Comparisons of their chemical structures revealed no common structural motifs that would explain their common effects on lymphocyte motility.
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Abbreviations

ATP: adenosine tris-phosphate

Bis.: bisindolylmaleimide

BSA: bovine serum albumin

BSS: balanced salt solution

cAMP: cyclic adenosine-3',5'-monophosphate

DAG: diacylglycerol

DMSO: dimethyl sulphoxide

DTT: dithiothreitol

EDTA: ethylenediamine tetracetic acid

EGF: epidermal growth factor

EGTA: ethyleneglycol-bis-(b-aminoethylether) N,N,N',N' tetra-acetic acid

FCS: fetal calf serum

fMLP: N-formyl-methionyl-leucyl-phenylalanine

GAP: GTPase activating protein

GDI: GDP dissociation inhibitor

GDP: guanosine 5'-diphosphate

GEF: guanine nucleotide exchange factor

HSA: human serum albumin

IP-10: inducible protein -10

MCP-1: monocyte chemotactic protein

MIP-1α: macrophage inflammatory protein

MIP-1β: " " "

MGSA: melanoma growth stimulating activity

Na-EDTA: ethylenediamine tetracetic acid (disodium salt)
NZ: nocadazole
PBS: phosphate buffered saline
PBTLs: peripheral blood T-lymphocytes
PDGF: platelet derived growth factor
PI: phosphatidylinositol
PI3K: phosphatidylinositol 3-kinase
PIP$_2$: phosphatidylinositol bis-phosphate
PKC: protein kinase C
PMSF: phenyl-methyl-sulphonyl-fluoride
RANTES: regulated on activation, normal T cell expressed and secreted
SDS-PAGE: sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TEMED: N,N,N',N-tetra-methylethylenediamine
TNF-α: tumour necrosis factor
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1. Introduction
The circulatory and migratory properties of white blood cells have evolved to allow efficient surveillance of tissues for infectious pathogens and rapid accumulation at sites of injury and infection. Lymphocytes exhibit complex migration pathways in the body. Resting blood lymphocytes which are predominantly non-motile recirculate selectively through specific lymphoid tissues; activated lymphocytes migrate selectively into inflammatory sites. Lymphocyte adhesion and extravasation appear to constitute a multistep phenomena (figure 1.1), in which the initial (stage 1), relatively low-affinity binding event ("rolling") is mediated by the selectin family of adhesion molecules\(^{1,2}\). Chemotactically activated lymphocytes, (stage 2) then induce a higher avidity binding, (stage 3) that is mediated by the lymphocyte integrins and their cognate endothelial ligands, the immunoglobulin superfamily glycoproteins including the intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecule-1 (VCAM-1)\(^{3}\).

Adherent cells then transmigrate, (stage 4) through the endothelium. However, adhesion alone cannot be sufficient to bring about the transendothelial migration of lymphocytes, a process which entails active movement of cells and shape change.

The specificity of lymphocyte migration is now beginning to be understood at the level of endothelial adhesion but the details of what causes the lymphocyte to subsequently become motile and traverse the endothelium are still shrouded in mystery. It is this latter crucial step in the multi-step procedure of lymphocyte extravasation that I shall especially concentrate on and the evidence so far accumulated on the signal transduction processes involved that link the extracellular signals originating at the plasma membrane
Figure 1.1: The four step model of lymphocyte transendothelial migration.

The four step model of lymphocyte transendothelial migration.

1. Tethering/attachment
2. Activation
3. Arrest and adhesion strengthening
4. Transendothelial migration
to the organisation of the cytoskeletal network which is involved in influencing cell shape and thus motility.

1.1: Background

In 1875 Ranvier was the first to suggest that lymphoid cells were motile\(^{216}\). This concept received enthusiastic support by others, however, objections were raised by several research workers, including P. Ehrlich, according to whom the lymphocyte had too little protoplasm to push the voluminous nucleus along\(^{217,218}\).

In 1921 Lewis and Webster and in 1923 Sabin clearly demonstrated lymphocyte motility with intermittent stops and starts and the presence of a trailing cytoplasmic tail\(^{219,220}\). The classic description of the morphology of moving lymphocytes was presented by Lewis in 1931\(^{221}\). Lewis described the motile lymphocyte as a polarised asymmetric cell in a configuration resembling that of a hand mirror with a thin advancing pseudopod, a rounded area enclosing the anteriorly placed nucleus and a trailing tail of cytoplasm. McFarland extended the studies of the cytoplasmic tail of the amoeboid lymphocyte and termed this extension of cytoplasm, the uropod\(^{222,223}\). The uropod was demonstrated to be a part of the cell not only associated with cell movement but with a variety of lymphocyte interactions with the environment, including other cells. The uropod is covered with microvilli and contains the golgi apparatus, mitochondria, rough endoplasmic reticulum, centrioles, microfilaments and microtubules\(^{224,225}\). In contrast to the uropod, the advancing edge of the lymphocyte, which was described by the early investigators to be the most substrate adherent area of the cell, is generally devoid of major cytoplasmic organelles.
The regulation of lymphocyte motility involves multiple control mechanisms, some of which are only partly understood at present. Within the organism, lymphocytes must move actively to specific sites in lymphoid and non-lymphoid tissues. This most likely requires that lymphocyte motility is switched on and off and guided in a precise way.

For a relatively long time, leukocyte motility and translocation, particularly chemo- and haptotaxis, were studied preferentially using granulocytes and monocytes\(^{226,227}\). This was despite the obvious fact that motility must be important for the function of the lymphocytes, both as recirculating cells and in immune responses. Thus, an abundance of information was accrued on granulocyte migration and chemotaxis, whereas relatively little was known about the corresponding events in lymphocytes.

A major reason why lymphocyte motility, migration, chemo- and haptotaxis did not receive major attention until "later" was poor lymphocyte \textit{in vitro} migration assays and the non-adhesiveness of lymphoid cells for noncellular substrates. Thus, lymphocytes maintain their spherical suspension shape and show poor motile behaviour under conditions in which fibroblasts and macrophages adhere and spread their cytoplasm over substrates. Lymphocytes can exhibit motile forms in suspension without anchorage to cellular and non-cellular substrates. Lymphocyte motility is often determined \textit{in vitro} when the cells are nonadherent using the number of polarised cells as a measure of motility\(^{129}\). However, it must be emphasised that the extension of pseudopods and migration of cells are separable because pseudopod formation is not always followed by migration\(^{228,229}\).
In conclusion, T lymphocyte motility depends on the capacity of the cells to form active cell edges, the binding capacity of lymphocyte adhesion receptors, the availability of adhesion ligands, and the capacity of the cells to de-adhere and perform repeated cycles of motile events leading to translocation.

1.2: Lymphocyte - endothelial recognition.

To enter the various lymphoid tissues involved in recirculation, blood lymphocytes have to cross the endothelial vascular lining (except in the spleen, where small penicillar arterioles end in the parenchyma, thus allowing unhindered access of blood leukocytes). The process of lymphocyte extravasation in lymphoid tissues occurs at specialised post-capillary vascular sites called high-endothelial venules (HEVs). Our understanding of the mechanisms of this selectivity has been advanced by the discovery that naive and memory lymphocytes prefer different recirculation pathways. When naive lymphocytes encounter antigen, those lymphocytes with receptors specific for the antigen are stimulated to expand clonally and are converted to memory lymphocytes that have altered expression of adhesion receptors and circulatory patterns. For peripheral tissues and lymph nodes, memory lymphocytes emigrate preferentially through tissue endothelium, whereas naive lymphocytes enter the lymph node through HEVs.

Recent research has started to identify the molecules involved in adhesion of lymphocytes to HEVs and endothelium (figure 1.2). L-selectin on the surface of the lymphocytes has been found to have various ligands, depending on the site in the body and recognises a carbohydrate epitope on several biochemically distinct molecules synthesised by HEVs. These include the proteins GlyCAM-1 and...
Figure 1.2: Adhesion molecules involved in lymphocyte-endothelial interactions.
CD34\(^{(7)}\). In mucosal lymphoid tissue, L-selectin (along with \(\alpha_4\)-integrins) binds to MAdCAM-1\(^{(8,20)}\) and it has been shown in vitro that glycolipids can interact with selectins in physiological flow conditions, thus contributing to rolling adhesions\(^{(231)}\). Recently it has been shown that \(\alpha_4\)-integrins can mediate both rolling and adhesion in the multistep recruitment of peripheral blood mononuclear cells in vivo and these interactions occur independently of the selectins and \(\beta_2\) integrins\(^{(308)}\). L-selectin is shed following T-cell activation\(^{(31)}\) and this may occur during interaction with endothelial cells to allow the T cells to migrate. Recent work has shown that the cytoplasmic domain of L-selectin interacts directly with the cytoplasmic actin binding protein \(\alpha\) actinin and forms a complex with vinculin and talin\(^{(232)}\). The HEV ligands for L-selectin and other putative homing receptors have been referred to as 'vascular addressins', signifying their role in mediating the tissue specific adhesion of lymphocytes expressing the appropriate homing receptors. The ligands for E-selectin (E-selectin is present on endothelia), include sialyl lewis X (sleX), which is present on neutrophils and macrophages, and there is a similar if not identical carbohydrate on a subset of memory T cells\(^{(32-35)}\). It has been found that mice with null mutations in both endothelial selectins (P and E) develop a phenotype of leukocyte adhesion deficiency\(^{(304)}\), thus providing strong evidence for the functional importance of selectins in vivo. Indeed, there is now direct evidence for the presence of distinct E- and P-selectin ligands on T-lymphocytes and it has been suggested that \(\gamma/\delta\) T cells may be preferentially recruited to inflammatory sites during the early stages of an immune response when P-selectin is upregulated\(^{(307)}\).
The integrin LFA-1 on blood lymphocytes requires activation for binding to its counterstructures ICAM-1 and ICAM-2, which are expressed on HEVs and endothelial cells\(^\text{(9,164)}\). Binding of L-selectin does not trigger activation of LFA-1, since lymphocytes attach and roll in flow on purified peripheral node addressin identically, whether or not purified ICAM-1 is present on the substrate. An additional stimulus is required before they will arrest and strengthen adhesion through LFA-1\(^\text{(3)}\). Indeed, recent work has shown that chemoattractant stimulation of neutrophils and lymphocytes, rolling on immobilised peripheral node addressin (PNAd) and ICAM-1 results in rapid arrest and firm sticking \textit{in vitro}\(^{\text{(233)}}\).

G protein-coupled receptors are required for lymphocyte recirculation and are likely to provide the signals required to activate the adhesiveness of LFA-1. In relation to this, some recent work has shown that transfecting fMLP receptors into lymphocytes and subsequently stimulating the cells with fMLP triggers rapid adhesion via VLA-4 and shape change, which is pertussis toxin sensitive\(^\text{(36)}\). Pertussis toxin profoundly depresses lymphocyte recirculation via the lymphatics, which suggests that G-protein coupled receptors of the class \(\alpha_i\) are required for lymphocyte emigration through the HEV\(^\text{(10)}\). This is seen in the condition known as ‘whooping cough’, whereby the infectious bacterium (Bordetella), secretes copious amounts of pertussis toxin into the blood system. One of the effects of this is the subsequent rise in the number of lymphocytes in the blood stream due to their inability to traverse the endothelia. Despite the lack of emigration, pertussis toxin treated lymphocytes bind normally to lymph node HEVs \textit{in vitro}. These findings provided the basis for an early proposal for a two step model in which G protein coupled receptors function subsequent to binding of lymphocytes to
This theory has also been alluded to in more recent in vitro work, which implies that cultured HEVs may stimulate lymphocyte motility by two mechanisms: one which is rapid and pertussis toxin sensitive and one which is slower, pertussis toxin insensitive and dependent on lymphocyte adhesion to the HEVs.

Investigations into lymphocyte-endothelial interactions has shown that functionally significant lymphocyte cell surface molecules (CD2, CD44, L-selectin and LFA-1) exist as organised complexes in the cell membrane. Redistributions and associations between them are triggered selectively by lymphocyte-endothelial cell contact. The enormous amount of research in this particular field has ultimately led to the discovery of other important molecules involved in lymphocyte-endothelial adhesion. It is now becoming clear that the interaction between VLA-4 on the lymphocytes and VCAM-1 is important in both constitutive migration of lymphocytes into lymphoid organs and also in immune mediated inflammation. Another important molecule currently being investigated is CD31. It has been suggested that in T cells, homophilic CD31 adhesion may be primarily involved in transmigration of naive T cells and that its role is complementary to that of ICAM-1. More evidence for the importance of CD31 was shown in a recent paper which suggests that it has an important role in the extravasation of natural killer (NK) T cells into tissues for constitutive surveillance and into sites of inflammation. Cross linking of CD31 molecules induces cytoskeletal rearrangement in human NK cells and this phenomenon is Mg\(^{2+}\), but not Ca\(^{2+}\) dependent, suggesting the involvement of an integrin. Also both cell spreading and cytoskeletal rearrangement, as well as CD31-mediated adhesion appears to be regulated by the intracellular cAMP content.
Another important adhesion molecule on T cells is cutaneous leukocyte-associated antigen (CLA)\(^{(29)}\), although theories for its role have yet to become conclusive.

CD44 is a glycoprotein which is found on the surface of most leukocytes including lymphocytes and has recently been found to be not necessary for normal lymphocyte circulation. However it is required for extravasation into an inflammatory site involving non-lymphoid tissue\(^{(30)}\). In addition, recent evidence has demonstrated that CD44 and its alternatively spliced isoforms (CD44R) endow some tumour cells with enhanced metastatic ability\(^{(290-292)}\). Recent work has shown that \textit{in vitro} there is a rolling interaction between lymphoid cells and endothelial cells that is not selectin mediated but is in fact mediated by CD44\(^{(305)}\).

Antigen injected into the tissue of sensitized individuals induces localised accumulation of lymphocytes. These lymphocytes (and those accumulating in tissues in autoimmune diseases) are almost all memory cells\(^{(13)}\). The phenotype of these cells is quite similar to that of lymphocytes trafficking through these sites under basal conditions, suggesting that the same molecular mechanisms that mediate basal trafficking may be up regulated in inflammation. Accumulation of lymphocytes induced by specific antigen or by injection of interferon \(\gamma\) or TNF\(\alpha\) is significantly inhibited by monoclonal antibodies (Mabs), to either the LFA-1\(\alpha\) or the integrin \(\alpha 4\) subunit\(^{(14,15)}\) and almost completely by a combination of Mabs to LFA-1\(\alpha\) and \(\alpha 4\)\(^{(16)}\). Mabs to E-selectin and VCAM-1 also inhibit lymphocyte accumulation in delayed type hypersensitivity in the skin\(^{(3)}\). However recent research has shown that this is not the full story and that an ICAM-, ELAM- and
VCAM-independent modulation in the early phase of lymphocyte attachment to endothelium, seems likely\(^{(21)}\).

Therefore, emigration of lymphocytes through peripheral node HEVs, originally thought to consist of two steps, has now been shown through recent evidence to require three sequential area code signals (L-selectin tethering, chemoattractant activation and subsequent integrin activation and binding), that are analogous to those involved in neutrophil emigration from the blood stream\(^{(3)}\). Identification of a putative lymphocyte chemoattractant secreted by peripheral lymph node HEVs and a chemoattractant receptor that is predicted to be selectively expressed on the naive subset of lymphocytes that recirculate through peripheral node HEVs will be the subject of intense research interest in coming years.

In conditions such as chronic inflammation and cell mediated hypersensitivity, lymphocytes make up a substantial part of local infiltrating leukocytes. In inflammation the endothelium may exert functions in lymphocyte recruitment from the blood, comparable to high endothelium in lymphoid tissues. In fact, HEV-like structures have been described in various conditions of chronic inflammation, including autoimmune lesions and immune reactivity around tumours\(^{(11,12)}\).

Inflammation also affects traffic through the HEVs. Antigen injected into tissue, drains to the regional lymph node and greatly increases blood flow to the node and naive lymphocyte traffic through the HEV\(^{(17)}\). Furthermore, memory lymphocytes now appear to enter the node directly; this is associated with induction of VCAM-1 on non-HEV
vascular endothelia within the node\(^{(17)}\). Entry is inhibited by Mab to the integrin \(\alpha 4\) subunit, and this suggests a role for interaction of VCAM-1 with \(\alpha 4\beta 1\)^{(18)}. Recent research has shown that the adhesion molecules topography on the surface of leukocytes is a big factor in the outcome of an adhesion cascade\(^{(237)}\), indeed various adhesion molecules are enriched in the uropod region of the polarised lymphocyte, particularly ICAM-1 and -3\(^{(238)}\).

The evidence so far, suggests that there are multiple adhesion molecules involved in extravasation and that multiple signals are also required for directing activated lymphocytes through the endothelia. Thus, a four step or area code model of leukocyte emigration from the blood stream, established and validated \textit{in vitro} and \textit{in vivo} with neutrophils\(^{(19)}\), appears extendible to all subclasses of leukocytes, including lymphocytes (\textbf{figure 1.1}). Combinatorial use of multiple adhesion and chemoattractant receptors in the four step model\(^{(3)}\) with distinct distributions on leukocyte subsets, regulates selection of the subclasses of leukocytes emigrating at inflammatory sites and the distinctive recirculation behaviour of lymphocyte subsets.

\textbf{1.3: Lymphocyte chemoattractants}

Lymphocyte chemoattractants are interesting candidates for the stage 2 signal for lymphocyte accumulation at inflammatory sites. Pertussis toxin treatment inhibits lymphocyte emigration in response to antigen\(^{(37)}\). Identification of lymphocyte chemoattractants has been hampered by the low motility of lymphocytes compared with that of monocytes or neutrophils. A number of chemokines, all of which were isolated based on chemoattractive activity for neutrophils or monocytes or by cloning genes of
unknown function, have subsequently been tested and found to be chemoattractive for lymphocyte subpopulations\(^{38,39}\).

Chemokines, also known as intercrines, comprise a superfamily of small, secreted proteins that mediate inflammation by inducing chemotaxis and activation of a variety of inflammatory cells. Members of the chemokine superfamily possess a conserved structural motif containing two cysteine pairs. Based on the arrangement of the cysteines within this motif, chemokines are divided into two subfamilies. The first cysteine pair of the C-X-C chemokines (α-intercrines), are separated by an intervening amino acid, while the first cysteine residues of the C-C chemokines (β-intercrines), are adjacent. C-X-C chemokines include IL-8, MGS, IP-10, ENA-78, platelet factor 4, platelet basic protein and thromboglobulin. Members of the C-C chemokine subfamily include MIP-1α and MIP-1β, MCP-1, -2, -3, RANTES and I-309. The two chemokine subfamilies demonstrate 20-45% homology to each other at the amino acid level and are basic heparin binding proteins.

Particular chemokines induce selective migration of leukocyte subsets which differ both in phenotypic markers and activation state. This has led to the view that the cellular composition at inflammatory sites depends on the combinatorial effects of multiple chemokines, each with selective chemotactic activities. For example, while the C-C chemokines RANTES, MIP-1α and MIP-1β all induce monocyte migration, they have distinct chemoattractant properties for lymphocytes. MIP-1α induces the preferential migration of activated CD8\(^+\) T cells and B cells (at higher concentrations the migration of these cells seems to be diminished and the migration of CD4\(^+\) T cells is enhanced),
while MIP-1β selectively induces chemotaxis of activated CD4+ T cells(41). RANTES induces migration of both activated and resting T cells, including, perhaps most significantly, resting memory T cells (CD4+ and CD45RO+) (42,43). Furthermore, IL-8 acts as a chemoattractant for about 10% of human peripheral blood T lymphocytes belonging to either the CD4 or CD8 subsets (50,51,69). Greater proportions of polyclonally activated, than of resting T lymphocytes, exhibit chemotactic responses IL-8(52-68). Recently, the C-X-C chemokine, IP-10, has been shown to induce chemotaxis of activated, but not non-activated, human peripheral blood T lymphocytes (65). Phenotypic analysis of the stimulated T cell population responsive to IP-10 demonstrated that stimulated CD4+ and CD29+ T cells migrated in response to IP-10. This resembles the biological activity of RANTES. Recent research has shown that recombinant human IP-10 is capable of inducing human T cell migration in vivo and thus provides more evidence for its role in inflammation (239).

This pattern of selective migration corresponds to the capacity of these chemokines to enhance the adhesion of specific subsets of activated T cells to IL-1 stimulated endothelial cells (65). MIP-1α and MIP-1β augment the attachment of activated CD8+ and CD4+ T cells respectively (42). It has now been reported that there is a new member of the C-C chemokine family, termed MIP-1γ (206), which is produced by dendritic cells and recruits T cells before activation.

Moreover, differences in the kinetics of the expression between these chemokines may further co-ordinate the regulation of the migration pattern and thus the composition of the lymphocyte population at inflammatory sites, at any given time. Chemokines have
been shown to induce T cell adhesion to purified recombinant human adhesion molecules and to extracellular matrix proteins, by stimulating the development of a high affinity state in the integrin molecules. T. Springer's lab have shown using a transendothelial chemotaxis assay with HUVECS (human umbilical vein endothelial cells) on transwells that only the C-C chemokines promote transendothelial chemotaxis of PBTLs and that the C-C chemokines selectively recruit a memory subset of T lymphocytes. Also, one of his latest papers shows that MCP-1, RANTES and MIP-1β induce T cell binding to fibronectin but not ICAM-1, suggesting that the chemokines may be most important, not in initiating integrin dependent firm adhesion of T cells to the vascular wall but rather in subsequent adhesive interactions during migration into tissue.

The endothelium may present chemoattractants to lymphocytes in a functionally relevant way, as well as providing a permeability barrier that stabilises the chemoattractant gradient. A new concept to emerge recently has been that of specialised chemokine binding proteins that act as clearance receptors to remove chemotactic and inflammatory peptides from the blood. This receptor/protein is also found on endothelial cells and thus it could potentially play a role of presenting chemokines to lymphocytes.

Since lymphocytes, responding to specific antigen in tissue, signal emigration of further lymphocytes into the site, a chemoattractant was sought in material secreted by mitogen stimulated mononuclear cells. Subsequent investigations revealed that MCP-1, previously thought to be solely a monocyte chemoattractant, is also a lymphocyte chemoattractant to an activated subset of memory lymphocytes. There is a clear distinction between the IL-8 and MCP-1 responsive T cell populations and that chemokine receptor expression on T cells may be regulated with respect to lineage as well as cellular activation.
A model of selective chemotaxis has been proposed for the C-C chemokine MIP-1β and other chemokines containing glycosaminoglycan binding sites\(^{(44)}\). In this model, endothelial cells at inflammatory sites present CD8\(^+\) T cells with a gradient of the chemokine immobilised on endothelial surface proteoglycans, such as CD44. The bound chemokine triggers functional activation of the lymphocyte integrins, enhancing attachment to the vascular endothelium and migration through the vessel into the surrounding tissue.

The chemokine receptors, like their ligands, form a family of structurally and functionally related proteins. They are members of the superfamily of hepta-helical, rhodopsin like, G-protein coupled receptors that can be defined by amino acid sequence homologies\(^{(45)}\). The C-C chemokines bind weakly, if at all, to human neutrophils. Nevertheless, MIP-1α and RANTES can induce small, transient elevations of intracellular calcium that can be homologously and heterologously desensitised by MIP-1α and RANTES, but not by other stimuli, suggesting a shared neutrophil receptor\(^{(46,47)}\). However, any functional importance is unclear, since MIP-1α and RANTES do not induce neutrophil chemotactic or microbicidal responses\(^{(47)}\).

To date, only the lymphocyte MIP-1β receptor (also known as the ACT-2 receptor)\(^{(48)}\) has been characterised biochemically, although the relationship of this protein to the monocyte receptors is unknown. A distinct receptor for multiple C-C chemokines has recently been cloned from monocytes\(^{(49)}\). This receptor, termed C-C CKR-1 induces a rapid, transient increase in intracellular calcium, but the binding affinity is not
necessarily predictive of signal strength. While MIP-1α binds to C-C CKR-1 with the highest affinity and induces the strongest calcium signal, RANTES transmits a more potent signal than MCP-1 and MIP-1β, which bind the receptor with higher affinities. Indeed, there are now at least seven human chemokine receptors that bind or respond to β-chemokines\(^{(294,295)}\). Recent research suggests that chemokines not only share receptors but also signal transduction pathways. The signal transduction pathway of MCP-1, RANTES and MIP-1α are similar, involving pertussis toxin sensitive G-proteins, an increase in intracellular calcium, a rapid activation of arachidonic acid release and possibly protein kinase activation\(^{(54-58,64)}\).

However, it is not only the recently discovered (and much publicised) chemokines that are lymphocyte attractants, as other molecules such as interleukins have been found to be chemoattractive for lymphocytes. For example, IL-1 has been reported to be a potent lymphocyte attractant \textit{in vitro}\(^{(59-61)}\). Its release from the epidermis in disease or following injury, may therefore constitute an important mechanism for the induction of pathological lymphocyte infiltrates. Low level release of epidermal IL-1 under normal conditions may also be responsible for physiological trafficking of lymphocytes in normal skin. Recombinant IL-6 has also been shown to induce lymphocyte migration \textit{in vitro}\(^{(62,63)}\). Other interleukins which have been reported to have chemotactic activity for T lymphocytes include IL-10\(^{(66,72)}\), which is specific for CD8\(^+\) T cells, IL-2 which is reported to be specific for activated CD4\(^+\) T cells\(^{(67,82)}\) and IL-15 which has just recently been proven to be a chemoattractant for T lymphocytes\(^{(244-246,249)}\). Furthermore, IL-10 inhibits the IL-8 chemotactic response of CD4\(^+\), but not that of CD8\(^+\) T cells, as well as inhibiting B cell motility induced by IL-4\(^{(170)}\). Another paper suggests that IL-1, IL-8 and
RANTES play important roles by inducing migration of T cells towards sites of inflammation, whereas the T cell derived cytokines IL-2, IFN-γ, IL-4, IL-10 and IL-13 seem to be important because of their modulatory effects on T lymphocyte chemotaxis\(^{(247)}\).

Clinical research has shown that IL-2 mediates the regression of certain malignancies, but clinical use is limited because of associated toxicities, including parenchymal lymphocytic infiltration with multiple organ failure. Recent research has shown that IL-2 toxicity involves organ-specific TNF-α and RANTES production with increased ICAM-1 and VCAM-1 expression as potential mechanisms facilitating lymphocytic infiltration and organ dysfunction\(^{(248)}\).

Recently, a source of T cell chemoattractants has been shown to be neutrophils, which upon stimulation with IL-8 release chemoattractants that mediate T-cell and monocyte accumulation at sites of inflammation\(^{(250)}\).

A lymphokine termed lymphocyte chemoattractant factor (LCF), which has no significant homology to any previously described lymphocyte chemoattractants, has been identified and cloned\(^{(70-72)}\) and membrane expression of CD4 functions to transmit the migratory signal induced by LCF. However, LCF has now been termed as interleukin-16 and is secreted from serotonin stimulated CD8\(^+\) T cells \textit{in vitro}, therefore serotonin may promote recruitment of CD4\(^+\) T cells via CD8\(^+\) T cells\(^{(251)}\). Eosinophils and CD4\(^+\) T cells are preferentially recruited into sites of inflammation and in a recent publication it was found that eosinophils are a source of two cytokines, IL-16 and RANTES, that are
chemotactic for both lymphocytes and eosinophils. Their data indicates that eosinophils could contribute to the recruitment of CD4+ T cells and more eosinophils. Also, it has been found that CD4-lck coupling is essential for IL-16 induced T cell migration.

Many more T cell chemoattractants are being discovered lately such as recombinant human growth hormone which is capable of inducing significant migration of resting and activated human T cells and their subsets. A new chemokine, called Mig, which is of the C-X-C family has been found and is likely to play a role in T cell trafficking. Also, serum amyloid A has been shown to be a T cell chemoattractant, as well as prostaglandin E2 and leukotriene B4.

It must also be noted that early research in the 1970's and '80's, reported that T lymphocytes are responsive in a chemotactic manner to casein, C5a, f-met-leu-phe and denatured proteins. Also, P.C. Wilkinson has quite recently shown that staphylococcal enterotoxin B stimulates motility in T cells over a period of 72 hours.

In summary, it is evident that there are many different types of chemoattractants for lymphocytes. The diverse binding affinities and signalling potentials that each chemoattractant possesses, as well as the differential expression of the chemoattractant receptors on target cells, may regulate the combinatorial effect of multiple chemoattractants on lymphocytes at localised sites of inflammation.
1.4: Signal transduction events

In the past few years major advances in our understanding of the signalling pathways involved in cell motility have been achieved. Unfortunately, little of this work has been done on lymphocytes, instead most cell motility research has tended to concentrate on fibroblasts, slime moulds (*Dictyostelium discoideum*) and neutrophils. Thus some of the literature reviewed here will incorporate relevant work done on neutrophils that can be considered as similar to the events occurring in lymphocytes.

**Polyphosphoinositides, intracellular calcium and protein kinase C.**

Binding of chemoattractants and other agonists to receptors generates intracellular signals\(^{(118-120)}\), leading to the alterations in the cytoskeleton involved in the motile response. Among the many potential signalling events, the two that have received most attention are alterations in polyphosphoinositides (ppIs), such as phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) and changes in intracellular calcium concentration\(^{(74)}\). There is a link between binding of chemoattractants to seven-transmembrane receptors and fluxes in ppIs and intracellular calcium. Occupancy of the receptors leads to activation, in a G protein dependent manner, of a phospholipase C (PLC), which is specific for PIP\(_2\)\(^{(75,76,78)}\). However, it must be noted that there are multiple potential ways of regulating the phosphoinositol cycle in lymphocytes and these could also be involved in the induction of motility\(^{(77)}\). The hydrolysis of PIP\(_2\) results in the generation of inositol 1,4,5-triphosphate (IP\(_3\)) and diacylglycerol (DAG), which has been implicated as a second messenger to induce shape change and altered actin polymerisation in lymphocytes\(^{(162)}\). IP\(_3\) binds to specific receptors on intracellular organelles and induces the liberation of sequestered calcium, while DAG in conjunction with calcium and
phosphatidyl serine, activates protein kinase C (classical isotypes - α, β and γ), which has been reported to be involved in regulation of the actin network in lymphocytes(132).

Research in this lab has shown that activation of a serine/threonine kinase, which may be a PKC isotype, is necessary for the constant shape changing required for motility of lymphocytes(130). Also, activation of a classical PKC isotype maintains lymphocytes in the non-motile state and inhibition of the same PKC switches the cell to a constantly shape changing, locomotory phenotype(131). This data suggests that the activation of a classical PKC isotype maintains the lymphocytes in a non-motile state. Once this PKC isotype has been inhibited, the cells would become motile with the activation of a second serine threonine kinase (another PKC isotype or related kinase which is not inhibited by the PKC inhibitors). A recent paper has shown the identification of a PKC substrate in B cells, known as lymphocyte specific protein-1 (LSP-1), which is an intracellular calcium binding protein that binds to F-actin and to the cytoskeleton(258).

Neutrophil stimulation by N-formyl peptides induces the rapid and transient activation of a group of ser/thr kinases(207,208). These kinases exhibit the ability to be renatured after polyacrylamide gel electrophoresis and retain their activation state under these circumstances. Activation is inhibited by pertussis toxin, but is not induced by phorbol myristate acetate (PMA) or blocked by staurosporine. Interestingly, activation of these kinases is also blocked by wortmannin and LY294002, inhibitors of PI 3-kinase, suggesting that the activities of the renaturable kinases may be dependent on the lipid messengers generated by PI 3-kinase(264). The renaturable kinases remain incompletely characterised, with their structure and regulatory properties still unknown. The identification of neutrophil p21-activated kinases, as members of this group of
renaturable kinases\(^{(265)}\), suggests that low molecular weight GTP-binding proteins are involved in the regulation of these signalling enzymes. The close correlation between activation of the renaturable kinases and acute leukocyte stimulation by chemoattractants makes it likely that they are participants in regulating early events in pathways leading to activation of the respiratory burst, cytoskeletal assembly and motility.

It is not certain at present, whether lymphocyte motility requires increases in intracellular calcium ([Ca\(^{2+}\)]\(_i\)). For reviews on the role of calcium in leukocyte motility see refs. 177, 178. It has been demonstrated in neutrophils that it is possible for the cells to polymerise actin\(^{(79,80)}\) and migrate in the presence of very low intracellular calcium levels and where transient increases in [Ca\(^{2+}\)]\(_i\) are buffered\(^{(81)}\). Also it has been shown that neutrophils in response to chemoattractants can polymerise actin and polarise with very low intracellular calcium levels\(^{(259)}\). Investigations on lymphocytes have also shown that Ca\(^{2+}\)-mediated signals seem relatively unimportant in motility, whereas PKC mediated signals are crucial\(^{(169)}\). Recent research has indicated that [Ca\(^{2+}\)]\(_i\) elevation rapidly causes rounding and immobilization in T cells\(^{(304)}\).

There is also evidence to suggest that there is a close molecular interaction between certain cytoskeletal proteins and a G\(_{ia}\)-like protein\(^{(106)}\). Specifically, this association appears to be required for the activation of PLC that results in IP\(_3\) production and subsequent internal calcium release.
TL-2 and IL-15 signal transduction.

However, not all chemoattractants act through G protein linked receptors and thus there are other alternative pathways to motility. For example, interleukin-2 which has been shown to be a potent lymphocyte chemoattractant\(^{82}\), has a receptor consisting of two chains, \(\alpha\) and \(\beta\)^\(^{83}\), the latter, which is associated with a number of protein tyrosine kinases\(^{84}\) (PTK). IL-2 induces strong tyrosine phosphorylation of PI 3-kinase, Raf, Shc (src homology 2 domain containing protein) and Vav in T cells\(^{85-90}\), as well as activating p21 ras via a PTK\(^{173}\). Products of the PI 3-kinase\(^{172}\) induced phosphorylation of membrane ppIs, such as PIP\(_3\), have been suggested as one possible signal for induction of cytoskeletal changes\(^{91}\), thus this could be one possible pathway (one of many!) for the induction of motility in lymphocytes by IL-2. Recently it has been discovered that both IL-2 and IL-15 which cause motility in T lymphocytes, have been found to cause tyrosine phosphorylation of proteins termed Janus kinases 1 and 3 (JAK-1 and -3) and also of STAT3 and STAT5\(^{212,213}\) (signal transducer and activator of transcription), which are members of the STAT family of transcription factors, downstream effectors of the JAK kinases. Also, another group found that IL-2 caused tyrosine phosphorylation of STAT3 and that herbimycin A blocked the nuclear translocation of STAT3\(^{260}\). IL-2 and IL-15 cause tyrosine phosphorylation of insulin receptor substrates (IRS)-1 and -2 in T cells and JAK-1 and JAK-3 associate with IRS-1 and -2 in T cells. This suggests that IRS-1 and -2 may be important docking molecules recruited in response to IL-2 and IL-15 in T lymphocytes\(^{261}\).
IL-2 has also been found to induce expression, translocation and association of PKC-ξ to a structure coincident with the actin cytoskeleton. Furthermore, PKC-ξ has a role in maintaining the integrity of the actin cytoskeletal structure in IL-2 stimulated cells\(^\text{(262)}\).

IL-8 which has been shown to be a chemoattractant for lymphocytes\(^\text{(50,51,69)}\), causes activation of phospholipase-C and -D in T lymphocytes\(^\text{(286)}\).

**Small molecular weight GTP-binding proteins.**

Recent cell motility research has focused on the pathway involving activation of the small molecular weight GTP-binding proteins\(^\text{(108-112,117,263)}\) ras and rho in cytoskeletal regulation. For example ras inhibition suppresses fibroblast migration towards PDGF-BB\(^\text{(266)}\). According to this schema, activation of ras via receptor coupled heterotrimeric GTP-binding proteins or via, as yet, unidentified tyrosine kinases binding to growth factor binding protein-2 (GRB-2) and SOS, leads to alterations in the cytoskeleton\(^\text{(93,94)}\). Although unconfirmed in lymphocytes, these pathways seem to be highly conserved so that data from other species and cell types are likely to be applicable. Research into ras in lymphocytes has shown that it is activated within minutes upon the cell being stimulated by mitogens and that this activation is apparently dependent upon PKC activation\(^\text{(95)}\). In particular, rhoA (a member of the rho family), has been implicated in growth factor induced formation of stress fibres and focal adhesions, whereas rac (a member of the rho family of small molecular weight GTP binding proteins) has been implicated in the formation of membrane ruffles\(^\text{(96,97)}\). Also, it has been shown that rho induced stress fibre formation is dependent on PKC activation and that rho-induced activation of a tyrosine kinase is required for the formation of stress fibres\(^\text{(99)}\). RhoA activation downstream of PKC is involved in LFA-1 activation and aggregation\(^\text{(107)}\).
Furthermore, microinjection of antibodies to GRB-2, block growth factor induced membrane ruffling and lamellipod formation in cultured epithelial cells\(^{98}\), thus linking upstream events close to, or at the level of the receptor with the downstream events resulting in cytoskeletal reorganisation. It must be stressed at this point that since lymphocytes do not express as many stress fibres as other cell types, such as fibroblasts, then if these small molecular weight G-proteins do play a role in lymphocyte motility, it is most certainly through rac rather than rho. However, recent work has shown that in lymphoid cells transfected with chemoattractant receptors, agonist stimulation activated rhoA in seconds and inactivation of rho by C3 transferase exoenzyme blocked agonist induced lymphocyte \(\alpha_4\beta_1\) adhesion to VCAM-1, suggesting that rho participates in signalling from chemoattractant receptors to trigger rapid adhesion in leukocytes\(^{267}\).

Recently it has been reported that CDC42, another member of the rho family, triggers the formation of filopodia, a third type of actin-based structure found at the cell periphery. Activation of CDC42 in Swiss 3T3 cells leads to the sequential activation of rac and then rho, suggesting a molecular model for the co-ordinated control of cell motility by members of the rho family of GTPases\(^{268}\). Another possible mechanism for the control of actin polymerisation by rho-like GTPases is suggested by the recent identification of WASP, the protein implicated in the Wiskott-aldrich immunodeficiency syndrome, as an effector of CDC42. Overexpression of WASP in a variety of cell lines causes ectopic actin polymerisation at sites that are enriched in WASP and this reorganisation of the actin cytoskeleton is CDC42 dependent\(^{269}\). In a separate study, activation of CDC42 was also shown to cause F-actin reorganisation and co-localise with the 85kDa regulatory subunit of PI 3-kinase\(^{270}\).
Between the initial receptor and the rho family member in the signal transduction pathway, specific kinases may be required. For example, PI 3-kinase is required for the activation of rac by the binding of agonists to tyrosine receptors\(^{271,272}\). However, PI 3-kinase is not required by agonists that induce ruffling via heterotrimeric G proteins, nor is it required for induction of ruffling by PMA\(^{273,274}\). A recent paper has shown that both GTP- and GDP-bound rac-1 associate with phosphatidylinositol-4-phosphate 5-kinase *in vitro* and *in vivo*. PI 3-kinase also bound to rac-1 and CDC42Hs, and these interactions were GTP-dependent. This suggests that the effects of rho family members on the actin cytoskeleton are mediated in part by phosphoinositide kinases\(^{279}\). Other data demonstrates that rho regulates 4,5-PIP\(_2\) synthesis and indirectly, 4,5-PIP\(_2\) hydrolysis. They also raise the possibility that PIP\(_2\) synthesis could mediate the effects of rho on the actin cytoskeleton\(^{280}\). Another paper shows that the induction of arachidonic acid release and leukotriene production is one of the major biochemical pathways by which rac can influence the cytoskeleton\(^{281}\).

A ser./thr. protein kinase called protein kinase N is a target downstream of rho-GTP and may therefore be also involved in motility\(^{282}\).

**Regulation of small molecular weight GTP-binding proteins**

Immediately upstream of each rho family member, a guanine nucleotide exchange factor (GEF) is apparently needed\(^{275}\). The family of GEFs for rho family members share common motifs, namely a Dbl homology region, which has GEF activity and a pleckstrin homology domain, which can bind PIP\(_2\) and the \(\beta\gamma\) subunits of heterotrimeric G proteins\(^{276}\). A GEF can have specificity for a particular member of the rho family.
Thus, transfection of fibroblasts with Tiam-1, a GEF for rac and CDC42, stimulates membrane ruffling presumably by activating rac\(^{277}\).

So far, the downstream elements of pathways that regulate cytoskeletal organisation have not been defined. The list of activities stimulated by CDC42, rac and rho is long and includes cascades of kinases that regulate gene transcription and cell growth\(^{276}\). But, none of these activities have been linked to F-actin rearrangements. CDC42 and rac directly activate ser./thr. kinases of the p65\(^{\text{PAK}}\) family (kinases homologous to STE20 of yeast and p120\(^{\text{ACK}}\) of rats)\(^{265}\). However, in neutrophils, inhibition of PI 3-kinase with wortmannin inhibits chemoattractant activation of p65\(^{\text{PAK}}\) and NADPH oxidase, but does not inhibit membrane ruffling\(^{265}\). Thus, activation of this particular PAK is not needed for membrane ruffling. A tyrosine kinase appears to be required downstream of rho for the formation of stress fibres, as rho mediated induction of stress fiber formation in Swiss 3T3 cells is inhibited by the tyrosine kinase inhibitor genistein\(^{99}\).

Also interacting with the rho family are proteins which can negatively regulate their activity by increasing the hydrolysis of their bound GTP; these negative regulators are the GTPase activating proteins or GAPs\(^{275,278}\).

A target of the B cell receptor - induced tyrosine phosphorylation is p190\(^{101}\), a GAP for rac and rho\(^{102}\). These ras proteins are important regulators of the actin network\(^{96,97}\), suggesting that the tyrosine phosphorylation of p190 may influence microfilament behaviour. Interestingly, Vav, which has been implicated in regulating ras\(^{103}\), has homologies to a GEF for rho in yeast, suggesting that it may also regulate rac and rho in...
lymphocytes. It may be that the phosphorylations of p190 and Vav lead to co-ordinated actions on rac and rho, for example, by inhibiting p190 action and stimulating Vav action or vice versa. In addition, it seems that there are multiple functions for the rhoGAP family members p190 and bcr\textsuperscript{(104)} (product of the breakpoint cluster gene), which may enable them to co-ordinate a network of signalling pathways linking protein tyrosine kinases\textsuperscript{(105)} to different rho family proteins and other GTPases involved in mediating organisation of the actin cytoskeleton in response to extracellular signals. Another regulatory molecule in this schema is rhoGDI (GDP dissociation inhibitor - this blocks the effects of GAPs and GEFs), which is an inhibitory GDP/GTP exchange protein for the rho family, although it can interact with rac p21 also. It has been shown that the rhoGDI protein is an integral part of the system that regulates cell motility in fibroblasts\textsuperscript{(113)}, presumably through the microfilament system. More detailed data has shown that the complexation of rhoGDI with both GDP and GTP bound forms of rac\textsuperscript{(113)} can be regulated by certain lipids generated in chemoattractant stimulated cells and thus this would be a path by which chemoattractants can cause actin regulation\textsuperscript{(114)}. Recently a rho-GDI was identified that was specifically expressed in lymphocytes and is downstream in the signalling cascade resulting from PKC activation\textsuperscript{(115)}. In addition, a lymphocyte protein was identified that has striking homology to a number of regulatory rho-like proteins, that affect motility\textsuperscript{(116)}.

Thus, the evidence is quite strong that rho can regulate actin microfilament organisation/assembly, although this has not been established in chemoattractant-stimulated leukocytes. Polymerisation of neutrophil actin can be induced by guanine nucleotides in permeabilised cells\textsuperscript{(283)}, and both rho and rac have been shown to regulate
the state of the actin cytoskeleton in mast cells\textsuperscript{(284)}. As with rho-induced actin assembly in stress fibres, little is known about the mechanisms by which rac regulates actin assembly associated with ruffling/cell motility, and there is no evidence yet that rac has similar effects in chemoattractant-stimulated leukocytes. However, it seems quite likely that these small molecular weight GTP-binding proteins and their regulatory counterparts play a significant role in the signal transduction of lymphocyte motility.

\textbf{Role of the second messenger cAMP.}

The role of cAMP (cyclic adenosine mono-phosphate), in transduction of motility is rather unclear so far, however recent research is indicating that an increase in intracellular cAMP ([cAMP]) concentration inhibits lymphocyte motility\textsuperscript{(121,124)}, and affects their adhesiveness\textsuperscript{(125)}. Elevation of [cAMP] induces a decrease of cellular filamentous actin and a stabilisation of microtubules\textsuperscript{(122)}. How increases in [cAMP] modulate the cytoskeleton is unknown but it could be via control of putative actin binding proteins, or it could be through intervention of transduction pathways that control cytoskeleton organisation. For example, it has recently been shown that cAMP-dependent protein kinase A (PKA) directly phosphorylates actin and reduces its polymerisability. In contrast, protein kinase C mediated phosphorylation of monomeric actin increases its polymerisability, thus having the opposite effect of PKA on actin\textsuperscript{(168)}. A recent study has shown that phosphodiesterase inhibitors inhibit the migration of human T lymphocytes by increasing the [cAMP] concentration\textsuperscript{(285)}.

Lymphocytes and their precursors are cells whose locomotor capacity varies at different stages of maturation or activation\textsuperscript{(129)}. A model has been proposed by P.C.
Wilkinson\(^\text{127}\), in which the two stages of lymphocyte locomotor activation can be seen as follows:- (a) acquisition of locomotor capacity which is growth determined, occupies a period of hours and may need expression of new genes; and (b) response by polarisation and locomotion to a chemoattractant, similar to the response of neutrophils and taking only minutes. These two stages can be distinguished pharmacologically. Two immunosuppressant drugs, cyclosporin and FK506, specifically inhibit mitogen activated lymphocyte growth, acting early in G\(_1\). These drugs inhibit the cell cycle related acquisition of locomotor capacity in lymphocytes\(^\text{127,128}\), but have no effect on the locomotor responses of already motile lymphocytes. Conversely, pertussis toxin has no effect on the acquisition of locomotor capacity but does inhibit the immediate response of lymphocytes to IL-8 and fetal calf serum\(^\text{127}\), their locomotion in filter assays\(^\text{37}\) and their entry into lymphoid tissues\(^\text{10}\). These observations suggest separate transduction pathways, one mediated by a pertussis toxin-sensitive G protein for chemoattractant induced lymphocyte motility; the other for growth activation and locomotor activation, the pathway for which is probably not directly mediated by a pertussis toxin-sensitive G protein.

Thus the second messengers involved in transduction of motility are beginning to emerge but it is clear that much of it is yet to be discovered and that factors such as state of lymphocyte activation and maturation are going to be important parameters in the signal transduction pathways used.
1.5: Actin Modulation

Whatever the nature of the molecular signal or signals, exposure to chemoattractants leads to highly ordered and spatially localised changes in the actin cytoskeleton that are directly responsible for lamellar protrusion and cell motility\(^{133-135,160}\). The control of the cellular microfilament system is mediated by second messengers through various actin binding proteins which have certain actin modifying functions. Actin polymerisation is correlated with protrusive activity in almost all cell types along with filament cross linking and filament severing.

There is very little literature published in the field of lymphocyte motility in connection with actin modulation by second messenger systems. I have thus included reports of systems that have been found in other cell types as it is thought that these systems are fairly conserved throughout evolution, they are therefore, of relevance to lymphocytes.

Recent studies have highlighted the importance of thymosin-β4 in regulation of the leukocyte cytoskeleton\(^{136-138}\). These cells contain up to 250µM of this protein, which quantitatively is sufficient to account for the majority of actin monomer sequestration. Consistent with this function, increasing intracellular levels of thymosin-β4 by either microinjection or by over expression in transfected cells reduces the amount of filamentous actin by decreasing the effective cytosolic concentration of actin monomers. This ultimately promotes monomer release from filament ends\(^{139}\). More importantly, thymosin-β4 can release monomer rapidly, thus large amounts of monomer can be released from this source in response to signals for filament assembly. There are two other notes to add about this important actin monomer binding protein. First, thymosin-β4 inhibits exchange of adenine nucleotide bound to actin monomer. Second, thymosin-
β4 has a 50-fold greater affinity for actin monomer bound to ATP (G-actin-ATP), than for monomer bound to ADP (G-actin-ADP). Therefore, monomer release from thymosin-β4 may be facilitated by exchange of ADP for ATP, by a local decrease in the ATP/ADP ratio, resulting from the activity of second messenger systems that have ATP-consuming activity. According to this scheme, the polymerisation of actin might in fact involve addition of ADP-actin monomers to filaments\(^{(140)}\).

Profilins are a group of 15kDa molecular weight basic proteins\(^{(141,142)}\) that are present in two interconvertible states; a high affinity state that binds actin monomers tightly and a low affinity state\(^{(143)}\) that may function to sustain high rates of filament assembly at the barbed end. Profilins can inhibit ATP hydrolysis by monomeric actin and speed exchange of ADP for ATP, thus facilitating microfilament assembly\(^{(136)}\). It is noteworthy that membrane ppIs including PIP and PIP\(_2\), lower the affinity of both forms of profilin for G-actin\(^{(144,145)}\) and that the levels of these ppIs are altered in response to chemoattractants providing a potential mechanism for dynamic regulation of actin assembly. The interaction between profilin and PIP\(_2\) prevents the hydrolysis of PIP\(_2\) by the phosphorylated form of PLC-γ\(^{(146,147)}\). Phosphorylation on tyrosine (by a receptor tyrosine kinase for example), of PLC-γ, allows the lipase to overcome profilin inhibition and to hydrolyse PIP\(_2\). While profilin seems to be able to regulate the activity of PLC-γ and to make it dependent upon tyrosine phosphorylation for activation, the hydrolysis of PIP\(_2\) by activated PLC-γ may, in fact regulate the interaction of profilin with actin\(^{(147)}\), as the subsequent binding of PIP\(_2\) to profilin inhibits the interaction between profilin and actin. Therefore, PIP\(_2\) turnover may link receptor tyrosine kinase activation (or any other system that can activate PLC-γ) with actin network reorganisation, by modulating the
availability of profilin, as the concentration and distribution of PIP$_2$ is altered in response to chemoattractant stimulation. There is also evidence to suggest that profilin is phosphorylated by PKC and that this phosphorylation is stimulated by PIP$_2^{(167)}$. In addition it has recently been shown that DAG, a product of PIP$_2$ can directly enhance the formation of actin nuclei at the membrane level by activating a nucleating protein factor$^{(148)}$, which is yet to be characterised. Small GTP-binding proteins like ras also have the ability to regulate inositol phospholipid metabolism$^{(118)}$. It is possible that regulation of the actin network by small GTP binding proteins requires specific modulation of the local inositol phospholipid concentration at the membrane level$^{(149)}$.

The ability of proteins to bind to actin filaments and prevent monomer exchange is termed capping and leukocytes contain several such proteins$^{(150)}$. Those that cap the barbed ends result in net filament depolymerisation. Gelsolin, an 82kDa protein, is able to bind to the barbed end of filaments and prevent monomer exchange as well as to sever filaments in a calcium dependent manner$^{(151)}$. Gelsolin is also able to bind actin monomers, an interaction that is decreased by interactions with membrane ppIs$^{(152)}$. For example, exposure of neutrophils to fMLP, decreases the number of gelsolin-G-actin complexes$^{(153)}$. A function of gelsolin that appears to be important in motility is its ability to sever filaments in a calcium dependent manner$^{(154)}$. Hence gelsolin is under dual regulation: calcium promotes its binding to actin, its severing of actin filaments and its blocking of monomer addition at the fast growing filament end - all effects leading to actin depolymerisation and to the solution of a cross-linked actin gel. Therefore it follows that the reversal of gelsolins tight binding to actin must be essential for assembly of the pseudopodial network. Thus, ppIs could be responsible for this reversal, with the
implication that gelsolin, which is regulated by the two known intracellular messengers (calcium and an intermediate of the phosphoinositide cycle), and is thus positioned as an integral component between second messenger systems and actin network regulation.

Other actin regulatory proteins include cofilin and destrin. Cofilin has the ability to bind along the side of F-actin and to depolymerise F-actin in a pH-dependent manner. Various ppIs inhibit the actions of cofilin in a dose dependent manner, while IP$_3$ has no effect on them$^{(156)}$. Furthermore, in the same study destrin, a pH independent actin depolymerising protein and deoxyribonuclease I, a G-actin-sequestering protein, were also functionally inhibited by ppIs. Thus it seems that the sensitivity to ppIs may be a common feature among actin binding proteins which can regulate the state of actin polymerisation. In recent years, a lymphocyte specific actin binding protein, termed LSP-1 was identified that only binds F-actin and is thought to be involved in mediating cell motility$^{(163)}$. However, this protein has now been found to be not lymphocyte specific but is present in all human leukocytes$^{(287)}$.

There are inevitably numerous more actin regulatory proteins and it must be stressed that apart from the microfilament network, there is also the microtubule and intermediate filament network which in some way are involved in cell movement$^{(159-161)}$. Although the general opinion is that fundamentally the initiation of shape change and motility is mostly controlled by actin$^{(135)}$, as it has been shown that in lymphoma cells, a high level of actin polymerisation is a prerequisite for the formation of pseudopodia and infiltration of the cells into tissues$^{(158)}$. Interestingly, rac has recently been found to interact with tubulin and this may have a role in controlling changes in cell morphology$^{(288)}$. Also, a new
unconventional myosin termed myosin IXb has been discovered with the highest levels in peripheral blood T lymphocytes\textsuperscript{(289)}. The tail region was found to contain a putative GTPase activating protein (GAP) domain of the rho/rac family of ras-like G proteins, suggesting a role for this myosin in actin-based processes in lymphocytes.

The actin regulatory proteins just described may be regulated by several different signalling pathways and stimulated pseudopod extension in lymphocyte motility will undoubtedly involve crosstalk between specific receptors and signal transduction systems.
1.6: Aims of the project.

The aim of this project was to elucidate whether there is a final common signal transduction pathway utilised by all agents that causes induction of motility in T lymphocytes. Therefore, this is an investigation into the penultimate step of the “four step model of lymphocyte transendothelial migration” (see fig. 1.1), in which the lymphocyte first changes shape.

The first step in investigating this question was to develop a model whereby freshly isolated peripheral blood T-lymphocytes (PBTLs) could be induced into a motile state by a number of agents. Therefore, agents will be tested for their ability to induce motility in PBTLs and once a number of these have been found, then the second messenger elements that they utilise will be examined in an attempt to observe whether there are any common signal transduction elements. If any common second messengers are found, then these would be contenders for part of a motility signal transduction pathway.

As well as investigating motility in PBTLs, a human lymphoid cell line, termed MOLT-4 cells will also be investigated as above. Two population variants of this cell line were available, a motile and non-motile population and differences between the two populations shall also be investigated.
2. Materials and Methods
2.1: Cells and media.

The MOLT-4 lymphoid cell line was obtained from ECACC (Porton Down, U.K.), and maintained in growth media which constituted RPMI-1640 (Gibco BRL, Life Technologies Ltd., Paisley, U.K.), with 10% fetal calf serum (heat inactivated, mycoplasma and virus screened), 10mM HEPES buffer, 2mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin, all obtained from Gibco. The cells were grown at 37°C. Motile and non-motile variants of MOLT-4 were isolated as described in ref. 131. In isolating peripheral blood T lymphocytes, a medium consisting of all the above but substituting the fetal calf serum with 2.5% human serum albumin (fraction V powder, 96-99% albumin, Sigma, U.K.), was used (2.5% HSA).

2.2: Solutions and buffers.

All materials from Sigma, U.K. unless otherwise stated.

Phosphate buffered saline (PBS) was prepared using deionised water (dH2O) and PBS Dulbecco A tablets (Unipath Ltd., Basingstoke, U.K.). The composition being, NaCl (8g/L), KCl (0.2g/L), disodium hydrogen phosphate (1.15g/L), potassium dihydrogen phosphate (0.2g/L). pH 7.3

Balanced salt solution (BSS) was prepared in dH2O by dissolving NaCl (8g/L), KCl (0.4g/L), CaCl2 (0.14g/L), MgCl2.6H2O (0.2g/L), glucose (1g/L), HEPES (2.388g/L) and adjusting to pH 7.4. All constituents were obtained from BDH Ltd., Glasgow, U.K.
Laemmli's sample buffer (1x) : 62.5mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulphate (SDS) (BIO-RAD, U.K), 5% β-mercaptoethanol, 8.5% glycerol, 2.5mM orthovanadate, 10mM paranitrophenylphosphate, 12μg/ml leupeptin, 12μg/ml aprotonin, 1.25mM PMSF, 0.025% bromophenol blue (BIO-RAD).

Western blot buffer : (2L) 70g glycine (BDH), 1.5g SDS, 24g tris-base, 1,600mls dH₂O and 400mls methanol (Fisher Scientific, Loughbrough, U.K)

Upper gel buffer : 500mls dH₂O, 30.25g tris-base, 2g SDS, 0.74g sodium EDTA, pH 6.8

Lower gel buffer : 500mls dH₂O, 90.75g tris-base, 2g SDS, 0.74g sodium EDTA, pH 8.8

Running buffer : 5L dH₂O, 15g Tris-base, 72g glycine, 5g SDS.

Gel destain solution : 1L dH₂O, 1L methanol and 200ml glacial acetic acid.

Gel overlay solution : 20ml methanol, 200mg amido black, 80ml dH₂O.

Coomasie blue stain : 2.2g Coomasie blue, 1L methanol, 1L dH₂O and 200ml glacial acetic acid,
2.3: General reagents.

All reagents were from Sigma (U.K.) apart from the following:
Bisindolylmaleimide GF109203X (Bis.) (Calbiochem, U.K), Human recombinant Interleukin-2, MIP-1α, MIP-1β, RANTES, MCP-1, Interleukin-8, Interleukin-1α, Interleukin-10, PDGF, EGF and TNFα are all from R&D systems (U.K). Interleukin-15 (Peprotech, USA). All amiloride compounds were from Research Biochemicals International (USA).

2.4: Isolation of human peripheral blood T-Lymphocytes (PBTLs).

Whole blood was obtained by venepuncture from healthy donors and anticoagulated by mixing with heparin at approximately 1-2 U/ml whole blood. After dilution 1:1 with PBS, 4ml volumes of the diluted blood were layered onto 3ml of ficoll-paque (Pharmacia Biotech, Sweden) in plastic, conical-based tubes. After centrifugation for 40 mins. at 400g, the mononuclear cells layer at the interface of the separation media was then collected by pasteur pipette and the mononuclear cells washed (i.e. resuspended in 20ml PBS and centrifuged 5-10 minutes at 400g) twice. Typically 1-2x10⁶ mononuclear cells were obtained per ml of blood. The cells were then resuspended at 1x10⁷ cells/ml in 2.5% human serum albumin (HSA). Then 1ml of the cell suspension was put in each plastic eppendorf tube (1.5ml volume) and to this was added 100μl of both mouse anti-human CD19 and CD14 monoclonal antibodies (murine IgG1κ, 200 tests/2ml, Serotec, U.K.). The mixture was then mixed by rotation on a rotar wheel at 25 revolutions per
minute at room temperature for 30 minutes. The cells were washed twice in 2.5% HSA and resuspended at 4x10⁷ cells/ml in 2.5% HSA. An equal volume of pre-washed goat anti-mouse IgG coated Dynabeads (DYNAL, U.K) was added before mixing for 30 minutes at room temperature on a rotar wheel as before. The contaminating B cells and monocytes with the attached magnetic beads were then removed using a Dyna-magnet (DYNAL). The remaining T cells had a mean purity of 92% with <2% monocyte and B cells as shown by fluorescence flow cytometry (data not shown). This method was derived from reference 53

2.5: Cell polarisation assay.

The cells were resuspended at 1x10⁶/ml in the relevant media (10% FCS for MOLT-4 cells and 2.5% HSA for PBTLs) and 90μl of cell suspension was added to each well of a 96 well cluster plate. The plate was then incubated 20 minutes at 37°C, 5% CO₂, before addition of 10μl of a 10x working concentration of the test reagent. After which the plate was incubated for 1 hour at 37°C, 5%CO₂. Cells were then fixed in a final concentration of 3.7% formaldehyde/PBS and then assessed microscopically, under 400X magnification, for the percentage of irregular, shape changed cells. The criterion for non-shape changed cells was that at least three-quarters of the cell approximated to a circle. Each experiment was set up in triplicate and results expressed as the mean % shape changed cells ± standard error of the mean (SEM). Since some of the test reagents were made up as stock solutions in dimethyl sulphoxide (DMSO), solvent controls were incubated in all assays. With the data shown in the RESULTS section, the corresponding concentrations of solvent were without effect.

This method was derived from and validated in previous literature²⁹,³⁰.
2.6: Time-lapse video microscopy\(^{(20)}\).

Cells were suspended in the relevant media (5x10^3 cells/ml), with 1mM HEPES and placed in the wells of 96-well cluster plates which were then sealed with tape to prevent evaporation. The cluster plate was placed on the stage of a Zeiss Axiovert 35 microscope and maintained at 37°C by means of a thermostatically controlled fan heater. For video analysis a Panasonic WV-BL600 camera was used with a Panasonic time-lapse video cassette recorder. Recordings were made over 1 hour and replayed at x 160.

2.7: Transmigration assay\(^{(28)}\).

For studies on PBTLs, Costar transwells (Cambridge, U.K.) of 24 well size with 6.5mm diameter polycarbonate filters and a 3μm pore size were used. Freshly isolated PBTLs were resuspended at 5x10^7 cells/ml in 2.5% HSA media. Then, 100μl of the cell suspension was added to the upper chamber of a Costar transwell insert. The insert was immediately placed in the well of a 24 well cluster plate containing 600μl of 2.5% HSA with the relevant concentration of the test reagent. Control wells contained only 2.5% HSA with no reagent. The plate was then incubated for 4 hours at 37°C, 5% CO\(_2\), after which the inserts were removed and the cells in the lower wells were fixed in a final concentration of 3.7% formaldehyde/PBS. The number of cells in the lower chamber was determined with the use of a Neubauer counting chamber. For each test reagent concentration, triplicate wells were set up and the results were expressed as the percentage cells transmigrated compared to the initial number of cells added to the transwell.
In experiments with MOLT-4 cells, the protocol was similar except inserts with 8μm pores were used.

2.8: Intracellular calcium [Ca$^{2+}$]$_{i}$ measurements$^{(342)}$.

Measurements of intracytoplasmic free Ca$^{2+}$ levels were performed with FURA-2/AM (Molecular Probes, USA.). Freshly isolated PBTLs or MOLT-4 cells were washed twice in BSS. The cells were then resuspended at 2.5 x 10$^6$/ml in BSS with a 5μM final concentration of FURA-2/AM and incubated at 37°C in a water bath for 45 minutes (in the dark) with occasional mixing. FURA-2/AM was obtained as special packaging in 50μg aliquots, which were reconstituted in DMSO to produce a 5mM solution. After incubation the cells were washed once in BSS and resuspended at 1.5 x 10$^6$/ml in BSS. The cells were kept in a water bath at 37°C before the experiments were started. A 2ml aliquot of the labelled cells was then transfered into a quartz cuvette and inserted into the spectrometer. Fluorescence of the cellular suspension was monitored with a Perkin-Elmer LS-50B luminescence spectrometer in quartz cuvettes thermostatically controlled at 37°C. Fluorescence of the cellular suspension was first done with unlabelled cells to correct experimental measurements for autofluorescence. The cell suspension was excited alternately and 380 nm and the fluorescence measured at 510 nm. Ten nanometer slit widths were used for both excitation and emission. After stabilization of the baseline, stimuli were added in small volumes (typically 20μl).

Graphic representations of [Ca$^{2+}$]$_{i}$ were computed by using the equation:
\[ [\text{Ca}^{2+}]_i = 224 \times (R - R_{\text{min}})/(R_{\text{max}} - R) \times \text{Sf380/Sb380}, \]

previously published by Grynkiewicz et al. \(^{(296)}\). \(R_{\text{max}}\) and \(R_{\text{min}}\) were evaluated in 1mM \(\text{Ca}^{2+}\) - containing media (BSS) by perforating the cells with 10\(\mu\)M ionomycin for \(R_{\text{max}}\) followed by the addition of an excess of EGTA at 5mM for \(R_{\text{min}}\).

2.9: Intracellular pH (\(pH_i\)) measurements\(^{(297)}\).

Measurements of \(pH_i\) were performed with BCECF/AM (Molecular Probes, USA). Freshly isolated PBTLs or MOLT-4 cells were washed twice in BSS and the cells then resuspended at 2.5 \(\times\) 10\(^6\)/ml in BSS with a final concentration of 5\(\mu\)M BCECF/AM and incubated at 37\(^\circ\)C in a water bath (in the dark) for 30 minutes, with occasional mixing. BCECF/AM was obtained as special packaging in 50\(\mu\)g aliquots, which were reconstituted in DMSO to produce a 5mM solution. After incubation the cells were washed once in BSS and resuspended at 1.5 \(\times\) 10\(^6\)/ml in BSS. The cells were then kept in a water bath at 37\(^\circ\)C before the experiments started. A 2ml aliquot of the labelled cells was then transferred to a quartz cuvette and is inserted into the Perkin-Elmer LS-50B luminescence spectrometer. Fluorescence of the cellular suspension was first done with unlabelled cells to correct experimental measurements for autofluorescence. Excitation wavelengths were adjusted alternatively to 440, or to 490 nm, while the emission wavelength was set to 530 nm. Ten nanometer slit widths were used for excitation and 5nm slit width used for emission.

After stabilization of the baseline, stimuli were added in small volumes (typically 20\(\mu\)l). Calibration of \(pH_i\) in cell suspensions \textit{in situ} is difficult and erroneous, since it is nearly impossible to change the buffer (and thereby the pH) without losing or damaging the
Therefore, ratios were calibrated by external measurements using BCECF free acid (1µM) (Molecular Probes, USA), in intracellular buffer titrated to different pH values between 6.6 and 7.4 (intracellular buffer consisted of 110mM KCl, 10mM NaCl, 2mM MgCl₂, 5mM KH₂PO₄, 2mM dithiothreitol, 2mM EGTA, 1% BSA and 20mM HEPES - all constituents obtained from BDH, Glasgow, except BSA and EGTA which were from Sigma). The ratios plotted against pH resulted in a highly linear correlation, with correlation coefficients > 0.98. A typical calibration curve is displayed in figure 2.1. This method of pH calibration has been validated previously in ref. 297.

Figure 2.1: Correlation between 490/440 nm ratio of BCECF and pH
2.10: D-\textit{myo-Inositol 1,4,5-trisphosphate (IP$_3$)} assay\textsuperscript{(343)}.

Assays were conducted using the $[^3H]IP$_3 binding assay kit provided by Amersham International following the instructions provided.

Fresh PBTLs were isolated and resuspended in 2.5\% HSA at a concentration of 5x10\(^6\)/ml. 500\(\mu\)l of the cell suspension was then aliquotted into each 1.5ml eppendorf tube and small volumes of the agonist were then added to the cells for 1 minute at 37\(^\circ\)C. The incubation was terminated by addition of 500\(\mu\)l ice cold 10\% perchloric acid. After leaving 10 minutes on ice, the samples were centrifuged for 5 minutes at 2,000g. 400\(\mu\)l of the supernatant from each sample was transferred to a separate tube containing 100\(\mu\)l of 10mM EDTA, (pH 7.0).

The samples were neutralised by adding 300\(\mu\)l of a 1:1 (v v) mixture of Freon (1,1,2-trichlorotrifluoroethane) and tri-n-octylamine, followed by vigorous mixing of the separate phases on a vortex mixer. After centrifugation for 1 minute at 2000g, three phases were obtained. The upper phase was the neutralised sample plus all the water-soluble components. A 400\(\mu\)l portion of the upper phase was removed for use in the IP$_3$ binding assay kit, which is based on the ability of IP$_3$ in the sample to displace fixed amounts of $[^3H]IP$_3 from the IP$_3$ receptor. A standard curve was constructed in the range 0.2 to 25 pmol and displacement values obtained were converted to pmol IP$_3$ by the use of this curve.

2.11: Preparation of acrylamide gels\textsuperscript{(298)}.

10\% acrylamide mini gels were made as follows using a Biorad minigel apparatus.
To a 50ml tube is added 8ml of lower gel buffer, 13.1ml dH₂O, 10.7ml 30% (w/v) Acrylamide/Bis-acrylamide stock solution (Anachem, U.K.), 200µls 10% Ammonium persulphate and 20µls TEMED (BIO-RAD). After gentle mixing the solution was slowly poured between the glass plates, up to the required mark. Then 200µl of gel overlay was layered over the top and the gel left to set. Once the lower gel was set, the overlay was washed off and the 5% stacking upper gel was poured on. This was prepared by mixing together, 5ml upper gel solution, 11.6ml dH₂O, 3.3ml Acrylamide/Bis. solution, 200µl ammonium persulphate and 200µl TEMED. Once the upper gel was poured on, the well-comb was inserted and the gel left to set, after which the comb was removed and the wells filled with running buffer.

### 2.12: Renaturable kinase assay.

Freshly isolated PBTLs were isolated and resuspended in 2.5% HSA. 250µl of the cell suspension was then aliquoted into each 1.5ml eppendorf tube and incubated at 37°C for 20 minutes. To each tube 250µl of 2x the final working concentration of the test reagent was then added, mixed well and the cells incubated for one hour at 37°C in a water bath. After incubation the cells were washed twice in serum free RPMI-1640 media, the supernatant aspirated and the cells solubilised in Laemmli sample buffer and boiled for 5 minutes. Samples were run on 10% (w/v) SDS-PAGE gels according to Laemmli along with rainbow molecular weight markers (High molecular weight range 14,300-220,000 Da, from Amersham, U.K).

The gels were processed for renaturation essentially as described by Kameshita and Fujisawa. After washing SDS from the gels (20% isopropanol, 15mM Tris buffer pH...
8.0) and denaturation for 1 hour in 6M guanidine-HCL, samples were renatured overnight at 4°C in 50mM Tris buffer pH 8.0, 50mM 2-mercaptoethanol and 0.04% Tween-20. Following equilibration in phosphorylation buffer (10mM HEPES pH 8.0, 2mM dithiothreitol, 0.1mM EGTA, 5mM MgCl₂ (BDH)) the gels were incubated with [γ³²P]ATP (Amersham) (1µCi/ml) for 1 hour at room temperature. Finally, the gels were washed extensively with 5% (w/v) trichloroacetic acid containing 1.0% (w/v) sodium pyrophosphate and dried. Autophosphorylation was visualised by autoradiography using Hyperfilm-MP from Amersham

2.13: Western blotting for tyrosine phosphorylation

Freshly isolated PBTLs were isolated and resuspended in 2.5% HSA. 250µl of the cell suspension was then aliquoted into each eppendorf and incubated at 37°C for 20 minutes. To each tube 250µl of 2x the final working concentration of the test reagent was then added, mixed well and the cells incubated for one hour at 37°C in a water bath. In experiments in which the cells were pretreated with herbimycin A, 0.5µl of 10⁻³M herbimycin A was added to the 250µl cell suspension to give a final concentration of 5 x 10⁻⁵M and the cells incubated for 45 minutes at 37°C, after which the test reagent was added as described above. After incubation the cells were washed twice in serum free RPMI-1640 media, the supernatant aspirated and the cells solubilised in Laemmli sample buffer and boiled for 5 minutes. Samples were run on 10% (w/v) SDS-PAGE gels according to Laemmli along with rainbow molecular weight markers (14,300-220,000Da, Amersham).
After electrophoresis, the gels were washed in western blot buffer x3 every 10 minutes whilst on a shaker (the Belly Dancer - Stovall, Life Science Inc, USA.) and then the proteins were transferred to nitrocellulose membranes (Hybond™ECL™, Amersham Life Science, U.K.) using a Trans-Blot semi-dry transfer cell (BIO-RAD). Nonspecific sites were blocked using 5% Bovine serum albumin in PBS/0.1% Tween-20 (PBS/tween) for 1 hour at room temperature. The membranes were washed 3 times in PBS/tween and then the primary antibody - antiphosphotyrosine, (monoclonal IgG2bκ - clone 4G10), (Upstate Biotechnology Incorporated, USA) was incubated with the membranes for 1 hour at a final dilution of 1/2000 in PBS/tween. The membranes were washed 3 times in PBS/tween for a period of 1 hour and then incubated with a horseradish peroxidase-labelled sheep anti-mouse IgG (Amersham, U.K.) at a final dilution of 1/2000 in PBS/tween. The membranes were washed 3 times in PBS/tween over a period of 1 hour and the phosphotyrosine bands were revealed using the ECL detection system (Amersham, U.K.) with ECL autoradiography film (Amersham, U.K.).

2.14: Immunofluorescence staining for actin and tubulin in PBTLs

PBTLs were isolated as normal and treated with either 10μM Bis. or 50μM nocadazole as described in section 2.14. After which the cells were fixed with 1% paraformaldehyde. The cells were then permeabilised by incubation at room temperature with 0.1% lysophosphatidylcholine for 45 minutes on a rotary wheel. The cells were then washed x1 in PBS/tween and resuspended in 400μl PBS/tween per treatment. Then 200μl from each treatment was put into a separate eppendorf tube. Either of the anti-tubulin antibodies was then added to the relevant tubes at a 1 in 1000 dilution (either, mouse
monoclonal antibody to polymerised β-tubulin, or depolymerised β-tubulin. Both from Affiniti Research, Exeter, U.K.). Also the cells were stained for F-actin by the addition of rhodamine-labelled phalloidin to a final concentration of 330nM. The cells were then left at room temperature for one hour on a rotary wheel (slow speed 25 revs/min.). Cells were then washed x3 in PBS/tween and resuspended in 200μl PBS/tween. The secondary antibody (anti-mouse polyvalent immunoglobulins - FITC conjugate) was then added to each tube at a 1 in 500 dilution. The cells were incubated at room temperature on a rotary wheel for one hour and then washed x4 in PBS/tween and resuspended in Citifluor (Citifluor Ltd. London), after which slides were prepared. The cells were viewed using a Zeiss Axioscop microscope equipped with epifluorescence and photographs taken using a Nikon FX-35DX camera with Ilford PAN F 50 black and white film.

2.15: Measurement of Taurine $^{14}$C efflux$^{(300,302)}$.

MOLT-4 cells were suspended at 1x10⁶/ml in 10% FCS media and incubated with Taurine [1,2-$^{14}$C] (Amersham), 0.1μCi/ml for 1.5 hours at 37°C. The cells were then washed x2 and resuspended at 1x10⁶/ml in 0.5% FCS media. 1ml aliquots were put in each eppendorf and the cells incubated at 37°C for 0 (control), 10, 20 or 30 minutes with or without 0.5mls dH₂O. The cells were then spun down in a microcentrifuge and 500μl of the supernatant was collected (control) or 750μl from the hypotonically shocked cells. The supernatant was then added to a scintillation vial along with 10ml Ultra gold scintillation fluid (Packard, USA), and counted in a Packard 2500 TR liquid scintillation analyzer. The cell pellets along with the rest of the supernatant were treated in the same
manner. The percentage efflux was calculated as follows [(DPM of supernatant of sample x 2) ÷ (total DPM of sample)] x 100.

To test the effects of chloride channel blockers on taurine efflux, the protocol was slightly different. The cells were labelled as above and then resuspended at 2×10^6/ml in 0.5% FCS media. 400μl of the cells were aliquoted into each eppendorf and to this was added 400μl of either medium (control) or 2x final concentration of the channel blocker. The cells were then incubated 5 minutes on a heating block after which they were incubated for a further 20 minutes with or without (control) the addition of 400μl of dH2O.

The percentage efflux was then calculated as above.

The chloride channel blockers tested were - NPPB [5-nitro-2-(3’phenylpropylamino) benzoic acid], (LC laboratories, Woburn, MA. USA). Tamoxifen [[trans-2-[4-(1,2,diphenyl-1-butenyl) phenoxy]-N,N-dimethylcethylamine]], (Aldrich-chemic), Niflumic acid and Quinidine.
RESULTS
Chapter 3

The Investigation for inducers of motility

Introduction

Before any investigations into the signal transduction mechanisms involved in PBTL motility could be carried out, it was essential to first of all acquire a model in which motility could be induced at will so that second messenger involvement could be investigated. As well as using fresh human PBTLs, a leukaemia cell line was also used, termed MOLT-4 cells. From this cell line, two sublines had previously been isolated, termed motile and non-motile (see methods 2.1).

Both PBTLs and non-motile MOLT-4 cells were used in experiments in which various factors were tested for their effect at inducing motility. In initial experiments a simple polarisation assay was used in place of a conventional transmigration assay. The degree of lymphocyte polarisation or shape change correlates with the degree of motility induced\(^{(129)}\) and the assay is a cheaper, better for screening and simpler assay than the conventional transmigration assay.

Polarisation assay.

A summary of all the factors tested and their effects can be seen in Table 3.1. All the factors tested were chosen because of reported effects on motility in lymphocytes or other cell types in the literature. The chemokines MIP-1\(\alpha\), MIP-1\(\beta\), MCP-1 and IL-8 had minimal effects on PBTLs, causing polarisation to a maximum of 10\% of the population (all at \(10^{-7}\)M concentration), whereas RANTES had no effect whatsoever in
this system (all factors tested at concentration range of $10^{-12}$M to $10^{-7}$M). The chemokines had no polarisation effect at all on the non-motile MOLT-4 cells.

Substance P, vasoactive intestinal peptide (VIP), lysophosphatidic acid (LPA) and platelet derived growth factor (PDGF), all caused polarisation in only 10% of the PBTL population (all at $10^{-7}$M concentration) and had no effects on the non-motile MOLT-4 cells. IL-1α, IL-10, epidermal growth factor and tumour necrosis factor α, all had no effects on either PBTLs or non-motile MOLT-4 cells.

Although factors like MCP-1 were found to cause polarisation in 10% of the PBTL population, it was decided that this would not provide sufficient sensitivity for investigating the second messengers involved. However, 5 factors were found to cause significant polarisation in PBTLs and the effects were significant enough to use these 5 as tools to induce motility in the subsequent investigations into the second messengers involved.

Freshly isolated PBTLs are virtually all spherical, non-motile cells as can be seen in figure 3.1. The biggest effect was seen with the protein kinase C inhibitor bisindolylmaleimide GF109203X (Bis), which caused up to 60% of the PBTL population to polarise (figure 3.2), with a leading edge and a trailing uropod. IL-2, IL-15 and fetal calf serum (FCS) also caused the PBTLs to change morphology in the same way as Bis. Nocodazole, the microtubule disrupting agent caused shape change in 20 - 30% of PBTLs, however, the morphology of the shape change was different from that induced by the four other factors in that there was often no typical head or tail structures (fig. 3.3). Interleukins-2 and -15 were found to cause polarisation in 20 - 30% of the PBTLs and FCS caused polarisation in 10 - 20% of the population.
Only Bis and nocadazole were found to have any effects on the shape of the non-motile MOLT-4 cells and because of these limited effects on these cells it was decided to use PBTLs as a model for inducing motility successfully.

The dose responses of the polarisation of PBTLs to these 5 inducers of shape change are shown below. As can be seen in figure 3.4, the maximum effect of Bis on the polarisation of PBTLs was at 10μM concentration, however, above this concentration Bis was found to be cytotoxic. Trypan blue tests showed that less than 5% of the PBTLs were non-viable after a 1 hour exposure to 10μM Bis. The polarisation effect of the microtubule disruptor, nocadazole, on fresh PBTLs is shown in figure 3.5. As can be seen from this graph, the optimum concentration for induction of shape change was 50μM. Above this concentration, nocadazole was found to be slightly toxic, nevertheless, trypan blue tests showed that at a 50 μM concentration of nocadazole, less than 5% of the PBTLs were non-viable after a 1 hour exposure.

The effects of interleukins-2 and -15 on the polarisation of PBTLs are shown in figure 3.6. As can be seen from these data, IL-15 is almost 10 times as potent as IL-2 at inducing shape change in PBTLs. From these experiments it was decided that the concentration to use IL-2 as a model for induction of polarisation would be 10^-7M and IL-15 would be used at a concentration of 10^-8M.

Finally, the data for the cell polarisation effects of FCS on PBTLs are shown in figure 3.7. As can be seen from these data the optimum effect was seen with 40% FCS, which caused about a 20% increase in PBTL polarisation. However this is a very high FCS
concentration and would not be practical to use in subsequent experiments as a model. Therefore, 20% FCS was chosen as the concentration to be used in subsequent experiments, as this still gave an increase of about 15 - 20% polarisation.

Transmigration assay.

Although 5 factors were found to induce polarisation in human PBTLs, this was not absolute proof that they were causing motility as polarisation of the cell is only the first stage of motility. To investigate motility, the transmigration assay was used (see methods). In each experiment the PBTLs were incubated for 4 hours in the Costar transfilter systems. Also, in concert with each transmigration assay, a polarisation assay was carried out on PBTLs from the same donor to assess the polarisation response of the cells to the factor being investigated.

Bis. was found to stimulate a significant increase in transmigration of the PBTLs as can be seen in figure 3.8. As the concentration of Bis. increased so too did the degree of transmigration, which correlated with the extent of cell polarisation. This same pattern was seen with IL-2 (figure 3.9) and IL-15 (figure 3.10) (this data has been published). However, nocadazole (figure 3.11) and FCS (figure 3.12) failed to cause any degree of PBTL transmigration, even though they still caused cell polarisation in these cells as normal. Therefore, it can be concluded that nocadazole and FCS do not cause actual motility, but they do induce the first stage of motility which is cell polarisation and thus they are still useful as tools in this project for investigating the second messengers involved in inducing shape change. All transmigration / polarisation
experiments were done in triplicate with 3 different donors and the results shown in figures 3 are representative of 3 experiments.

Videomicroscopy of PBTLs treated with each of the five inducers of shape change, confirmed the results of the transmigration assays, in that Bis, IL-2 and IL-15 caused the cells to constantly change shape, whereas the FCS or nocadazole treated cells changed shape initially but then remained frozen in this shape.

To summarise this stage of the project:- 5 factors were found to cause significant cell polarisation in human PBTLs and these were Bis, nocadazole, IL-2, IL-15 and FCS. However, only Bis., IL-2 and IL-15 caused transmigration of the cells across nitrocellulose filters. Nevertheless, nocadazole and FCS can still be used to compare the induction of motility (ie: polarisation) with the other three. Using these 5 factors as tools, the next step was to analyse the second messengers induced to elucidate if there were any common elements, which would then be contenders for part of a final common pathway of motility.
Table 3.1: Summary of lymphocyte polarisation assay results.

<table>
<thead>
<tr>
<th>Factor tested</th>
<th>Non-m. MOLTs</th>
<th>PBTLs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GF109203X (Bis.)</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Nocadazole</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Interleukin-2</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Interleukin-15</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Fetal calf serum</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>MIP-1 α</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MIP-1 β</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>RANTES</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MCP-1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Interleukin-8</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Interleukin-1 α</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Interleukin-10</td>
<td>N.D</td>
<td>-</td>
</tr>
<tr>
<td>Substance P</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VIP</td>
<td>N.D</td>
<td>+</td>
</tr>
<tr>
<td>LPA</td>
<td>N.D</td>
<td>+</td>
</tr>
<tr>
<td>PDGF</td>
<td>N.D</td>
<td>+</td>
</tr>
<tr>
<td>EGF</td>
<td>N.D</td>
<td>-</td>
</tr>
<tr>
<td>TNF α</td>
<td>N.D</td>
<td>-</td>
</tr>
</tbody>
</table>

**Key:**
- +++ + + + + = 50 - 60% of cells shape changed
- +++ + + + = 40 - 50% shaded
- +++ = 20 - 30% shaded
- ++ = 10 - 20%
- + = 0 - 10%
- = no effect
- N.D = not done
Figure 3.1: Freshly isolated Peripheral Blood T Lymphocytes (magnification x630).

Figure 3.2: PBTLs treated with 10μM Bis. (magnification x630).
Figure 3.3: PBTLs treated with 50μM Nocodazole.

Figure 3.4: Dose response of PBTLs polarisation to Bis.
Figure 3.5: Dose response of PBTLs polarisation to Nocodazole

![Graph showing the dose response of PBTLs polarisation to Nocodazole concentration (μM).]

Figure 3.6: Dose response of PBTLs polarisation to IL-2 & IL-15.

![Graph showing the dose response of PBTLs polarisation to IL-2 and IL-15 concentration (Molar).]
Figure 3.7: Dose response of PBTLs polarisation to Fetal calf serum.

Figure 3.8: The effect of Bis. on the transmigration and polarisation of PBTLs
Figure 3.9: The effect of IL-2 on the transmigration and polarisation of PBTLs

Figure 3.10: The effect of IL-15 on the transmigration and polarisation of PBTLs
Figure 3.11: The effect of Nocodazole on the transmigration and polarisation of PBTLs

Figure 3.12: The effect of FCS on the transmigration and polarisation of PBTLs
Chapter 4

Investigations into the roles of intracellular calcium and phosphoinositides in lymphocyte motility

As mentioned in the introduction section, intracellular calcium ([Ca^{2+}]_i) is an important regulator in many signal transduction events\(^{(177)}\), however its role in regulation of leukocyte locomotion is not yet fully understood\(^{(178)}\).

**Intracellular calcium studies.**

These experiments were undertaken by labelling freshly isolated human PBTLs with the molecular probe FURA-2/AM and upon agonist stimulation, measuring the fluorescence via a luminescence spectrometer. Complexing with [Ca^{2+}]_i causes an increased fluorescence emission by FURA-2, (see methods).

The aim of this study was to assess whether any of the 5 inducers of shape change would affect the [Ca^{2+}]_i levels. As well as testing these 5, other factors such as the chemokines were tested as they have been reported in the literature to affect the [Ca^{2+}]_i levels in monocytes and lymphocytes\(^{(54-58,64)}\). The results of these experiments are summarised in table 4.1. All experiments in this chapter were done in triplicate unless otherwise stated. Also, all experiments with PBTLs were not only done in triplicate, but with 3 different donors. As a positive control for the [Ca^{2+}]_i measurements, an aliquot of the labelled cells were stimulated with 10μM ionomycin (used also as part of the calibration method - see methods), which causes an influx of extracellular calcium into the cell (figure 4.1).
Of the five inducers of polarisation tested, only Bis showed an effect on $[Ca^{2+}]_i$ (figure 4.2). However, the increase in intracellular calcium observed seemed to be a fluorescent artifact as the $[Ca^{2+}]_i$ increase was not a typical calcium transient which rises and then falls back to baseline (as in figure 4.7). As can be seen in fig 4.2, Bis was not fluorescing in the absence of PBTLs. Therefore, a possible answer was that once Bis entered the cells it was binding to something intracellularly and this complex was autofluorescing and giving a false signal in this system. To determine if this was indeed the case, an experiment was carried out whereby, the PBTLs were labelled with FURA-2/AM as usual and then before the experiment was run the cells were pretreated with 0.1% Triton X-100, which is a detergent which permeabilises the cells, thus allowing the cells contents to be released into the extracellular media. Then the experiment is run and Bis is added to the system. A representative experiment is shown in figure 4.3. As can be seen, Bis is causing a response even with the cells pretreated with Triton, which proves that Bis is not actually causing an increase in $[Ca^{2+}]_i$, but is in fact fluorescing non-specifically when it is in the presence of intracellular components of the cell.

Three of the chemokines, MIP-1α, MIP-1β and MCP-1, seemed to exhibit very small effects on the PBTLs $[Ca^{2+}]_i$ levels, as can be seen in figures 4.4 - 4.6. These small effects could be due to the fact that the chemokines are specific for subsets of T cells and these experiments contain large populations of all PBTLs. However, some clear results were obtained by using non-motile MOLT-4 cells in this system. An example can be seen in figure 4.7, in which $10^{-7}$M MCP-1 causes a transient increase in $[Ca^{2+}]_i$ of about 200 nM. It must be noted here also that MCP-1 has no polarisation effect at all on the non-motile MOLT-4 cells. Similar $[Ca^{2+}]_i$ fluxes in non-motile MOLT-4 cells were seen
with MIP-1α, MIP-1β and RANTES, but these results were not as reproducible as the MCP-1 effect. These 3 chemokines also had no effect on the polarisation of the MOLT cells. Hence, it can be concluded from this data that an increase in \([Ca^{2+}]_i\) does not seem to be essential for the induction of motility in lymphocytes.

Indeed, further experiments provided evidence for the theory that an increase in \([Ca^{2+}]_i\), actually prevents the induction of motility. In the first set of these experiments, the motile variant of the MOLT-4 cells were used in an experiment in which they were exposed to various concentrations of ionomycin, a compound which permeabilises the cell membranes and allows an influx of extracellular calcium into the cell. A representative experiment can be seen in Figure 4.8. The motile MOLT-4 cells were incubated with various concentrations of ionomycin for 30 minutes. As can be clearly seen, as the concentration of ionomycin is increased, the cells become less polarised and round up. The non-motile MOLT-4 cells were also tested for their response to ionomycin exposure. As with the motile variant, the non-motile cells were incubated with various concentrations of ionomycin \((10^{-7} \text{M to } 10^{-12} \text{M})\) for 30 minutes, but no effects on the shape of the cells were observed (data not shown), thus indicating that an elevation in \([Ca^{2+}]_i\) is not enough to cause shape change. Ionomycin was also tested to see whether after induction of polarisation in PBTLs, ionomycin would reverse the shape change and revert the cells back to a spherical, non-motile state. As can be seen in Figure 4.9, this was indeed the result. Ionomycin reversed the polarisation induced by each of the five factors after 30 minutes. Thus it seemed at this point that by increasing the \([Ca^{2+}]_i\), this in turn prevented motility.
The endosomal Ca\(^{2+}\) - ATPase inhibitor, thapsigargin has been reported in the literature to increase [Ca\(^{2+}\)], levels in lymphocytes\(^{(179)}\). Thapsigargin was then tested to see if it, like ionomycin reduced the extent of polarisation in motile MOLT-4 cells and prevented the polarisation induced in PBTLs. Thapsigargin was found to have no effect on the polarisation of motile MOLT-4 cells and no effect on their [Ca\(^{2+}\)], levels (data not shown). In experiments in which PBTLs were pretreated with thapsigargin for 15 minutes and then stimulated with either of the five inducers of shape change for a further 45 minutes, it was observed that thapsigargin significantly prevented the induction of polarisation in these cells (figure 4.10). To prove that thapsigargin does indeed cause an increase in [Ca\(^{2+}\)], levels in PBTLs, experiments with FURA-2 labelled PBTLs were carried out in which they were stimulated with 10uM thapsigargin. A representative experiment can be seen in figure 4.11, in which thapsigargin induces a [Ca\(^{2+}\)], increase of about 135nM. However when the cells are exposed to 5mM EGTA for 5 minutes before the experiment is started there is no increase in [Ca\(^{2+}\)], upon thapsigargin stimulation. This shows that the majority of the increase in [Ca\(^{2+}\)], is not from the release of Ca\(^{2+}\) from intracellular stores but a concomitant influx of Ca\(^{2+}\) from the extracellular medium due to increases in plasma membrane Ca\(^{2+}\) permeability. It is thought that a second messenger known as Ca\(^{2+}\) influx factor (CIF) is released or generated from the endoplasmic reticulum or adjacent regions once the [Ca\(^{2+}\)], concentration in this organelle falls beneath a critical level\(^{(180)}\). Further evidence to support the notion that an increase in [Ca\(^{2+}\)], actually prevents the induction of motility is shown in figure 4.12. Again PBTLs that are pretreated with thapsigargin are unable to polarise upon stimulation with the 5 inducers of shape change. However when the same experiment is done again but the cells are pretreated with 5mM EGTA (to chelate the extracellular
Ca\(^{2+}\)) before treatment with thapsigargin, then the PBTLs are able to polarise as normal, thus indicating that the increase in [Ca\(^{2+}\)]\(_i\) induced by thapsigargin is of extracellular origin.

**Phosphoinositide studies.**

In recent years phosphoinositides have been shown to play a key role in signal transduction\(^{181,182}\). As mentioned in the introduction section, there are links between alterations in polyphosphoinositides (pPI's) and changes in [Ca\(^{2+}\)]\(_i\) levels. Stimulation of cell surface receptors initiates hydrolysis of a membrane-bound inositol lipid, which produces at least two second messengers, - diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP\(_3\)). These messengers are generated by a membrane transduction process comprising 3 main components: a receptor, a coupling G protein and phosphoinositidase C. DAG acts by stimulating PKC whereas IP\(_3\) releases calcium from internal stores.

The first investigation into the role of pPI’s in the induction of motility was by assaying IP\(_3\) production in PBTLs upon stimulation with inducers of shape change. This work was done using the IP\(_3\) assay kit from Amersham (see methods). A representative experiment can be seen in **figure 4.13.** It is clear from this data that there are no significant changes in IP\(_3\) levels detected with this methodology when the PBTLs are stimulated by the inducers of shape change. In these experiments the cells were stimulated with the agonists for a standard time of one minute. From this data it seems that IP\(_3\) is not involved in a motility signal transduction pathway.

The involvement of the phosphoinositide 3-kinase (PI 3-kinase) in the induction of motility was investigated by using specific inhibitors of the kinase. A fungal metabolite
known as wortmannin has proved to be a selective inhibitor of PI 3-kinase, if used at sub-100nM concentration\textsuperscript{(183)}. PBTLs were preincubated with various concentrations of wortmannin for 30 minutes and then stimulated with the 5 inducers of polarisation for 1 hour. A representative experiment can be seen in figure 4.14. Wortmannin has prevented polarisation of the PBTLs but only at concentrations above 100nM. It must be noted at this point that wortmannin has an IC\textsubscript{50} ~3nM, therefore at concentrations above 100nM, wortmannin is no longer specific for PI 3-kinase and affects other systems such as phospholipase D, myosin light chain kinase and pleckstrin. Wortmannin was found to have no effect at all on the motile MOLT-4 cells (data not shown).

Another specific PI 3-kinase inhibitor is a compound known as LY294002\textsuperscript{(184)}, which inhibits PI 3-kinase activity with an IC\textsubscript{50} of 1.4μM. This compound was also tested to see whether it would prevent the induction of polarisation in PBTLs. PBTLs were preincubated for 15 minutes in the presence of various concentrations of LY294002 and then stimulated with the 5 inducers of polarisation for 1 hour. A representative experiment can be seen in figure 4.15. The inhibitor was found to significantly inhibit the induction of polarisation by all five inducers at concentrations as low as 100nM. Therefore these data suggest that PI 3-kinase may be involved in a motility pathway. However, the LY294002 compound was found to have no effect whatsoever on the polarisation of the motile MOLT-4 cells (data not shown). This seems to be a conflicting result to the experiments with wortmannin, however the fact is that wortmannin is less stable than LY294002 and this could explain why wortmannin did not inhibit motility.
When administered to animals, lithium induces subtle alterations in neural activity (for example in manic-depressive illness and diurnal rhythms) and early development (teratogenesis). Lithium is known to reduce the supply of inositol, the key substrate for the phosphoinositide cascade by inhibiting some of the enzymes which hydrolyse the inositol phosphates\(^\text{185}\). Thus lithium inhibits signal transduction indirectly by slowing down the supply of the precursor lipid required to generate messengers such as IP\(_3\) and DAG.

Therefore, as an additional approach to investigate the involvement of phosphoinositides in motility, lithium chloride was tested for effects on the polarisation of PBTLs and MOLT-4 cells. In figure 4.16 is a representative experiment in which PBTLs were preincubated with lithium chloride for 30 minutes prior to stimulation with the various inducers of polarisation for 1 hour. A concentration of 100mM lithium was found to inhibit polarisation by all five agonists, however at lower concentrations only the effects of IL-2, IL-15 and FCS were blocked. Lithium chloride was found to have no effect whatsoever on the polarisation of the motile variant of the MOLT-4 cells. Reports in the literature suggest a concentration of 10mM for 30 minutes is sufficient to disrupt the phosphoinositide cascade\(^{181,199}\).

To summarise this chapter; it was found that an increase in \([\text{Ca}^{2+}]_i\) was not essential for the cells to polarise, in actual fact it was found that an increase in \([\text{Ca}^{2+}]_i\) levels inhibited polarisation. PBTLs polarisation did not increase intracellular IP\(_3\) levels and the role of PI 3-kinase in the signal transduction of motility was uncertain as one PI 3-kinase
inhibitor (wortmannin) did not inhibit motility, whilst another PI 3-kinase inhibitor (LY294002) did.

However, experiments with lithium suggest that ppIs may be involved in motility, as it prevented polarisation by all five shape change inducers. It seems that the ppl’s are more important for the signal transduction events utilised by IL-2, IL-15 and FCS. The results obtained with the MOLT-4 cells did not always back up those of the PBTLs, but these are transformed cells and they are already motile, therefore it would be expected that compounds which inhibit the induction of motility, would have no effect.
### Table 4.1: Summary of intracellular calcium studies on PBTLs

<table>
<thead>
<tr>
<th>Factor Tested</th>
<th>Increase in $[\text{Ca}^{2+}]_{i}$ (nM)</th>
<th>% Cells polarised</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis. (GF109203X) (10μM)</td>
<td>0 (autofluorescence)</td>
<td>↑ 50 - 60%</td>
</tr>
<tr>
<td>Interleukin-2 (10^{-7}M)</td>
<td>0</td>
<td>↑ 20 - 30%</td>
</tr>
<tr>
<td>Interleukin-15 (10^{-8}M)</td>
<td>0</td>
<td>↑ 20 - 30%</td>
</tr>
<tr>
<td>Nocodazole (50μM)</td>
<td>0</td>
<td>↑ 20 - 30%</td>
</tr>
<tr>
<td>Fetal calf serum (10%)</td>
<td>0</td>
<td>↑ 10 - 20%</td>
</tr>
<tr>
<td>MIP-1α (10^{-7}M)</td>
<td>0 - 10</td>
<td>↑ 10 - 20%</td>
</tr>
<tr>
<td>MIP-1β (10^{-7}M)</td>
<td>0 - 10</td>
<td>↑ 0 - 10%</td>
</tr>
<tr>
<td>MCP-1 (10^{-7}M)</td>
<td>0 - 10</td>
<td>↑ 0 - 10%</td>
</tr>
<tr>
<td>Interleukin-8 (10^{-7} to 10^{-9}M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RANTES (10^{-7} to 10^{-9}M)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Figure 4.1: The effect of 10μM Ionomycin on PBTLs [Ca$^{2+}$]$_{i}$ levels

[Graph showing the effect of Ionomycin on calcium levels]
Figure 4.2: The effect of Bis. on PBTLs $[Ca^{2+}]_i$ levels

A = No cells and 10uM BIS.
B = PBTL's and 1uM BIS.
C = PBTL's and 10uM BIS.

Figure 4.3: The effect of Bis. on PBTLs $[Ca^{2+}]_i$ levels, +/- pretreatment with Triton X-100
Figure 4.4: The effect of MIP-1α on PBTLs [Ca^{2+}]_i levels

Figure 4.5: The effect of MIP-1β on PBTLs [Ca^{2+}]_i levels
Figure 4.6: The effect of MCP-1 on PBTLs \([\text{Ca}^{2+}]_i\) levels

Figure 4.7: The effect of MCP-1 on non-motile MOLT-4 cells \([\text{Ca}^{2+}]_i\) levels
Figure 4.8: The effect of Ionomycin on the polarisation of motile MOLT-4 cells

![Graph showing the effect of Ionomycin on the polarisation of motile MOLT-4 cells. The x-axis represents the Ionomycin concentration (control, 0.1uM, 0.5uM, 1uM, 5uM) and the y-axis represents the percentage of polarised MOLT-4 cells. There is a significant decrease in polarisation with increasing Ionomycin concentration.]

Figure 4.9: The effect of Ionomycin on the polarisation of PBTLs

![Graph showing the effect of Ionomycin on the polarisation of PBTLs. The x-axis represents the treatment (control, 10uM BIS, ZN 400, 10(7)M IL-2, 10(8)M IL-15, 20% FCS) and the y-axis represents the percentage of polarised PBTLs. There is a decrease in polarisation with Ionomycin treatments, with 10uM Ionomycin being the most effective.]
Figure 4.10: The effect of thapsigargin on the polarisation of PBTLs

- No Thapsigargin
- 0.01uM Thapsigargin
- 0.1uM Thapsigargin
- 1uM Thapsigargin
- 10uM Thapsigargin
Figure 4.11: The effect of thapsigargin on the $[Ca^{2+}]_{i}$ levels of PBTLs

Figure 4.12: The effect of thapsigargin on the polarisation of PBTLs pretreated with 5mM EGTA
**Figure 4.13:** Assay of IP₃ production in PBTLs upon polarisation

![Graph showing IP₃ production](image)

**Figure 4.14:** The effect of wortmannin pre-treatment on induction of polarisation in PBTLs

![Bar graph showing the effect of wortmannin](image)
Figure 4.15: The effect of LY294002 pre-treatment on the induction of polarisation in PBTLs

Figure 4.16: The effect of lithium chloride pre-treatment on the induction of polarisation in PBTLs
Chapter 5

Investigations into the roles of intracellular pH and ion channels in lymphocyte motility

Introduction.

Maintenance of intracellular pH (pH$_i$)\(^{(186)}\) within eukaryotic cells is dependent on the concerted action of a number of specific transporters and ion channels\(^{(186-88)}\). Together these proteins act to regulate pH$_i$ to specific and characteristic values for a given cell type\(^{(187)}\). The need to control intracellular pH reflects the exquisite pH sensitivity of many biological processes, such as protein synthesis, ion conductivities and DNA replication\(^{(189)}\). Recent studies have also suggested that many cellular activation processes mediated by growth factors and other exogenous stimuli involve changes of pH$_i$, as part of the activation process\(^{(190,191)}\). Neutrophils, for example, change their intracellular pH after they encounter chemotactic factors\(^{(192-94)}\). When this pH change is prevented pharmacologically, neutrophils do not respond to the chemotactic agent, indicating the importance of this ionic alteration\(^{(192)}\).

The major components of the pH regulatory apparatus involve both sodium-dependent and sodium-independent processes. The Na$^+$/H$^+$ antiporter is a ubiquitous transporter involved in pH homeostasis, sodium concentration and regulation of cell volume\(^{(186-88)}\). Activity of this transporter is affected by various effector molecules, including serum factors\(^{(190,191)}\) and is inhibited selectively by certain amiloride derivatives\(^{(195)}\). In addition
to the Na\(^+\)/H\(^+\) antiporter, both sodium-dependent and sodium-independent bicarbonate exchangers have been identified as participants in pH homeostasis\(^{(186-88)}\).

**Intracellular pH measurements**

In view of the importance of pH in the regulation of cellular activities, agents that alter pH\(_i\) would be expected to influence a number of cell functions. Therefore, it was investigated, if by inducing motility in PBTLs, this would then affect the pH\(_i\). These experiments were carried out by labelling fresh PBTLs with BCECF/AM and then measuring the fluorescence emitted upon agonist stimulation in a luminescence spectrometer (see methods). All five inducers of polarisation were tested in this system. None of these showed any effects whatsoever on the pH\(_i\) of PBTLs or non-motile MOLT-4 cells. A representative result is shown in figure 5.1, in which PBTLs were stimulated with 10μM Bis. The positive control for these experiments was 40mM sodium propionate (figure 5.2). Within each experiment, an aliquot of the BCECF labelled cells was tested with 40mM sodium propionate as a positive control. Weak organic acids such as sodium propionate lower pH\(_i\) within eucaryotic cells by their passive diffusion as free acids across the plasma membrane and their subsequent dissociation within the cytosol\(^{(186)}\). The ability of mammalian cells to recover from this acute acid load is the result of the Na\(^+\)/H\(^+\) exchanger\(^{(186)}\).

**Role of ion channels in motility**

There is evidence in the literature which suggests that the Na\(^+\)/H\(^+\) exchanger has an important role to play in the locomotion of neutrophils\(^{(198)}\). Therefore it was investigated by using specific inhibitors of the Na\(^+\)/H\(^+\) antiporter, whether these would inhibit
motility in lymphocytes. The inhibitors used were amiloride and its derivatives\(^\text{195}\). The effect of these Na\(^+\)/H\(^+\) antiporter inhibitors on the polarisation of motile MOLT-4 cells is shown in figure 5.3. The most potent compounds were amiloride 5-((N,N-hexamethylene) \((A130)\), amiloride 5-((N-methyl-N-isobutyl) \((A149)\) and amiloride 5-((N-ethyl-N-isopropyl) \((A171)\), which virtually rounded up all the cells at 100\muM. The other two inhibitors, amiloride 5-((N,N-dimethyl)-hydrochloride \((A125)\) and amiloride hydrochloride \((A113)\) were less potent as they only had effects at 500\muM and above. In these experiments, the cells were incubated with the inhibitors for a period of 1 hour. All experiments in this chapter were done in triplicate and all experiments with PBTLs were also done with 3 different donors, unless otherwise stated. In figures 5.4 - 5.8 are representative results of experiments in which PBTLs were pre-incubated with the particular amiloride derivative for 30 minutes and then the PBTLs were incubated with an inducer of shape change for 1 hour. A130 (fig. 5.4), A149 (fig. 5.5) and A171 (fig. 5.6), all inhibited polarisation of the PBTLs at a concentration of 100\muM. A125 inhibited shape change at a higher concentration of 500\muM (fig. 5.7) and A113 was the least potent as it inhibited shape change significantly at a concentration of 1mM (fig. 5.8).

To prove that the amiloride compounds were indeed preventing motility by inhibiting the action of the Na\(^+\)/H\(^+\) antiporter, it was investigated whether the amilorides would decrease the pH\(_i\) of motile MOLT-4 cells. In theory, if the amilorides were indeed blocking the antiports, this would lead to a build up of H\(^+\) ions within the cell and a decrease in pH\(_i\). A representative experiment can be seen in figure 5.9, in which BCECF labelled motile MOLT-4 cells were stimulated with 100\muM A130 after 60 seconds.
Although there is an increase in fluorescence upon stimulation with A130, there is also an increase in the negative control (i.e., in the presence of no cells). This autofluorescence was seen with all the amiloride derivatives. Attempts at the same experiment were made using an alternative pH$_i$ probe termed SNARF-1 which works at different wavelengths to BCECF. Although the amilorides were found not to autofluoresce at the wavelengths used by SNARF-1, this probe was found not to be sensitive enough to pick up any changes in pH$_i$ (data not shown).

Therefore it could not be proved directly that the amilorides were blocking the antiports. However an indirect way of investigating whether the antiport is being blocked is by testing the ability of the cells to recover from an acute acid load. In figure 5.10 can be seen a representative experiment in which control motile MOLT-4 cells were subjected to a 40mM dose of sodium propionate which caused an acute drop in pH$_i$ which then returned to normal over time due to the activity of the Na$^+$/H$^+$ antiporter which pumps out the H$^+$ ions. However, MOLT-4 cells which have previously been treated for 5 minutes with 100μM A171, have their recovery from the sodium propionate load impaired resulting in a sustained acidification, suggesting that the antiporters have been blocked. However, it must be noted at this point that these experiments were very difficult to do and the result shown in fig. 5.10 is not very convincing, but there is a better example of this type of experiment in fig. 5.19.

A question which arises from this data is whether the decrease in pH$_i$, caused by inhibition of the antiporter is the reason that amiloride is blocking polarisation of the cells. Thus, an experiment was set up whereby motile MOLT-4 cells were exposed to various concentrations of sodium propionate and the resulting pH$_i$ measured and extent
of cell polarisation quantified. As can be seen in figure 5.11, as the concentration of sodium propionate increases from 1mM, at which there is no effect, to 40mM, the extent of intracellular acidification increases. In figure 5.12 is a representative experiment in which motile MOLT-4 cells are subjected to various doses of sodium propionate for 5, 15 and 30 minutes. A dose of 1mM has little effect on cell polarisation as on pH_i, but a concentration of 20mM causes the cells to round up and to a further extent with 40mM. This pattern correlates with the effects on pH_i. It does seem however that the effects are transient, with a more pronounced effect after 5 minutes but after 30 minutes exposure to sodium propionate there is no effect on the cells polarisation, which is probably due to the cells recovery from the acidification. This data suggests that it is the intracellular acidification which is responsible for making the cells non motile.

Is there a difference in the pH_i levels between motile and non motile MOLT-4 cells? Also is there a difference in their ability to recover from an acute acid load? To answer these questions a number of experiments were carried out whereby both motile and non-motile MOLT-4 cells were subjected to 40mM sodium propionate and then the recovery to basal pH_i was measured over a 30 minute period. A representative result is shown in figure 5.13. In this experiment there is a difference in basal levels of pH_i, between the two cell types, however this varied from one experiment to another, with the motile cells exhibiting a higher pH_i in one experiment and the non-motiles exhibiting a higher one in another, therefore, very little can be read into this. Also, both types of MOLT-4 cells were identical in their ability to recover from an acute intracellular acidification. Hence, it can be concluded that pH_i, is not an important difference between motile and non-motile MOLT-4 cells.
As well as the amiloride compounds, a number of proprietary compounds were tested which are more potent than commercially available amiloride derivatives and with IC_{50}'s for antiport inhibition of sub-μM concentrations in other experimental systems (N. Matthews, personal communication). These inhibitors were termed inhibitor 1, inhibitor 2, etc.. As in the previous experiment with the amiloride derivatives, the inhibitors were incubated at 37°C with motile MOLT-4 cells for a period of 1 hour. A representative experimental result can be seen in figure 5.14. As can be clearly seen, only inhibitor 3 had any effect on the motile MOLT-4 cells polarisation, rounding up all the cells at 100μM. The antiport inhibitors were then investigated in experiments with PBTLs, to see if they could block the induction of shape change. PBTLs were incubated for 30 minutes with the inhibitor being tested and then the PBTLs were stimulated by one of the inducers of polarisation for 1 hour. Inhibitor 1 had some effects at reducing the extent of polarisation (figure 5.15). Inhibitor 2 had no effect whatsoever (data not shown). Inhibitor 3 was found to be the most effective of the six inhibitors, as it nearly blocked the effects of the inducers of polarisation at a concentration of 100μM (figure 5.16). Inhibitor 4 was slightly more potent than inhibitor 1 (figure 5.17) and inhibitor 5 had a slight effect at 100μM concentration (figure 5.18). Inhibitor 6 had no effect at all (data not shown).

From this data, inhibitor 3 proved to be the most potent compound and as with the amiloride A171, it was investigated whether inhibitor 3 was actually blocking the antiporter, by testing the ability of inhibitor 3 treated motile MOLT-4 cells to recover from an acute acid load. A representative experiment is shown in figure 5.19, in which the control cells (not treated with an antiporter inhibitor) recover from the acid load as
normal. However motile MOLT-4 cells that were treated with 100μM inhibitor 3 for 5 minutes prior to the start of the experiment, were unable to recover from the acid load as quickly as control cells, thus indicating that the inhibitor 3 is indeed targeting the antiporter.

To summarise, this data indicates that the induction of polarisation in MOLT-4 cells and PBTLs does not affect the pH$_i$ of the cells, however upon intracellular acidification, motile MOLT-4 cells lose their polarity. Using inhibitors, it was found that inhibition of the antiports, which in turn caused intracellular acidification, prevented the cells from polarising.

**Chloride channels**

Research in the past has shown that chloride movements, which occur via Cl$^-$ channels or a Cl$^-$ transporter, have many physiological roles in various cells, such as pH control$^{(201,202)}$ and cell volume control$^{(201,203,204)}$. Also it has been found that upon agonist stimulation, there is a Cl$^-$ efflux from human neutrophils$^{(205)}$.

It was therefore decided, to investigate the role if any of Cl$^-$ channels in lymphocyte motility. A number of Cl$^-$ channel blockers were used in polarisation assays to assess whether they could block or reverse shape change. In figure 5.20 is a representative experiment in which motile MOLT-4 cells were incubated for 1 hour with one of 3 inhibitors, quinidine, niflumic acid or 5-nitro-2[3'-phenylpropylamino]benzoic acid (NPPB). As can be seen, NPPB was the most effective as it virtually rounded up all the cells at 100μM. At the same concentration niflumic acid and quinidine only rounded up
half the cells. Tamoxifen (trans-) which as well as being an anti-oestrogen, is a chloride channel blocker and significantly inhibited motile MOLT-4 polarisation as can be seen in **figure 5.21**. At a concentration of 12.5µM, tamoxifen inhibited all polarisation of the motile MOLT-4 cells. Tamoxifen had the same potency at blocking induction of polarisation in PBTLs, as can be seen in **figure 5.22**. This is a representative experiment in which tamoxifen was incubated with the PBTLs for 15 minutes and then the cells were stimulated with one of the five inducers of polarisation for 1 hour. Using this same experimental protocol, the other Cl channel inhibitors were tested for their ability to block polarisation in PBTLs. NPPB was effective at a concentration of 100µM (**figure 5.23**), whereas quinidine (**figure 5.24**) and niflumic acid (**figure 5.25**) were less effective, both only blocking the extent of polarisation by about 25%, at a 100µM concentration.

To determine if these chloride channel blockers were indeed targeting the chloride channels, a number of experiments were done whereby MOLT-4 cells were labelled with 14C Taurine (see methods). It has been validated in the literature that the physiological role of volume regulated chloride channels relates not only to their permeability to the inorganic Cl but to their permeability to larger organic osmolytes such as taurine and so therefore the measurement of taurine efflux from hypotonically shocked, taurine labelled cells is an indicator of volume regulated chloride channel activity. In **figure 5.26** is a representative experiment in which both motile and non-motile MOLT-4 cells were compared in terms of taurine efflux after hypotonic shock. As can be seen from this data, taurine uptake by both types of cells were roughly the same and upon hypotonic shock to increase the cell volume, the volume regulated chloride channels are activated.
in both cell types. Also the volume regulated chloride channel (as measured by taurine efflux) does not appear to be spontaneously activated in motile MOLT-4 cells. Upon hypotonic shock, the amount of taurine released did not differ that much between 10 minutes and 30 minutes in the presence of water. In figure 5.27, is a representative experiment, whereby motile MOLT-4 cells were treated with one of the Cl\(^-\) channel blockers for 5 minutes and then the cells were hypotonically shocked to test whether the blocker could indeed block the taurine efflux. In these experiments the cells were hypotonically shocked for 20 minutes. As can be seen from fig. 5.27, the control cells all released a small amount of taurine, however it was observed that tamoxifen (12.5\(\mu\)M) seemed to cause some spontaneous activation (or toxicity) of the channels as even without hypotonic shock the tamoxifen treated cells were releasing more taurine than control cells. Upon hypotonic shock, the only compound which seemed to have any effect was NPPB which blocked half of the taurine efflux. Niflumic acid and Quinidine had no effect on blocking the taurine efflux and tamoxifen actually caused more taurine efflux than control cells. The concentrations of the blockers used in these experiments was the concentration that gave maximum inhibition of motility in motile MOLT-4 cells (figures 5.20 and 5.21).

Therefore, this data suggests that blocking the volume regulated chloride channels does not seem to be the mechanism by which these compounds (except tamoxifen) inhibit motility and they must be targetting other elements of the cell machinary.

It was also found that none of the Cl\(^-\) channel blockers had any effect on the pH\(_i\) of either PBTLs or motile MOLT-4 cells (data not shown). Hence, these data suggest that the Cl\(^-\)
channel blockers can inhibit lymphocyte motility by a mechanism independent of pH regulation.

**Figure 5.1: The effect of Bis on the pH of PBTLs**

![Graph showing the effect of Bis on pH of PBTLs over time](image)
Figure 5.2: The effect of sodium propionate on pH, of PRTLs

![Graph showing the effect of sodium propionate on pH of PRTLs.]

Figure 5.3: The effect of amilorides on the polarisation of motile MOLT-4 cells

![Graph showing the effect of amilorides on the polarisation of motile MOLT-4 cells.]

% motile MOLT-4 cells polarised (+/ SEM)

concentration (μM)
Figure 5.4: The effect of amiloride A130 on the induction of polarisation in PBTLs

Figure 5.5: The effect of amiloride A149 on the induction of polarisation in PBTLs
Figure 5.6: The effect of amiloride A171 on the induction of polarisation in PBTLs

Figure 5.7: The effect of amiloride A125 on the induction of polarisation in PBTLs
Figure 5.8: The effect of amiloride A113 on the induction of polarisation in PBTLs

![Bar chart showing the effect of amiloride A113 on polarisation in PBTLs.]

- control
- 1μM A113
- 10μM A113
- 100μM A113
- 1mM A113

Figure 5.9: The effect of amiloride A130 on the pH of motile MOLT-4 cells

![Graph showing the pH of motile MOLT-4 cells with and without cells.]

- + Cells
- No Cells
- 100μM A130
Figure 5.10: The effect of A171 on the ability of motile MOLT-4 cells to recover from an acute acid load

Figure 5.11: The effect of sodium propionate on the pH of motile MOLT-4 cells
Figure 5.12: The effect of sodium propionate on the polarisation of motile MOLT-4 cells

Figure 5.13: Comparison between motile and non-motile MOLT-4 cells in their ability to recover from an acute acid load
Figure 5.14: The effect of Na\(^+\)/H\(^+\) antiport inhibitors on the polarisation of motile MOLT-4 cells

![Graph showing the effect of Na\(^+\)/H\(^+\) antiport inhibitors on MOLT-4 cells.](image1)

Figure 5.15: The effect of antiport inhibitor-1 on the induction of polarisation in PBTLs

![Graph showing the effect of antiport inhibitor-1 on PBTLs.](image2)
Figure 5.16: The effect of antiport inhibitor-3 on the induction of polarisation in PBTLs

Figure 5.17: The effect of antiport inhibitor-4 on the induction of polarisation in PBTLs
Figure 5.18: The effect of antiport inhibitor-5 on the induction of polarisation in PBTLs

Figure 5.19: The effect of antiport inhibitor-3 on the ability of motile MOLT-4 cells to recover from an acute acid load
Figure 5.20: The effect of Cl⁻ channel inhibitors on the polarisation of motile MOLT-4 cells

![Bar chart showing the percentage of MOLT-4 cells polarised with various concentrations of Cl⁻ channel inhibitors.](image)

- NPPB
- Niflumic acid
- Quinidine

Concentration (uM)

Figure 5.21: The effect of tamoxifen on the polarisation of motile MOLT-4 cells

![Bar chart showing the percentage of MOLT-4 cells polarised with various concentrations of tamoxifen.](image)

Concentration (uM)
Figure 5.22: The effect of tamoxifen on the induction of polarisation in PBTLs

Figure 5.23: The effect of NPPB on the induction of polarisation in PBTLs
Figure 5.24: The effect of quinidine on the induction of polarisation in PBTLs

Figure 5.25: The effect of niflumic acid on the induction of polarisation in PBTLs
Figure 5.26: Comparison of $^{14}$C Taurine efflux from motile and non-motile MOLT-4 cells.

Figure 5.27: Do the Cl$^{-}$ channel blockers block volume regulated chloride channels in motile MOLT-4 cells as assayed by $^{14}$C Taurine efflux.
Chapter 6

Investigations into the roles of renaturable kinases in lymphocyte motility

Introduction

In 1989, Ferrell and Martin (206), utilized a method to search for novel protein kinases in platelets that involved subjecting lysates to SDS-PAGE, transferring the proteins to nitrocellulose and renaturing the blotted enzymes. The protein kinases were detected by autoradiography after autophosphorylation with $[\gamma^{32}P]ATP$. These renaturation kinase assays have since demonstrated the activation of multiple protein kinases in a variety of cell types, including chemoattractant treated neutrophils (207-209) and chronic treatment of large granular lymphocytes and T cells with okadaic acid (210).

Renaturable kinase assay

A modified version of the method above (see methods) was used to determine if there was any activation of renaturable protein kinases upon induction of polarisation in freshly isolated human PBTLs. PBTLs were treated for 1 hour at $37^\circ C$, with an inducer of polarisation, then lysed and the proteins separated by SDS-PAGE. The gel was then renatured as described in methods section and the activities of autophosphorylated protein kinases were located by autoradiography after exposure to $[\gamma^{32}P]ATP$. It must be noted at this point that with every experiment, an aliquot of the treated PBTLs was fixed and scored for the extent of polarisation induced. In the results shown, the inducers of
polarisation were found to induce shape change to the expected degree as shown in chapter 1.

The results shown in figures 6.1 and 6.2 are representative of 4 experiments, all done with different PBTL donors. In figure 6.1, the most prominent event is the Bis induced increase in autophosphorylation of a 58kDa band (indicated by the arrow). This same band was induced in all four experiments by Bis. Also in figure 6.1, there is a significant increase in autophosphorylation of a doublet (indicated by the arrows), of 98 and 92kDa by IL-2, IL-15 and nocadazole. This same doublet seems to be increased also in figure 6.2 by IL-2, Bis and FCS, however these were not found to be reproducible in further experiments.

Collaborative work with C. Southern in this Institute has shown that activation of the 58kDa kinase by Bis occurs after 1 minute and increases up to a maximum level after 30 minutes. This time scale also correlates with the degree of polarisation of non motile MOLT-4 cells induced by Bis. In the case of the 58kDa band, phosphoamino acid analysis revealed autophosphorylation on threonine residues and recent work has identified the 58kDa kinase as being mammalian ste20-like kinase-1 (MST-1) (personal communication, C. Southern).

Hence, there is some evidence to suggest that this 58kDa kinase (MST-1) may be involved in a motility pathway, however, it is not conclusive as IL-15, IL-2, FCS and nocadazole do not seem to affect the kinase as Bis does.
**Figure 6.1:** The effect of induction of polarisation in PBTLs on renaturable kinases autophosphorylation

A = Control  
B = 10μM Bis  
C = 10^{-7} M IL-2  
D = 10^{-8} M IL-15  
E = 50μM Nocadazole

(Refer to the figure for the protein bands' approximate molecular weights in kDa indicated on the left of the picture.)

**Figure 6.2:** The effect of induction of polarisation in PBTLs on renaturable kinases autophosphorylation

A = Control  
B = 10^{-7} M IL-2  
C = 10^{-8} M IL-15  
D = 10μM Bis.  
E = 20% FCS

(Refer to the figure for the protein bands' approximate molecular weights in kDa indicated on the left of the picture.)
Chapter 7

Investigations into the roles of tyrosine phosphorylation in lymphocyte motility

Introduction

Tyrosine phosphorylation is now recognised as a key mechanism by which cytokine receptors and antigen receptors of lymphocytes initiate intracellular events\(^{211}\), also, the phosphorylation of signalling proteins on tyrosine is essential for cellular regulation of growth and differentiation.

Tyrosine phosphorylation studies

These experiments were undertaken (as detailed in methods section) by incubating freshly isolated human PBTLs with one of the five inducers of polarisation at 37°C for one hour. Then the cells were lysed and the proteins separated by SDS-PAGE, after which the gels were subjected to Western blotting with the nitrocellulose blots being probed for phosphorysine. With each experiment, an aliquot of the treated cells was checked for extent of polarisation induced. In all experiments shown, there was a normal degree of polarisation induced as shown in chapter 1.

As can be seen in figure 7.1 (representative of 3 experiments with 3 different donors), only IL-2 caused any change in tyrosine phosphorylation levels in whole cell lysates of treated PBTLs. There is a significant increase in tyrosine phosphorylation of a protein band of \(~110\text{kDa}\) molecular weight (indicated by the arrow). There is unequal loading
of protein in the FCS treated lane, however other experiments showed FCS to have no
effect on tyrosine phosphorylation levels (data not shown). Later experiments with IL-15
showed it to have the same effect as IL-2, (figure 7.2), (representative of 3 experiments
with 3 different donors), in that it also caused tyrosine phosphorylation of a protein band
of ~110kDa. To further test the involvement of tyrosine phosphorylation in lymphocyte
motility, experiments were carried out in which established tyrosine kinase inhibitors
were incubated with the PBTLs prior to them being induced to polarise by Bis, etc.

Genistein, tyrphostin A25 and tyrphostin A47, were all tested for inhibiting induction of
polarisation in PBTLs by all five inducers of polarisation, by incubating the cells with
the relevant inhibitor for 30 mins at 37°C and then stimulating the cells with one of the
inducers of polarisation for one hour at 37°C. The inhibitors were tested at
concentrations from 100nM to 1mM and all experiments were done in triplicate with
cells from 3 different donors. None of these inhibitors had any effect at preventing the
extent of polarisation induced (data not shown). However, similar experiments with the
tyrosine kinase inhibitor herbimycin A (IC₅₀ = 1µM), proved otherwise (figure 7.3).
PBTLs were incubated with 5x10⁻⁵ herbimycin A for 30 minutes (as done previously by
P.C.Wilkinson(82)) and then stimulated with the inducers of polarisation for 1 hour at
37°C. As can be seen from figure 7.3, herbimycin A has significantly reduced the extent
of polarisation induced by Bis, nocadazole, IL-2, IL-15 and FCS. The experimental data
shown is representative of 3 experiments done with 3 different blood donors.
Thus, this data suggests that there is something common between the 5 different
treatments that induces polarisation, that is in turn inhibited by herbimycin A.
The next obvious experiment to do was to investigate whether IL-2 and IL-15 could still cause tyrosine phosphorylation of the 110kDa protein if the cells were pretreated with herbimycin A (thus reducing the extent of motility). Representative results of these experiments are shown in figures 7.4 and 7.5. The experiments as usual were done 3 times with different blood donors. The cells were treated as in figure 7.3. As can be seen in figure 7.4, herbimycin A has not prevented the tyrosine phosphorylation of the 110kDa band by IL-2 or IL-15 as can be seen in figure 7.5. This data suggests that herbimycin A is not preventing the 110kDa protein (p110) from being tyrosine phosphorylated, so therefore herbimycin A must be inhibiting its target downstream from the p110. This theory does not rule out the possibility that the p110 might be part of a motility signal transduction pathway, common to both IL-2 and IL-15.

So what is the identity of the p110? Evidence from the literature suggests the closest possibility is Janus kinase-3 (JAK-3), which has a molecular weight of ~120kDa. However, attempts to immunoprecipitate JAK-3 from PBTL cytoplasmic fractions with anti-JAK-3 antibodies (see methods), proved unsuccessful (data not shown).

To summarise this chapter, no common changes in tyrosine phosphorylation levels of PBTLs, were found to be induced by the 5 inducers of polarisation. However, IL-2 and IL-15 were found to both cause an increase in tyrosine phosphorylation of p110, which could be JAK-3. The tyrosine kinase inhibitor, herbimycin A was found to significantly reduce the extent of polarisation induced by all five of the inducers, but it did not affect the tyrosine phosphorylation of p110 by IL-2 and IL-15.
Figure 7.1: The effect of induction of polarisation in PBTLs on tyrosine phosphorylation in PBTLs

A = EGF receptor (positive control)
B = Control (no agonists)
C = 20% FCS
D = HUVEC conditioned media
E = $10^{-7}$M IL-2
F = 10$\mu$M Bis
G = 50$\mu$M nocadazole

Figure 7.2: The effects of IL-2 and IL-15 on tyrosine phosphorylation in PBTLs

A = Control (no agonists)
B = $10^{-7}$M IL-2
C = $10^{-8}$M IL-15
D = Control (no agonists)
E = $10^{-7}$M IL-2
F = $10^{-8}$M IL-15

(approximate molecular weights kDa of the protein bands are indicated on the right of the pictures)
Figure 7.3: The effect of herbimycin A pre-treatment on induction of polarisation in PBTLs
Figure 7.4: The effect of herbimycin A pre-treatment on tyrosine phosphorylation induced by IL-2 in PBTLs.

A = Control
B = 10^{-7} M IL-2
C = 5 \times 10^{-5} M herbimycin A
D = 5 \times 10^{-5} M herbimycin A
and 10^{-7} M IL-2

Figure 7.5: The effect of herbimycin A pre-treatment on tyrosine phosphorylation induced by IL-2 and IL-15 in PBTLs

A = Control (no agonists)
B = 10^{-7} M IL-2
C = 10^{-8} M IL-15
D = 5 \times 10^{-5} M Herbimycin A
E = 5 \times 10^{-5} M Herbimycin A & 10^{-7} M IL-2
F = 5 \times 10^{-5} M Herbimycin A & 10^{-8} M IL-15
G = 5 \times 10^{-5} M Herbimycin A
H = 5 \times 10^{-5} M Herbimycin A & 10^{-7} M IL-2
I = 5 \times 10^{-5} M Herbimycin A & 10^{-8} M IL-15

(approximate molecular weights kDa of the protein bands are indicated on the right of the pictures)
Chapter 8

Investigations into the roles of microtubules in lymphocyte motility

Introduction

All the three major cytoskeletal fibres - microfilaments, microtubules and intermediate filaments - and their associated proteins seem likely to contribute to the establishment of cell polarity and the process of cell translocation across surfaces. As discussed in the main introduction, the microfilament system is known to be intimately linked to the mechanism of movement and force generation\(^\text{(133)}\). The contributions of the microtubule and intermediate filament systems, however, are less clear\(^\text{(159)}\).

Microtubules studies

As tools for investigating the importance of microtubules in lymphocyte motility, a number of microtubule - directed drugs were utilised in polarisation assays with fresh human PBTLs. Amongst the many microtubule - directed drugs, the taxol family are unusual in that they stabilise cytoskeletal microtubules against depolymerisation\(^\text{(215)}\) and induce polymerisation and bundling. It is now known that taxol inhibits the dynamic reactions at microtubule ends, suppressing both treadmilling and dynamic instability\(^\text{(309)}\).

A representative experiment can be seen in figure 8.1, in which PBTLs were incubated with various concentrations of taxol for 30 minutes at 37°C and then stimulated with one of the 5 inducers of polarisation for 1 hour. All experimental results in this chapter were done in triplicate with 3 different blood donors. As can be seen from figure 8.1, taxol has significantly inhibited the extent of polarisation induced, even at concentrations as
low as 0.1μM. This data suggests that microtubules must be remodelled, before shape change can take place.

Vinblastine binding to tubulin occurs rapidly and binding is rapidly reversible. Beginning with concentrations higher than approximately 1μM, vinblastine depolymerises microtubules by causing splaying and peeling of protofilaments at both microtubule ends in vitro\(^{310}\). Also unlike colchicine (described below), free vinblastine binds directly to microtubule ends without first forming a complex with soluble tubulin\(^{310}\). Also vinblastine does not become incorporated into the tubulin lattice of the microtubule, but incorporates strictly at the microtubule ends\(^{311}\). As can be seen in figure 8.2, fresh PBTLs incubated with various concentrations of vinblastine for 1 hour (at 37°C) exhibit rounding up of the cells at high concentrations and did not induce polarisation. Trypan blue tests showed that PBTLs incubated with 100μM vinblastine were less than 10% non-viable (data not shown).

In figure 8.3, can be seen a representative experiment whereby PBTLs were incubated for 1 hour at 37°C in various concentrations of colchicine. As the concentration increases to 100μM the extent of polarisation increases. In contrast with vinblastine, which binds directly to the ends of the microtubules, colchicine either cannot bind at all to microtubule ends or it does so very inefficiently. Instead, it first binds to soluble tubulin and forms a final-state tubulin-colchicine complex, which then incorporates at the microtubule ends through a polymerisation-dependent pathway\(^{312}\).
Colchicine binding to tubulin is competitively inhibited by nocadazole\(^{313}\) and in figure 8.4, can be seen a representative experiment in which PBTLs were incubated as above with various concentrations of nocadazole. The results here are similar to colchicine treated cells in that there is an increase in the polarisation of the cells up to approximately 35% at higher concentrations of the drug.

Vincristine differs from vinblastine only by the exchange of a methyl group for an aldehyde group, however, this has a major effect on the charge of the molecule and its effect on PBTL polarisation was different from vinblastine as shown in figure 8.5. Vincristine caused an increase in PBTLs polarisation of approx. 20% at low concentrations.

Colcemid (also known as demecolcine), is very similar to colchicine both structurally and functionally, in fact, colchicine only differs from colcemid by an extra carbon and oxygen. Colcemid, depolymerises microtubules and limits microtubule formation\(^{314}\) and it was also found to increase polarisation in PBTLS (~10%) (fig. 8.6) but not to the extent that was caused by nocadazole, colchicine or vincristine.

Thus, nocadazole, colchicine and colcemid have similar modes of action on microtubules, but vincristine is structurally very different from these molecules, however both types of molecules induce polarisation of PBTLs, indicating that these molecules do indeed target the microtubule system. Another piece of evidence to suggest that their effects on polarisation are via the microtubule system is the fact that taxol was found to neutralise the polarisation effects of nocadazole on PBTLs (see figure 8.1).
Therefore, these data suggest that microtubules are playing an important role in the induction of polarisation of PBTLs, as when they are stabilised by taxol, polarisation cannot be induced, however when they are disrupted by various microtubule targeting drugs, the cells change shape, suggesting that microtubule disruption is a precursor to cell shape change.

In addition to observing the shape change effects of microtubule disrupting agents on PBTLs, the distribution of polymerised and depolymerised β-tubulin in PBTLs before and after polarisation was investigated using immunofluorescence techniques (see methods). In figure 8.7, can be seen photographs of untreated fresh PBTLs stained for polymerised β-tubulin. The polymerised β-tubulin is organised in spindle structures radiating from the microtubule organising centre. It must be noted at this point that all these pictures were taken at a 630x magnification, however the PBTLs are very small and not much detail can be attained in the immunofluorescence. Figure 8.8 shows PBTLs treated with 10μM Bis. and stained for polymerised β-tubulin. As can be seen in the polarised cells, the polymerised β-tubulin is located in spindles as before but is located behind the leading edge which is rich in filamentous actin (not shown). Thus, the polymerised β-tubulin seems to be confined to the main cell body and excluded from the cellular protrusions (see black arrows).

Depolymerised β-tubulin has a different cellular distribution from polymerised, as shown in figure 8.9, in which PBTLs (untreated) show a diffuse localisation of depolymerised β-tubulin with some capping of the distribution in some cells. PBTLs treated with 10μM Bis and stained for depolymerised β-tubulin (figure 8.10) exhibit a tubulin distribution
which can only be described as more diffuse than in figure 8.8 but also more confined to
the main cell body and excluded from the leading edge.

In summary, investigations into the roles of microtubules in lymphocyte motility, have
shown that taxol blocks polarisation, therefore microtubule disassembly is essential for
induction of polarisation in PBTLs. Also depolymerisation of microtubules by
nocodazole, colchicine, colcemid and vincristine causes PBTL polarisation but not
motility (as shown for nocodazole in chapter 3). Therefore, microtubule disassembly is
required, but in itself is not sufficient for induction of motility.

Figure 8.1: The effect of taxol on the induction of polarisation in PBTLs
Figure 8.2: The effect of vinblastine on the polarisation of PBTLs

![Vinblastine concentration (uM)]

Figure 8.3: The effect of colchicine on the polarisation of PBTLs

![Colchicine concentration (uM)]
Figure 8.4: The effect of nocodazole on the polarisation of PBTLs

![Figure 8.4](image)

Figure 8.5: The effect of vincristine on the polarisation of PBTLs

![Figure 8.5](image)
Figure 8.6: The effect of colcemid on the polarisation of PBTLs
Figure 8.7: Untreated PBTLs stained for polymerised β-tubulin (x630)
Figure 8.8: PBTLs treated with 10µM Bis, stained for polymerised β-tubulin (x630)
Figure 8.9: Untreated PBTLs stained for depolymerised β-tubulin (x630)
Fig 8.10: PBTLs treated with 10μM Bis. stained for depolymerised β-tubulin (x630)
Chapter 9

Structure-activity relationship of inhibitors of lymphocyte motility.

During this project a number of compounds were found to have inhibitory effects on lymphocyte motility as measured by the polarisation assay. Most of these compounds are different from each other in their proposed targets (ie: herbimycin A is a tyrosine kinase inhibitor, whereas taxol is a microtubule stabiliser), however, they have all been found in this study to have one thing in common, which is the capability to inhibit, to varying degrees, the polarisation of PBTLs by shape change inducers. Therefore, it was decided to investigate whether there were any structural similarities between all these compounds which might be the reason for their common effects on PBTLs.

In figure 9.1 is shown the chemical structure of ionomycin which is a calcium ionophore and was found to significantly prevent the induction of shape change in PBTLs at a concentration of 10μM as seen in fig. 4.9. Thapsigargin, the Ca^{2+}-ATPase inhibitor was also found to inhibit PBTL polarisation at a concentration of 10μM (figure 4.12), and its chemical structure is depicted in figure 9.2. The structure of these compounds are very different and it seems unlikely that they have a common mechanism.

All the amiloride compounds (Na^{+}/H^{+} antiport blockers) which were tested in chapter 5 for their ability to prevent PBTL polarisation are depicted in figure 9.3 (see chapter 5 for full names). Their order of potency in inhibiting polarisation was
A171 > A149 > A130 > A125 > A113, which is consistent with their known effects on Na\(^+\)/H\(^+\) antiport blockade\(^{195}\). As can be seen from their chemical structures, they all have an aromatic core with amino groups in side chains.

In figure 9.4 can be seen the structures of the chloride channel blockers, NPPB, niflumic acid, quinidine and tamoxifen. From the data shown in chapter 5, the compounds NPPB and tamoxifen were the most potent polarisation inhibitors, however their structures are dissimilar and it may be that their effect on chloride channels are by different mechanisms (eg: direct blockade versus inhibition of a regulatory molecule).

The microtubule targetting drugs, taxol, vinblastine and vincristine are large polycyclic compounds and their structures are shown in figure 9.5. Taxol was found to be very effective at inhibiting polarisation in PBTLs (see figure 8.1) and vinblastine was found to round up freshly isolated PBTLs. Vincristine actually caused polarisation in PBTLs but its structure is shown here to show how similar vinblastine and vincristine are, as they differ only by a methyl group for an aldehyde group.

Wortmannin and LY294002 have been reported in the literature to be potent PI 3-kinase inhibitors and were shown in figures 4.14 and 4.15 to inhibit PBTL polarisation. However as mentioned in chapter 4, wortmannin inhibited polarisation at concentrations which are non-specific for PI 3-kinase. Their structures are shown in figure 9.6 and as you can see there is very little similarity between their structures.
Herbimycin A, the tyrosine kinase inhibitor, was shown in figure 7.3 to inhibit a large percentage of PBTL polarisation and was later shown not to inhibit the tyrosine phosphorylation of p120 by IL-2 and IL-15 (see chapter 7). It is a large cyclic compound as can be seen in figure 9.7 and has no similarity to any of the other compounds described in this chapter.

Trifluoperazine (TFP) is a compound which has been reported in the literature to inhibit motility in lymphocytes and is a member of the phenothiazine class of compounds which have been shown to have neuroleptic as well as immunosuppressive effects possibly by disruption of the mechanisms regulating actin polymerisation. The chemical structure of TFP is shown in figure 9.8 and its inhibitory effects on the induction of PBTL polarisation can be seen from the data in figure 9.9. This is a representative experiment from 3 experiments with 3 different blood donors. All tests were done in triplicate. In the experiments shown in figure 9.9, fresh PBTLs were incubated at 37°C with the relevant inducers of polarisation (eg: IL-2) for 40 minutes and then an appropriate concentration of TFP was added to the system and the PBTLs incubated for a further 20 minutes at 37°C, after which the cells were fixed and assessed for polarisation (see methods section). TFP was found to virtually abolish all polarisation at a 20nM concentration and trypan blue tests showed that less than 5% of PBTLs were non-viable after a 20 minute exposure to 20μM TFP.

There does seem to be a superficial similarity between the structures of TFP (figure 9.8) and quinidine (figure 9.4), however their inhibitory effects on PBTL polarisation are not comparable as quinidine is virtually ineffective at 25μM (see figure 5.24) and only has
a small effect at concentrations of 100μM. The only other vague similarities that can be described are those between TFP and the amiloride compounds (Figure 9.3), in that they are all basic compounds with benzene rings, however TFP again proved to be much more potent than the amiloride compounds (figures 5.4 - 5.8).

In summary, it seems as though there is no strong structural motifs or similarities that are common to any of the compounds found to inhibit polarisation in PBTLs and therefore it must be assumed that they are all targeting separate systems within the cells.
**Figure 9.1: Ionomycin**

![Ionomycin structure](image)

**Figure 9.2: Thapsigargin**

![Thapsigargin structure](image)
Figure 9.3: Amiloride compounds

A-149

A-130

A-171

A-113

A-125
Figure 9.4: Chloride channel blockers

NPPB

Niflumic Acid

Quinidine

Tamoxifen
Figure 9.5: Microtubule targeting drugs

Taxol

Vinblastine

Vincristine
Figure 9.6: Phosphoinositide 3-kinase inhibitors

Wortmannin

LY294002
Figure 9.7: Herbimycin A

Figure 9.8: Trifluoperazine
Figure 9.9: The effect of trifluoperazine on induction of polarisation in PBTLs
10. Discussion
In this project the aim was to investigate the second messengers involved in T-lymphocyte motility. The reason behind this being that, lymphocyte motility is an integral step in the multistep action of extravasation of T cells from blood vessels into sites of inflammation. Thus, if any information could be gained upon the signal transduction pathways used in the induction of motility, then these would be possible targets for pharmacological intervention, so as to prevent motility and the subsequent transmigration of the PBTLs through the endothelial walls into the surrounding tissue to cause an inflammatory reaction. Of course, this would also create problems in the normal recirculation of lymphocytes, so any potential anti-inflammatory compound which acts by inhibiting the induction of polarisation / motility, would be likely to be profoundly immunosuppressive.

**The investigation for inducers of motility**

A model of lymphocyte motility had to be established and various agonists were tested for their ability to cause polarisation (the first stage of motility) in fresh PBTLs and non-motile MOLT-4 cells. Surprisingly, the much publicised chemokines had very little effect on the polarisation of freshly isolated PBTLs, with MIP-1\(\alpha\), MIP-1\(\beta\), MCP-1 and IL-8 only affecting a maximum of 10% of the population of cells. This could be due to the fact that each chemokine tends to be specific for a certain subset of T cells. The activation state of the lymphocytes also governs whether the cells respond to a certain factor, and this could also explain why factors such as substance P, VIP, LPA and PDGF, only had an effect on 10% of the PBTLs, as PBTLs are mostly unactivated in the peripheral blood.
The MOLT-4 cells proved to be unresponsive to all of the factors tested except, Bis. and nocadazole. The reasons for this could be that either the MOLT-4 cells do not possess the receptors for any of these factors or it could be that pieces of the signal transduction machinery needed to relay the signals are missing or unable to function (which is a strong possibility, since these are transformed cells). For example, although the MOLT-4 cell line is classified as a T cell lymphoblastic leukaemia it is actually negative for the CD3 marker. Hence, it was decided to concentrate on using PBTLs as a model for induction of motility in lymphocytes as five factors were found to cause significant polarisation in PBTLs, these being, Bis.- a PKC inhibitor, nocadazole- a microtubule disrupting agent, FCS - a mixture of unknown quantities of proteins and growth factors and finally the two interleukins-2 and -15, which are physiological agents involved in T cell regulation.

However, cell polarisation is only an indicator of motility as it does not always lead to cell locomotion. Therefore, these five factors were tested in transmigration assays and IL-2, IL-15 and Bis., were found to cause significant transmigration across polycarbonate filters, whereas FCS and nocadazole had no effect whatsoever. Nevertheless, FCS and nocadazole were still useful as tools for inducing polarisation in PBTLs as this is the crucial first step in motility - cells must change shape before they can become motile, therefore, nocadazole and FCS presumably use the same intracellular machinery as Bis. IL-2 and IL-15 to cause shape change

Role of intracellular calcium

Having established a model for the induction of motility in PBTLs, the next step was to investigate whether these 5 inducers of shape change utilised any common second
messengers, that would then be contenders for involvement in a motility signal transduction pathway. It was found that none of the 5 factors caused any significant increases in \([Ca^{2+}]_i\) that could be detected with the system used. Therefore, there could have been very small local fluctuations in \([Ca^{2+}]_i\) within the cells that escaped detection. Also tested for effects on PBTLs \([Ca^{2+}]_i\) levels, were some of the chemokines which have been reported in the literature to cause transient \([Ca^{2+}]_i\) increases. However, MIP-1\(\alpha\), MIP-1\(\beta\) and MCP-1 had minimal effects on PBTLs in this system, with only an increase of about 10nM \([Ca^{2+}]_i\) being detected. This could be due to the fact that within each experiment there are 2x10^6 PBTLs and only a subpopulation of these will respond to the chemokine in question, thus diluting the signal. Interleukin-8 and RANTES were found to have no effect whatsoever, but interestingly the non-motile MOLT-4 cells responded dramatically to MCP-1 and a few of the other \(\beta\)-chemokines with classical \([Ca^{2+}]_i\) transients, proving that this system does indeed work, but more importantly showing that a physiological factor such as MCP-1 which causes a \([Ca^{2+}]_i\) transient increase does not affect the morphology of the cells.

Further investigations into \([Ca^{2+}]_i\) and motility showed that an increase in \([Ca^{2+}]_i\) actually inhibits polarisation. First of all motile MOLT-4 cells and PBTLs that had previously been treated to cause polarisation were found to round up on exposure to ionomycin. At low concentrations (0.5\(\mu\)M), the calcium ionophore ionomycin preferentially inserts in the membrane of the intracellular stores\(^{317}\) and causes an increase in \([Ca^{2+}]_i\) levels due to emptying of intracellular calcium stores. At higher concentrations (10\(\mu\)M), the ionophore ionomycin, inserts into the cell membrane causing an influx of extracellular calcium into the cytosol. Thapsigargin treated PBTLs were unable to polarise upon exposure to the inducers of shape change due to the increase in
[Ca$^{2+}$]$_i$ levels elicited by the Ca$^{2+}$-ATPase inhibitor. These results are supported by other workers$^{303,318}$, who have also found that a [Ca$^{2+}$]$_i$ elevation in T-lymphocytes causes rounding up and immobilisation of the cells.

The [Ca$^{2+}$]$_i$ dependence of lymphocyte motility is opposite to that described for other leukocytes where [Ca$^{2+}$]$_i$ increases are associated with increased motility. For example, [Ca$^{2+}$]$_i$ elevation speeds up neutrophil migration on various substrates$^{319}$, via a calcineurin dependent mechanism$^{320}$. Since stimuli (ionomycin and thapsigargin) that led to a [Ca$^{2+}$]$_i$ rise also provoked a rounding of the lymphocyte, then there must be a link between the cytoskeleton and calcium. Fragmentation of filamentous actin by calcium-dependent proteins (such as gelsolin$^{321}$) can explain some of these events. In neutrophils, a Ca$^{2+}$-induced depolymerisation of actin has been shown$^{322}$. A correlation between [Ca$^{2+}$]$_i$ augmentation and retraction of protrusions has been described in other cell types, for example cytotoxic T cells after interaction with a target cell$^{323}$, or endothelial cells stimulated with thrombin$^{324}$. However, this is not necessarily true in all cell types. For instance, in neuroblastoma cells, although lysophosphatidic acid elicits both a [Ca$^{2+}$]$_i$ rise and a neurite retraction, in this case the two events are not causally related$^{325}$.

Local signalling events at the leading edge may be responsible for protrusion of a pseudopod by regulating actin-binding proteins. Although the studies with Ca$^{2+}$-depleted cells show that changes in [Ca$^{2+}$]$_i$ are not a required part of the signal at the leading edge, localised brief increases in [Ca$^{2+}$]$_i$ could play an auxiliary role in protrusion by generating additional actin-nucleation sites and by breaking crosslinks. With increased ability to measure [Ca$^{2+}$]$_i$ while observing morphological changes, and with improved
methods to manipulate \([\text{Ca}^{2+}]_i\), it should be possible to determine whether such local \([\text{Ca}^{2+}]_i\) transients do play a functional role.

**Role of phosphoinositides**

The first set of experiments which investigated the role of phosphoinositides in lymphocyte motility were assays of IP\(_3\) levels within the cells. IP\(_3\) is an important second messenger as it can signal the endoplasmic reticulum to release its stores of \text{Ca}^{2+} and thus elevate the \([\text{Ca}^{2+}]_i\) levels. It was found that none of the five inducers of shape change had any effects on the IP\(_3\) levels after an arbitrary one minute period. The results from experiments whereby inhibitors of PI 3-kinase were used were unclear as wortmannin was only inhibiting polarisation in PBTLs at concentrations at which is unspecific for PI 3-kinase. However, another PI 3-kinase inhibitor, LY294002 was found to significantly block the induction of motility in PBTLs at low concentrations. Wortmannin is known to be less stable than LY294002 and this could explain why it had less of an effect than LY294002, which then lead to the conclusion that PI 3-kinase could possibly be involved in the signal transduction of motility. Further evidence for the role of phosphoinositides in motility came from the experiments involving lithium chloride, which was found to inhibit the induction of shape change by IL-2, IL-15 and FCS (and Bis. and nocadazole, but at higher concentrations.). These results are not conclusive but they do suggest that phosphoinositides could be playing a role in motility signal transduction.

Although no direct role for PI 3-kinase in actin polymerisation has been demonstrated, it is necessary for some forms of cell motility and adherence. PDGF receptor mutants that do not bind PI 3-kinase, do not ruffle or undergo chemotaxis in response to
It has also been found that both PDGF receptor mutants that do not bind PI 3-kinase and wortmannin inhibit binding of GTP to rac in response to PDGF\(^{272}\). These data place PI 3-kinase upstream of rac in fibroblasts and, consistent with this idea, injection of fibroblasts with V12 rac circumvents inhibition of ruffling by wortmannin\(^{327}\). Finally, although the biochemical mechanism is obscure, there is growing evidence both from receptor mutants and from inhibitors that PI 3-kinase is required for stimulus-dependent activation of integrins and cell adherence\(^{328,329}\).

**Role of intracellular pH and ion channels**

Induction of shape change in PBTLs was found to have no effect on the intracellular pH of the cells as measured in this system. However, a decrease in pH\(_i\) caused rounding up of motile MOLT-4 cells and blocked polarisation in PBTLs. One of the main pH\(_i\) regulatory mechanisms is the Na\(^+\)/H\(^+\) antiporter and blockage of these was found to cause inhibition of motility in PBTLs and motile MOLTs.

Cytosolic pH\(_i\) is a candidate to regulate cell motility, since certain steps in the actin polymerisation sequence and the binding of actin filaments to membrane-anchoring proteins are pH-dependent events\(^{330}\). Indirect observations are consistent with this notion: the ability of neutrophils to polarise and perform chemotaxis is reduced when the extracellular pH (pH\(_o\)) is made more acidic, which is expected to lower pH\(_i\)\(^{192}\). More importantly, it is possible to induce cytoskeletal reorganisation in neutrophils in a receptor-independent manner by the addition of weak electrolytes, which can modify pH\(_i\) at constant pH\(_o\)\(^{192}\). Therefore, pH\(_i\) must be given consideration as a regulator and possible mediator of cell shape change and chemotaxis.
In a recent publication\textsuperscript{331}, neutrophil spreading on adhesive substrates caused a rapid and sustained cytosolic alkalinisation. This pH\textsubscript{i} increase was prevented by the omission of external Na\textsuperscript{+}, suggesting that it results from the activation of Na\textsuperscript{+}/H\textsuperscript{+} exchange. It was also found that neutrophil motility was prevented by selectively blocking the NHE-1 isoform of the Na\textsuperscript{+}/H\textsuperscript{+} antiporter. Support for the results obtained in this project were also observed in that neutrophil spreading was strongly inhibited when pH\textsubscript{i} was clamped at acidic values\textsuperscript{331}. Interestingly, the inhibition of neutrophil shape change required pre-acidification of the cells\textsuperscript{331}, since neutrophils spread normally when acidified shortly after contact with the substrate. This suggests that the pH-sensitive step is an early event, and that adherence and spreading, once initiated, can proceed independently of pH\textsubscript{i}.

It has been shown that there are interactions between actin filaments and the Na\textsuperscript{+}/H\textsuperscript{+} antiporter\textsuperscript{332}. Also, microtubules have been suggested to have links with the Na\textsuperscript{+}/H\textsuperscript{+} antiporter, which were shown to be regulated in a mechanosensitive manner in lymphocytes\textsuperscript{333}. In addition, it has been shown in human B-lymphoid cells that the cAMP-mediated signal transduction pathway and pertussis toxin-sensitive GTP-binding proteins act synergistically to regulate amiloride-sensitive sodium channels\textsuperscript{334}.

Thus, it would seem that the the Na\textsuperscript{+}/H\textsuperscript{+} antiporters play a vital role in lymphocyte motility, probably due to its role in pH\textsubscript{i} regulation which has effects on all enzymes within the cell and also it would seem through its interactions with the cytoskeleton.

Investigations into the roles of chloride channels in lymphocyte motility showed that various chloride channel blockers could inhibit PBTLs and motile MOLTs polarisation effectively, however it was found that the blockers were not actually exerting their
motility inhibitory effects via chloride channel blockage but through other mechanisms which are unknown. Therefore, the role of chloride channels in lymphocyte motility remains unclear at this point, however it can be assumed that they would play at least some minor role in the complicated entanglement that is the signal transduction regulation of motility, as chloride channels are important in cell volume control\(^{201,203,204}\), as well as pH\(_i\) control via the Cl\(^-\)/HCO\(_3^-\) antiport exchanger\(^{201,202}\). A Cl\(^-\)/HCO\(_3^-\) antiport exchanger, similar to the band 3 protein in the membrane of red blood cells, is thought to play an important part in pH\(_i\) regulation in many nucleated cells. Like the Na\(^+\)/H\(^+\) exchanger, the Cl\(^-\)/HCO\(_3^-\) antiport exchanger is regulated by pH\(_i\), but in the opposite direction. Its activity increases as pH\(_i\) rises, increasing the rate at which HCO\(_3^-\) is ejected from the cell in exchange for Cl\(^-\), thereby decreasing pH\(_i\) whenever the cytosol becomes too alkaline. Also, it must be noted at this point that the importance of chloride channels in motility may not be just due to their physiological role in Cl\(^-\) transport but to their permeability to larger organic osmolytes such as taurine\(^{300,302}\).

**The role of renaturable kinases**

Investigations into the role of renaturable kinases in PBTL motility, showed that of the five inducers of motility, only Bis. seemed to have any effects, in that it repeatedly activated a renaturable autophosphorylating kinase of molecular weight 58kDa. Collaborative work in the same laboratory has identified this 58kDa kinase to be MST-1 (mammalian Ste20-like), a serine/threonine protein kinase. Little is known about this kinase at the current time, however its role in the signal transduction of motility in PBTLs cannot be considered too important as the other four inducers of motility failed to activate it in this assay.
The role of tyrosine phosphorylation

In this study it was found that of the five inducers of polarisation in PBTLs only IL-2 and IL-15 caused tyrosine phosphorylation of proteins that were detected in this system. Both IL-2 and IL-15 were found to cause tyrosine phosphorylation of a protein of molecular weight approximately 110kDa (p110). Of the tyrosine kinase inhibitors used, only herbimycin A was found to block polarisation of PBTLs and more importantly it was capable of inhibiting shape change in PBTLs by all five of the inducers of motility effectively. This suggested that herbimycin A was targeting a point in the signal transduction system that was utilised by all five factors. Similar results have been obtained with neutrophils pre-treated with herbimycin A, in that it blocks their chemotactic response to fMLP\(^{337}\). It should be noted at this point however, that herbimycin A is not necessarily blocking shape change by inhibition of a tyrosine kinase, it could be due to other adverse effects. Further evidence to support this notion was obtained in the experiments in which it was found that PBTLs pre-treated with herbimycin A before exposure to IL-2 or IL-15 were still found to cause tyrosine phosphorylation of p110. Thus, if herbimycin A is indeed inhibiting polarisation by targeting a tyrosine kinase then it must be downstream from the p110 protein. Identification of the p110 protein has been unsuccessful. Evidence from the literature would suggest the most likely candidate to be Janus kinase-3 (JAK-3), as this protein has been shown to be tyrosine phosphorylated by both IL-2 and IL-15 in T cells\(^{213}\) and has a molecular weight of approximately 120kDa. However, experiments to immunoprecipitate the p110 protein using anti-JAK-3 antibodies proved unsuccessful. Therefore, it is uncertain at this point whether the p110 protein is indeed JAK-3, however, whatever the
identity of the protein it is possible that it is part of a motility signal transduction pathway utilised by IL-2 and IL-15.

Indeed, it has been shown in neutrophils treated with chemotactic agents such as fMLP, that they also cause tyrosine phosphorylation of a protein of molecular weight 120kDa\(^{(336)}\) - (identity unknown at time of publication).

**Role of microtubules**

To evaluate the involvement of microtubules in PBTL polarisation, a number of microtubule targeting drugs were used on the PBTLs. From these results it was clear that before the PBTLs could change shape, the microtubular system must be rearranged. This was deduced from experiments with taxol. The effect of taxol at the molecular level is opposite to that of the other microtubule targeting drugs used, such as colchicine. Taxol stabilises microtubules in a polymerised state\(^{(340)}\). Due to this effect, taxol promotes polymerisation of free microtubules not associated with any organising centers, so that gradually the system of microtubules radiating from the perinuclear center is replaced by numerous aggregates of free microtubules\(^{(341)}\). This disintegration of the microtubule system was found to be accompanied by the inability of the PBTLs to polarise in response to the five inducers of shape change. Also, it was found that agents such as colchicine and nocadazole which disrupt microtubules, could cause shape change in PBTLs themselves.

Immunofluorescence photographs of unactivated PBTLs stained for polymerised and depolymerised β-tubulin show that they have different cellular distribution profiles but in polarised PBTLs both types of tubulin seemed to be excluded from the leading edge of
the cell which is rich in F-actin microfilaments forming a cortical meshwork. Indeed, it has been found that polarised motile fibroblasts are characterised by trailing processes rich in microtubules\(^{338}\).

What then is the role of microtubules in PBTLs motility? Unfortunately, there is little published work on this subject concerning PBTLs, however there are theories on the subject. One hypothesis, originally proposed in 1982\(^{339}\), is that the microtubules transport new membrane and cortical components from the golgi apparatus to the leading edge. One reason that the microtubules need to be disrupted prior to polarisation is a structural one, in that the intracellular scaffolding has to be re-arranged before the cell can change shape. Also, it could be that the disruption of the microtubule network allows the release of second messengers that are bound to the microtubules, so that they are then free to take part in signal transduction.

Therefore, it would seem that PBTL polarisation is stabilised first by reorganisation of the actin cortex induced by the extension of pseudopods; in the next stage polarisation is enhanced and further stabilised by the microtubule-dependent redistribution of organelles

**Inhibitors of lymphocyte polarisation**

After assessing the chemical structures of all the compounds which inhibited PBTL polarisation, it was found that there were no significant structural similarities between any of the compounds that may explain their common effects on PBTLs polarisation. Therefore, it must be assumed that they are all targeting separate systems within the cells.
Conclusion

Thus, five different factors were found to cause significant polarisation in PBTLs, but no common second messenger elements were found to be utilised by the five. However, a number of pharmacological agents were found, that prevented induction of polarisation in PBTLs by all five factors and these it would seem may be targeting unknown second messenger elements involved in the signal transduction of T-lymphocyte motility, or the intracellular motility machinery itself.
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12. List of publications
