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THE DIFFERENTIAL REGULATION OF CYCLIC AMP
PHOSPHODIESTERASES IN
T LYMPHOCYTES.

Alison Mary Michie B.Sc.

This thesis is presented for the degree of Doctor of Philosophy.

Department of Biochemistry and Molecular Biology,
University of Glasgow.


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Dedicated to my parents
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<th>Definition</th>
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<tr>
<td>145-2C11</td>
<td>Anti-CD3 monoclonal antibody</td>
</tr>
<tr>
<td>A&lt;sub&gt;280&lt;/sub&gt;</td>
<td>Absorbance at 280nm</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AET</td>
<td>2-aminoethyl isthiourium</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AKAP</td>
<td>A-kinase anchoring protein</td>
</tr>
<tr>
<td>Amrinone</td>
<td>[5-amino-(3',4'-bipyridin)-6(H)-one]</td>
</tr>
<tr>
<td>Approx.</td>
<td>Approximately</td>
</tr>
<tr>
<td>ANF</td>
<td>Atrial natriuretic factor</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATF</td>
<td>Activation transcription factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMPK</td>
<td>Calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>cyclic AMP</td>
<td>Adenosine-3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD2/4/8/28</td>
<td>Co-receptors</td>
</tr>
<tr>
<td>CD25</td>
<td>Interleukin 2 receptor α chain</td>
</tr>
<tr>
<td>CD44</td>
<td>Extracellular matrix receptor</td>
</tr>
<tr>
<td>CD45</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cyclic GMP</td>
<td>Guanosine-3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
</tr>
<tr>
<td>Cl</td>
<td>Curie</td>
</tr>
<tr>
<td>Cilostamide</td>
<td>3-[4,5-dihydro-6-[4-(1H-imadazol-1-yl)phenyl]-5-methyl-3(2H)-pyrazone]</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxy-terminal of a protein</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CRE</td>
<td>Cyclic AMP responsive element</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic AMP responsive element binding protein</td>
</tr>
<tr>
<td>CREM</td>
<td>Cyclic AMP responsive element modulator</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>Diethylaminoethyl-cellulose column.</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPD</td>
<td>Dnp-like phosphodiesterase (PDE4B)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EC50</td>
<td>Concentration at which 50% activation ensues</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis (b-aminoethyl ether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EHNA</td>
<td>Erythro-9-(2-hydroxy-3-nonyl)-adenine</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>Fig.</td>
<td>Figure</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast performance liquid chromatography</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>(g_{av})</td>
<td>Average gravitational force</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>Genistein</td>
<td>4',5,7-Trihydroxyisoflavone</td>
</tr>
<tr>
<td>(G_i)</td>
<td>Inhibitory G protein to adenylyl cyclase activity</td>
</tr>
<tr>
<td>(G_s)</td>
<td>Stimulatory G protein to adenylyl cyclase activity</td>
</tr>
<tr>
<td>G protein</td>
<td>GTP binding protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine trisphosphate</td>
</tr>
<tr>
<td>h6.1</td>
<td>Human PDE4A</td>
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<tr>
<td>FL89</td>
<td>N-[2-((p-Bromocinnamy1)amino)ethyl]-5'-isoquinolinesulfonamide</td>
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<td>Hepes</td>
<td>N-2-Hydroxyethylpiperazine-N'-2-ethane-sulphonic acid</td>
</tr>
<tr>
<td>IBMX</td>
<td>1-isobutylmethyl-3-xanthine</td>
</tr>
<tr>
<td>IC(50)</td>
<td>Concentration at which 50% inhibition ensues</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>ICER</td>
<td>Inducible cyclic AMP element repressor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G antibody</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-2R</td>
<td>Interleukin 2 receptor</td>
</tr>
<tr>
<td>IP(3)</td>
<td>Inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>(K_i)</td>
<td>The dissociation constant for the enzyme-inhibitor complex</td>
</tr>
</tbody>
</table>
kg  Kilogram
$K_m$  Michaelis constant, equal to the substrate concentration at
which the reaction rate is half the maximum value
l  Litre
LFA-3  Lymphocytes function associated antigen-3 (CD58)
M  Molar
mA  Milliamps
MAPK  Mitogen activated protein kinase
MEP-1  Erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA)
MHC  Major histocompatibility complex
Milrinone  \( [1,6\text{-Dihydro-2-methyl-6-oxo-(3,4’-bipyridine)-}5\text{-carbonitrile}] \)
ml  Millilitre
mg  Milligram
NF-AT  Nuclear factor of activated T cells
NF-κB  Nuclear factor κB
N-terminus  Amino terminus of protein
NK cells  Natural killer cells
NO  Nitric oxide
°C  Degrees celcius
PBS  Phosphate buffered saline
PDE  Phosphodiesterase
PE  Phycoerythrin
pH  \(-\log_{10}[\text{H}^+]\)
PHA  Phytohaemagglutinin
PI-3-K  Phosphatidylinositol-3-kinase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Cyclic AMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>Cyclic GMP-dependent protein kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-myristate, 13-acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>PPT</td>
<td>Protein phosphatase</td>
</tr>
<tr>
<td>Pre-TCR</td>
<td>Pre-T cell receptor (β-gp33 heterodimer)</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylinerine</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PTPase</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RACKs</td>
<td>Intracellular receptors for PKC</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombinase-activating gene</td>
</tr>
<tr>
<td>RD1</td>
<td>Rat dnc-like 1 (PDE4A)</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Ro20-1724</td>
<td>4-(Butoxy-4-methoxybenzyl)-2-imidazolidinone</td>
</tr>
<tr>
<td>Rolipram</td>
<td>4-{3-(cyclopentoxy)-4-methoxyphenyl}-2-pyrrolidone</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SRBCs</td>
<td>Sheep red blood cells</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethanolamine</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′-Tetramethylethylene diamine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>Tc</td>
<td>Cytotoxic T cell</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell antigen receptor</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol 13-acetate (PMA)</td>
</tr>
<tr>
<td>TRE</td>
<td>PMA-responsive element</td>
</tr>
<tr>
<td>Tyrophostin A1</td>
<td>[α-Cyano-(4-methoxy)cinnamionitrile]</td>
</tr>
<tr>
<td>Tyrophostin A25</td>
<td>[α-Cyano-(3,4,5-trihydroxy)cinnamionitrile]</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)methylamine</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>V_{max}</td>
<td>Maximum reaction rate</td>
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Summary

Cyclic AMP phosphodiesterases were studied in T lymphocytes from different maturation stages, tissues and species. Although cyclic AMP phosphodiesterases were characterised in lymphocytes isolated from murine spleens, human tonsils, a murine thymoma cell line and a human mature T cell line, the main focus of this study was cyclic AMP PDE activities from murine thymocytes. With the use of selective phosphodiesterase inhibitors, the relative contribution that particular phosphodiesterase isoforms made towards total phosphodiesterase activity in the cells was assessed. Thus, cyclic GMP stimulated, PDE2 activity and cyclic AMP specific PDE4 activity were shown to provide the major cyclic AMP hydrolysing activities in murine thymocytes. PDE4 activity predominated (~80% total) in the absence of cyclic GMP. However, on addition of low (10μM) concentrations of cyclic GMP, PDE2 activity constituted the major PDE activity in thymocytes (~80% total).

The PDE4 selective inhibitor rolipram inhibited murine thymocyte PDE4 activity in a dose-dependent manner (IC_{50} ~65nM). PDE2 activity was stimulated in a dose-dependent fashion (EC_{50} ~1μM) by cyclic GMP and was selectively inhibited by the compound, EHNA (IC_{50} ~4μM). No calcium/calmodulin stimulated, PDE1 activity or cyclic GMP-inhibited, PDE3 activity was found to be present in murine thymocytes. The non-selective PDE inhibitor IBMX elicited >98% inhibition of PDE activity in thymocytes indicating the absence of IBMX-insensitive, PDE7 activity in these cells. FPLC analysis confirmed these findings, revealing a rolipram inhibited PDE4 activity and a cyclic GMP stimulated, EHNA inhibited, PDE2 activity, but no PDE1, PDE3 or PDE7 activity.

The selective inhibitors identified the profile of phosphodiesterase activities in T cells from other sources. Thus, PDE2 activity was also found to be present in
murine splenic lymphocytes, proliferating T3.2 thymoma cell line derived from murine thymus and the human leukemic T cell line, Jurkat. In contrast to murine thymocytes, PDE3 appeared to be present in human tonsillar T lymphocytes, the human T cell line Jurkat and the thymoma cell line, T3.2. Similar to murine thymocytes, PDE4 activities were found to be present in the murine thymoma cell line, T3.2 and the human T cell line, Jurkat. Moreover, primary lymphocytes isolated from murine thymuses, spleens and human tonsils did not contain Ca²⁺/CaM stimulated, PDE1 activity. In contrast, the proliferating human Jurkat T cell line contained calcium/calmodulin-stimulated PDE1 and an IBMX insensitive PDE activity. The differential distributions of phosphodiesterase activities within these lymphoid cells indicated species and possibly maturation-dependent differences of phosphodiesterase expression within these cells.

Upon ligation of the TCR/CD3 complex with either the mitogenic lectin phytohaemagglutinin or anti-TCR/anti-CD3 monoclonal antibodies, phosphodiesterase activities and cyclic AMP levels were found to be rapidly and differentially regulated in murine thymocytes. Within 5 minutes of challenge with PHA, there was a transient decrease (~83%) in PDE4 activity and in PDE2 activity (~40%), returning almost to basal in 20 minutes. Both anti-CD3 and anti-TCR antibodies also caused an initial reduction in the PDE4 activity (~50%) which was then followed by a sustained increase in activity. In contrast to that observed with PHA, anti-TCR/CD3 antisera had little effect on PDE2 activity. Cyclic AMP levels were also differentially regulated upon stimulation of thymocytes with either PHA or anti-CD3 monoclonal antibodies. Hence, there was a transient 2-fold elevation in cyclic AMP levels upon stimulation of thymocytes with PHA, which reached basal levels again within 30 minutes. In contrast anti-CD3 antibody stimulation of thymocytes led to a slow sustained elevation in cyclic AMP levels over a 30 minute period.
Study of the mechanism controlling the anti-CD3-stimulated elevation in PDE4 activity in murine thymocytes revealed that the increase in this phosphodiesterase activity was dependent on protein tyrosine kinase and protein kinase C-mediated signals. This interpretation was based on findings that the tyrosine kinase inhibitor, genistein partially inhibited the TCR-mediated elevation in PDE4 activity. Moreover, the protein kinase C selective inhibitor chelerythrine blocked the rise of PDE4 activity and the phorbol ester, phorbol-12-myristate, 13-acetate caused an increase in PDE4 activity which was of a similar magnitude to the elevation seen in the presence of anti-CD3 antibodies. Interestingly, the elevation in PDE4 activity was blocked upon stimulation of thymocytes with TPA or anti-CD3 antibody in the presence cyclohexamide or actinomycin D. This indicated that a rapid induction of PDE4 activity occurred in murine thymocytes upon crosslinking of the TCR/CD3 complex. These data identify 'crosstalk' between cyclic AMP and PKC mediated signalling pathways.
CHAPTER 1.

INTRODUCTION.
1.1. T cell development biology.

T lymphocytes provide the cellular arm of the immune system and are responsible for the helper (combating bacterial infection) and cytotoxic (protecting against viral pathogens) T cell functions of an immune response. Mature T lymphocytes respond to antigen via cell surface T cell antigen receptors (TCR), which specifically recognise proteolytically processed peptide antigens bound in the grooves of self major histocompatibility complex (MHC) proteins on antigen presenting cells (APCs).

Mature T cells which are tolerant to self antigen develop in the thymus. Pre-T cells originating from haematopoietic sites such as foetal liver and adult bone marrow enter the thymus at the cortico-medullary junction/subcapsule of the thymus via the blood. These cells express a very low concentration of the coreceptor CD4, which is quickly lost thus giving the CD4⁺/CD8⁻ or “double negative” phenotype. Immature thymocytes rearrange and express TCRβ which promotes the development of thymocytes (Mallick et al., 1993; Raulet et al., 1985). The positive selection of cells expressing a productive TCRβ avoids the accumulation of thymocytes with nonproductive TCRβ rearrangements. TCRβ is expressed on the surface of immature T cells with a surrogate α chain (gp33) (Crozetrap et al., 1993). These peptide chains are linked by disulphide bonds to form a pre-T cell receptor (pre-TCR) in association with the CD3γδε complex (a collection of non-covalently associated proteins involved in receptor assembly, membrane trafficking and transduction of transmembrane signals in lymphocytes, see section 1.2.1). Further maturation occurs via signalling through the β chain, the CD3 complex and the non-receptor protein tyrosine kinase (PTK) p56lck (see section 1.4).

β chain expression promotes maturation of CD4⁺/CD8⁻ thymocytes beyond this “double negative stage” (Kishi et al., 1991). Following expression and allelic exclusion of TCRβ, the cells are selected for clonal expansion and maturation, with the induction of CD4 and CD8 co-receptor expression producing
the CD4+/CD8+ "double positive" phenotype (Boyd and Hugo, 1991). At this stage TCR$\alpha$ rearrangement can occur, and gp33 expression is down-regulated, allowing for the expression of the mature TCR/CD3 complex (Groettrup et al., 1993). CD4$^+$/CD8$^+$ cells may temporarily become TCR negative at this transition, because $\beta$ chains can only be exported to the surface in a $\alpha\beta$ dimeric conformation. However, once productive $\alpha$ chains are formed, the $\alpha\beta$ heterodimer is expressed.

At the CD4$^+$/CD8$^+$ stage, negative selection leading to the deletion of auto-reactive clones occurs (Boyd and Hugo, 1991; Boyd et al., 1993) (fig. 1.1). Indeed, programmed cell death (apoptosis) during negative selection plays an important role during thymic education since the vast majority of cells (95%) die in the thymus and only 5% are rescued from cell death and are positively selected (Bill and Palmer, 1989; Hueber et al., 1994; Kaye and Ellenberger, 1992; Swat et al., 1991) (see section 1.4.1). In addition, clonal anergy can also occur, whereby the cells do not react upon recognition of a peptide antigen (Nossal, 1994).

Positive selection for self MHC restriction, and functional maturation of the cells also occurs at the CD4$^+$/CD8$^+$ stage. This selection procedure occurs in order to produce a T cell repertoire that only recognizes foreign peptide antigen in the context of self MHC. The essential event during this process is the binding of $\alpha\beta$ TCR to self MHC molecules (Bill and Palmer, 1989). Binding rescues the short-lived CD4$^+$/CD8$^+$ thymocytes from cell death, and in doing so, signals whether CD4 or CD8 co-receptor will be down-regulated. Generally, this determines the class of effector cell to which they will differentiate and specificity for MHC class-I or II molecules. Positive selection is associated with the termination of TCR rearrangement as gene rearrangement of the $\alpha$ gene locus continues until the cell is positively selected or the cell dies. Thus positive selection not only selects cells with functional self restricted antigen receptors, but also rescues cells from programmed cell death.
Figure 1.1. Simplified scheme of T cell development from stem cell to mature T lymphocyte.
Stem cell
Sca1+
Tdt±
CD2-

Bone Marrow

Pre-T cell
Sca1-
Tdt+
CD2+

Committed to T cell lineage

Thymus migration

Double negative thymocyte
CD3+/TCR+
CD2+
CD4-/CD8-

Double positive thymocyte
CD3+/TCR+
CD2+
CD4+/CD8+

Explosive proliferation

Massive apoptosis

CD4+ CD8-
Single positive thymocytes

CD3+/TCR+/CD2-

Thymus

Exit

Periphery

Helper T cell

Mature T lymphocytes

Cytotoxic T cell
As a result of positive selection, CD4+/CD8+ thymocytes up-regulate their TCR levels (Borgulya et al., 1991; Ohashi et al., 1990). The development from the CD4+/CD8+ stage to the expression of CD4 or CD8 "single positive" cells is also thought to be dependent on TCR-MHC interaction (Scott et al., 1989; Sha et al., 1988). If the TCR is not ligated within 4-5 days, the cells simply die (Egerton et al., 1990). CD4 or CD8 is down-regulated before entry into the periphery, via the medulla or cortico-medullary vascular sites (Savino et al., 1993).

1.2. T cell antigen receptor/CD3 complex.

The TCR/CD3 molecule can be subdivided into three units; the clonotypic αβ heterodimer, responsible for mediating antigen recognition, the CD3 complex and the ζ family (made up of ζ and η subunits) responsible for transmitting the signals from the αβ heterodimer intracellularly (Weiss, 1991).

Antigen recognition for T lymphocytes is effected through the variable domains of TCR molecules. Genes that encode these variable regions are assembled during the early stages of T cell differentiation from germline variable (V), diversity (D) and joining (J) gene segments by a process known as V(D)J recombination. This allows the production of a maximal number of receptors from a relatively small amount of genetic material. V(D)J recombination occurs at four different gene loci: α, β, γ, and δ (Davis and Bjorkman, 1988). For α or γ, one of many domain-sized V regions is juxtaposed to one of several short J gene segments; while for the other chains, β or δ, an additional short D segment is interposed between the V and J segments. Imperfect joining of recombining gene segments with the loss or gain of nucleotides (under the action of terminal deoxytransferase (TdT)) enhances the variability of TCR proteins and superimposes junctional diversity on to combinatorial diversity (Strominger, 1989). Initial β chain rearrangement and expression is followed by a termination of β gene rearrangement, which contributes to allelic exclusion. The α chain then rearranges, but is not allelically excluded (Raulet et al., 1985; Snodgrass et al.,
1985). The recombinase-activating gene (RAG) genes are required to anneal DNA regions together in this recombination event (Mombaerts et al., 1992; Shinkai et al., 1992). These genes are found to be expressed in early lymphocyte progenitors, with their expression levels tightly regulated (Neale et al., 1992). Knockout mutations of RAG1, RAG2, or TCRβ in murine models lead to an accumulation of immature TCRγ/CD4−/CD8− thymocytes. Thus demonstrating the importance of these genes during T cell maturation (Mombaerts et al., 1992; Shinkai et al., 1992).

1.2.1. Structure of TCR/CD3 complex.

The TCR homodimer of the TCR/CD3 complex is composed of a clonotypic antigen recognition unit (αβ or γδ heterodimers). Following ligand binding, signals are transmitted to one or more of the CD3 components. The CD3 complex consists of two CD3ε chains, one CD3δ, one CD3γ. The invariant, non-covalently associated CD3 (δ, γ, ε) and ζ family (ζ and η) chains are involved in receptor assembly, membrane trafficking and the transduction of transmembrane signals (Weiss, 1991) (fig. 1.2). This multicomponent structure has no intrinsic catalytic activity, but non-receptor PTKs are found to be recruited to the receptor complex, via specialised motifs contained within the TCR/CD3 complex. The CD3 structure facilitates the transmission of signals from the membrane to the nucleus of the cell, via multiple and complex signalling cascades (Weiss and Littman, 1994).

The αβ-TCR comprises a disulphide-linked heterodimer, the α (40-50kDa) chain being more acidic than the β (35-47 kDa) chain (Marrack and Kappler, 1987). Each polypeptide chain comprises two immunoglobulin-like domains of approximately 110 amino acids, which are anchored into the plasma membrane by a transmembrane peptide and a short cytoplasmic tail. The amino acid sequence polymorphisms reside in the N-terminal domains of α and β polypeptides, which are homologous with the variable domains of immunoglobulins (Davis and
Figure 1.2. Schematic representation of the T cell antigen receptor.

The diagram shows the components of the TCR: the clonotypic heterodimer, CD3 chains (δ, ε and γ), and the ζ family (ζ and η). Associated with these molecules are the non-receptor tyrosine kinases p70s6k and p59fyn(T). The co-receptors CD4, CD8 and CD2 are also represented with the tyrosine kinase p56lck associated to CD4/CD8.
Bjorkman, 1988). It is this domain that is responsible for conferring antigenic specificity to the T cell activation process.

The CD3-γ (25-28kDa), -ε (21kDa), and -δ (21kDa) components are non-polymorphic, and are therefore not involved in the generation of diversity which is associated with the αβ TCR component of the receptor complex (Tan et al., 1991). The cytoplasmic tails of invariant chains are considerably longer than the α and β tails (40 to 100 residues as opposed to 5 residues) of the heterodimer, suggesting that they play a role in coupling the antigen receptor to the signal transduction machinery. The CD3γ, δ and ε chains are the products of three closely linked genes and are related in their primary sequences.

The ζ family is a related, but genetically distinct group from CD3. It consists of three polypeptides ζ and η, which are products of a single gene and the γ chain. ζ and η differ at their C-terminal ends, due to alternative splicing. The ζ chain is expressed as a homodimer or as a heterodimer with η (Samelson et al., 1985). ζ (16kDa) and η (21kDa) chains comprise a small extracellular domain of only nine residues, a transmembrane segment and a large cytoplasmic tail. The C-terminal end of η is about 40 amino acids longer than that of ζ. The ζ and η polypeptides exist at the cell surface, as disulphide linked dimers (ζ-ζ, ζ-η and η-η) (Baniyash et al., 1988), and have been postulated to have different functions at distinct developmental stages (Bauer et al., 1991).

There appears to be a certain amount of redundancy between CD3 chains, since chimeric molecules of cytoplasmic regions of CD3ε, ζ, η and FcεRIγ joined to extracellular and transmembrane domains of cell surface receptors, such as CD4, CD8 or CD16 results in the initiation of early and late signalling pathways, independently of TCR (Irving and Weiss, 1991; Le tourneur and Klausner, 1991; Romeo and Seed, 1991). This redundancy has been explained by the presence of a recurrent functional motif of about 20 amino acids which exist in the cytoplasmic domain of the CD3 and ζ family chains (Weiss, 1993). This motif consists of six conserved amino acid residues spaced precisely over a 26-amino
acid sequence (D/E\textsubscript{X}7D/EX\textsubscript{2}YX\textsubscript{2}LX\textsubscript{7}YX\textsubscript{2}L) (where X corresponds to a variable residue). It is common to each of the CD3 proteins (γ, ε, δ) and ζ family, and has come to be known as the immunoreceptor tyrosine-based activation motif (ITAM) (Cambier, 1995; Reth, 1989) (fig. 1.3). This consensus sequence can be found in the cytoplasmic tail of CD3-δ, CD3-ε, CD3-γ and TCR-ζ family subunits (Wegener \textit{et al.}, 1992). Furthermore, TCR-ζ polypeptide appears to be unique among this set of signalling molecules as it displays three copies of the ITAM motif (Irving and Weiss, 1991). These sequence motifs are essential for intracellular signalling in T cells (Weiss and Littman, 1994), enabling the recruitment of signalling components such as non-receptor PTKs to the T cell receptor. The number and exact sequence of ITAMs that are present in the cytosolic domain appears to have a direct bearing on the efficiency and specificity of signal transduction (Irving \textit{et al.}, 1993). It has become clear recently that ITAM motifs are not however, completely redundant. Indeed, analysis of chimeric receptor signalling indicates that there are distinct differences in their capabilities. For example, TCR-ζ and CD3ε chimeras mediate partially distinct protein tyrosine phosphorylation responses (Letourneur and Klausner, 1992).

1.2.2. Non receptor protein tyrosine kinases.

Associated with the TCR/CD3 complex are three members of the src-related kinases, p\textsubscript{56}\textsuperscript{ck}, p60\textsuperscript{es} and p59\textsuperscript{hm(1)}, of which, p56\textsuperscript{ck} is the best documented. These each contain a unique N-terminal region with a myristylation site for membrane association (Resh, 1994), a catalytic tyrosine kinase domain (SH1 domain), single SH2 and Src-homology-3 (SH3) domains and a negative regulatory C-terminal tyrosine (Mustelin and Burn, 1993). They associate with the cytoplasmic regions of the TCR/CD3 complex, being critical for T cell development (see section 1.4), and have been implicated in the regulation of TCR signal transduction. p70\textsuperscript{cap} and p72\textsuperscript{zuk} are two related non-receptor tyrosine kinases (zeta chain associated protein family (ZAP-70 family)) that are distinct
Figure 1.3. Diagrammatic representation of the oligomeric TCR.

The black rectangles represent the ITAMs present in the cytoplasmic domains of the ζ and CD3 chains. The \((YXXL)_2\) motif is shown, with on each of the ITAM domains, with a schematic illustration of p70zap binding via its N-terminal SH2 domains upon TCR ligation and phosphorylation of tyrosine to give \(Y(PO_4)XXL\) sequence.
from other src-related kinases in possessing two tandem N-terminal SH2 domains. However, they lack the unique N-terminal region with myristylation site, SH3 domain and negative regulatory C-terminal tyrosine phosphorylation site (fig. 1.4). These molecules have also been shown to strongly associate with the cytoplasmic region of the zeta chain upon ligation of the TCR/CD3 complex and may play a role in TCR-mediated intracellular signalling (Chan et al., 1991; Chan et al., 1992).

SH2 domains are sequences of about 100 amino acids originally identified in cytoplasmic tyrosine kinases v-Src and v-Fps (Pawson, 1988; Sadowski et al., 1986). SH2 regions recognise and bind to phosphotyrosine containing peptide sequences (generally denoted as Y(PO$_4$)-X-X-L/I) (Valius and Kazlauskas, 1993). SH3 regions are small protein modules of about 60 amino acids that are found in intracellular signalling proteins such as cytoplasmic PTKs as well as in cytoskeletal and adaptor proteins (Koch et al., 1991; Pawson and Gish, 1992), which bind to proline-rich peptides of about 10 amino acids (generally denoted as X-X-X-P-P-X-P-X-X-) (Ren et al., 1993; Yu et al., 1994). Proteins containing these domains have been shown to be critical for recruiting specific proteins to receptors, so that they can take part in signalling cascades, and allowing signalling networks to be built within the cell (Pawson, 1995; Valius and Kazlauskas, 1993). SH2 and SH3 domains vary slightly around the consensus sequences outlined above, thus bestowing specificity to a particular protein-protein interaction (Songyang et al., 1995).

These protein binding domains have been found to play an essential role in the organisation within the cell upon TCR/CD3 complex ligation. SH2 and SH3 domains enable multimeric signalling complexes to form, recruiting PTKs (p56$^{fos}$, p59$^{fyn}$, p70$^{sca}$) to the receptor complex. Proteins which act as adaptor molecules displaying no intrinsic catalytic activity, such as grb-2 and guanine nucleotide exchange proteins or guanine-nucleotide releasing factors for p21$^{ras}$ such as p120$^{GAP}$ and Vav, as well as phosphatidylinositol-3-kinase (PI-3-K) and
Figure 1.4. Comparison of the structure of src and ZAP-70 family kinases.

The structure of p56\textit{lck} and p59\textit{fyn(T)} are compared with that of p70\textit{zap} and p72\textit{cyk}. The diagonal lined area in all of the kinases represents the catalytic (SH1) domain. The vertical lined area in p56\textit{lck} and p59\textit{fyn(T)} is the regulatory site, which when phosphorylated by p50\textit{cak} is inhibitory to the catalytic activity of the kinases.
phospholipase C-γ1 (PLC-γ1) are integral proteins within these signalling complexes. The interactions between these molecules appear to link T-cell receptor activation to downstream signalling pathways such as PLC-γ1 and the p21ras/mitogen activated protein kinase (MAPK) cascade.

p56^lck^ has been shown to be essential for normal signal transduction through the TCR (Strauss and Weiss, 1992). p56^lck^ was found to be associated to the cytoplasmic region of CD4 and CD8α in mature T cells (Barber et al., 1989; Rudd et al., 1988). The interaction between CD4 and p56^lck^ was mediated by cysteine motifs present in the unique N terminal domain of p56^lck^ in the sequence CXCP (Turner et al., 1990). On the other hand, p59^myc(T)^ was found to be associated directly with TCR-ζ chain and the CD3 γ, δ, ε chains through its N-terminal region (Samelson et al., 1990; Timson-Gauen et al., 1992). The associations of p56^lck^ and p59^myc(T)^ do not require stimulation of the cell. p70^src^ is a 70kDa protein expressed exclusively in T cells and NK cells (Chan et al., 1992), which is found associated to the tyrosine-phosphorylated ζ chain of the TCR/CD3 complex. However, unlike p56^lck^ and p59^myc(T)^, the interaction of p70^src^ requires receptor ligation (Chan et al., 1991; Wange et al., 1992). p72^yk^ is preferentially expressed in B cells and myeloid cells (Law et al., 1994; Taniguchi et al., 1991) but it is found to be expressed in low levels in thymocytes and at even lower levels in mature T cells (Hutchcroft et al., 1992).

A third class of PTK associated with TCR activation is responsible for phosphorylating the C-terminal regulatory tyrosine residue of p56^lck^ (Y^505^) and p59^myc(T)^ (Y^528^) and thus down-regulating their activities. p50^ck^ resembles the src family kinases in possessing the kinase domains, SH2 and SH3 domains. However, it lacks the N-terminal membrane-attachment motif, tyrosine autophosphorylation site and a C-terminal regulatory sequence (Okada et al., 1991).
1.2.3. TCR/CD3 complex ligation.

Ligation of the TCR by antigen leads to the production of interleukin-2 (IL-2), and the proliferation of that specific T cell clone (Altman et al., 1990). Immediately following crosslinking of the TCR/CD3 complex there is an increase of tyrosyl phosphorylation in cytoplasmic and membrane bound proteins within the cell (Hsi et al., 1989). Targets include the ζ and ε chain of the TCR/CD3 complex, and PLC-γ1. The phosphorylation of tyrosine residues within ITAM domains of the TCR/CD3 chains is essential for signalling. Indeed mutation of these tyrosine residues for a non-phosphorylatable residue results in a lack of signalling. Following tyrosine phosphorylation of the ITAMs, signalling proteins are able to associate with the TCR via their SH2 motifs. p70^rap appears to require the modification of both tyrosines in the ITAM domain of TCR-ζ, as the SH2 domains of p70^rap will only bind to tyrosine-phosphorylated TCR-ζ when the phosphorylated residues are present in tandem (Howe and Weiss, 1995) (fig. 1.4).

A problem arises when one considers that an activated kinase is required in order to phosphorylate the TCR/CD3 complex, thus initiating the signalling cascade. The PTPase, CD45 plays an essential role in T cell signalling, having a positive role in T cell signal transduction processes, regulating the activities of p59^fyn(T) and p56^lck, opposing p59^ck (Biffen et al., 1994; Ledbetter et al., 1988). CD45 appears to mediate its effects by dephosphorylating the negative regulatory tyrosine phosphorylation site in the C-terminal of src-homologous kinases. In a CD45^- cell line, the TCR/CD3 complex was found to be completely uncoupled from intracellular pathways (Koretzky et al., 1991), showing CD45 to have an obligatory role in lipid signalling and activation in T cells.

With these considerations, a model has emerged regarding the initiation of T cell signalling. Upon TCR/CD3 ligation of MHC bound antigen, CD4 or CD8 form a ‘bridge’ between these molecules and the MHC molecules increasing the stability of the interaction. (Parnes, 1989). It is evident that co-crosslinking of CD4/CD8 with TCR/CD3 enhances the effectiveness of T cell stimulation, thus
suggesting that the increased avidity and signalling capability in the presence of these co-receptors are critical in the response to antigen (Emmrich, 1988; Saizawa et al., 1987). This interaction possibly localises CD4- or CD8-associated p56\(^{ck}\) at the cytoplasmic domains of the TCR/CD3 complex (Diez-Orejas et al., 1994), thus enabling the phosphorylation of ITAM motifs in the \(\zeta\) and \(\varepsilon\) chains. These phosphorylation events would promote the recruitment of p70\(^{sap}\) to the TCR/CD3 complex. Upon binding, this kinase could become a substrate for p56\(^{ck}\) and this phosphorylation could activate p70\(^{sap}\) (Weiss, 1993). In support of this model, Chan et al. (1992) demonstrated that association of activated p70\(^{sap}\) with TCR-\(\zeta\) is dependent on the co-expression of p56\(^{ck}\) or p59\(^{59k}\). Indeed, p56\(^{ck}\) has been found to be associated with p70\(^{sap}\) (Duplay et al., 1994), but to date no phosphorylation of p70\(^{sap}\) has been described.

Tyrosine phosphorylation events are essential for T cell activation (June et al., 1990b). Upon activation of PTKs, there are a series of signalling cascades which are activated, ultimately leading to transcriptional activation of the IL-2 gene, and T cell proliferation. The main signalling pathways recruited are highlighted below (fig. 1.5).

1.2.4. Phosphoinositide signalling.

TCR ligation causes a rapid increase in phosphatidylinositol-4,5-bisphosphate (PIP\(_2\))-specific PLC-\(\gamma1\) activity (Imboden and Stobo, 1985). This elevation of activity is observed within 30 seconds of stimulation, and is due to tyrosine phosphorylation of PLC-\(\gamma1\). It is this event which is associated with functional coupling of the TCR to the inositol phospholipid pathway (Mustelin et al., 1990; Secrist et al., 1991; Weiss et al., 1991). p56\(^{ck}\) has been found to be associated with PLC-\(\gamma1\) in stimulated T cells, suggesting that p56\(^{ck}\) plays a central role in coupling the TCR to phosphoinositide signalling (Weber et al., 1992). Activation of PLC-\(\gamma1\) elicits the hydrolysis of PIP\(_2\) to generate the second messengers, inositol-1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG), leading
Figure 1.5. Signalling pathways activated upon ligation of the TCR/CD3 complex.
to the release of calcium from intracellular stores and the activation of PKC (Rana and Hokin, 1990) (fig. 1.5). Rapid, sustained increases in intracellular calcium levels \([\text{Ca}^{2+}]_{\text{i}}\), are achieved initially by the release of intracellular calcium stores. Their depletion signals calcium influx across the plasma membrane through calcium channels (Cardenas and Heitman, 1995). This elevation in \([\text{Ca}^{2+}]_{\text{i}}\), is thought to influence calcium/calmodulin-dependent events, such as the activation of the calcium-dependent phosphatase, calcineurin resulting in the modulation of transcription factor activities, such as NF-AT (Clipstone and Crabtree, 1992), which in turn controls transcriptional activity of promoter regions of genes such as IL-2.

DAG stimulation of PKC isoform(s) results in the activation of most of these serine/threonine protein kinases (Berridge and Irvine, 1989; Nishizuka, 1988). In total, twelve mammalian isoforms of the PKC family have so far been identified, all varying in their tissue distribution, primary amino acid sequence, effector sensitivities and substrate specificities \textit{in vitro} (Dekker and Parker, 1994; Hug and Sarre, 1993). Pharmacological evidence has shown that PKC is very important in T cell signalling, promoting the induction of IL-2 receptor expression and IL-2 production and initiation of DNA synthesis via the modulation of activator protein-1 (AP-1) activity (Gupta et al., 1991; Hirano et al., 1984; Isokov and Altman, 1987) (see section 5.1).

1.2.5. \(p21^{ras}\) signalling pathway.

\(p21^{ras}\) is a member of the family of low molecular weight GTP-binding proteins (G proteins), which also includes Rho and Rac. The proteins bind GTP and have an intrinsic GTPase activity that catalyses GTP to GDP. The GTP-bound form of \(p21^{ras}\) is the biologically active form (Satoh et al., 1992), therefore upon hydrolysis of GTP, \(p21^{ras}\) is inactivated. By virtue of its GTPase activity, \(p21^{ras}\) functions as a molecular switch in signal transduction.
This protein appears to be a very important signalling component in T cells (Downward et al., 1992). It is apparent that GTP-bound p21ras can accumulate after TCR crosslinking, via phytohaemagglutinin (PHA) or anti-CD3 stimulation, through a tyrosine kinase linked activation which is independent of calcium and PKC (Izquierdo et al., 1992). This is important, as it indicates that a second tyrosine kinase controlled signalling pathway originates from the TCR involving the p21ras GTPase (Downward et al., 1992; Downward et al., 1990) (fig. 1.6). The activity of p21ras is regulated by the rate of hydrolysis of bound GTP and the rate of exchange of bound GTP (Downward, 1992). Proteins which are responsible for GTPase activity include p120GAP and neurofibromin (McCormick, 1989). Proteins which control guanine nucleotide exchange in T cells include the mammalian homologue of the Drosophila son of sevenless gene (SOS) (Shou et al., 1992), She and Vav whose activity is known to be stimulated by tyrosine phosphorylation (Gulbins et al., 1993; Schlessinger, 1993). In fibroblasts, Grb-2 (an adaptor molecule, which contains two SH3 domains and one SH2 domain (Lowenstein et al., 1992)) has been shown to localise SOS to the cell membrane via the C-terminal SH3 domain of Grb-2. This complex associates with tyrosine phosphorylated motifs, and promotes an increase the activity of p21ras (Buday and Downward, 1993; Gale et al., 1993) (fig. 1.6). This signalling complex formation has also been identified in a B cell line, where She/Vav and Grb-2 are required for p21ras activation (Gulbins et al., 1994; Kumar et al., 1995). However, in T cells She is substituted for Vav (Gupta et al., 1994; Ramos-Morales et al., 1994).

p21ras activation leads to the activation of the MAPK or extracellular signal regulated protein kinase (ERK) cascades (Pelech and Sanghera, 1992; Robbins et al., 1992). T lymphocytes express at least two MAPKs, ERK1 and ERK2, that are stimulated in response to TCR ligation (Nel et al., 1991; Whitehurst et al., 1992). Two intracellular pathways for ERK2 regulation coexist in T cells: one is mediated by p21ras, and the other by PKC (Izquierdo et al.,
Figure 1.6. Schematic representation of p21^ras/Raf-1/MAPK signalling pathway upon ligation of the TCR/CD3 complex.
Plasma membrane TCR

SH2 SH3 Grb2

pSH3

GDP

Pi

p21ras

GTP

p120GAP

Raf-1

PKC

MEK

ERK2

NF-AT, c-jun, c-myc
gene transcription
The TCR stimulates both PKC and p21ras, but upon stimulation of the TCR in cells transfected with the inhibitory p21ras mutant, N17ras, which prevents activation of p21ras, TCR does not activate ERK2 (Izquierdo et al., 1993). This indicates that p21ras and not PKC couples the TCR to MAP kinase regulation.

A downstream effector of p21ras, raf-1 (a serine threonine kinase) physically associates with GTP-bound p21ras via its N-terminal regulatory domain, causing raf-1 activation (Avruch et al., 1994; Hailberg et al., 1994). Recently, raf-1 has been reported to be physically linked to the T cell receptor, associating with δ and γ chains of the CD3 complex in unstimulated cells (Loh et al., 1994). Raf-1 (in some cells it is identified as MAPKKK) is responsible for activating MAPKK (MEK), which upon phosphorylation stimulates ERK2. The activation of p21ras appears to mediate the action of the transcription factors NF-AT, c-Jun, and c-Myc via the MAPKs thus linking this GTPase with the transcriptional activation of the IL-2 gene (Northrop et al., 1993; Pulverer et al., 1991; Seth et al., 1992; Woodrow et al., 1993) (fig. 1.6).

In addition to activation by p21ras, PKC has also been shown to phosphorylate and activate raf-1 in T lymphocytes (Siegel et al., 1990; Whitehurst et al., 1995), opening up the possibility of p21ras-independent activation of the MAPK cascade in T lymphocytes (fig. 1.6). Raf-1 has also been shown to be regulated negatively by cyclic AMP in fibroblasts, PC12 cells and T cells (Chen and Iyengar, 1994; Lange Carter and Johnson, 1994; Whitehurst et al., 1995). The implications of this will be discussed in section 1.6.1.

1.3. Co-receptors of the TCR/CD3 complex.

Accessory molecules play an essential role during T cell interactions with, and activation by APC. They have been shown to augment the biochemical signals provided by the TCR, as well as being implicated in T cell adhesion. The role of CD4/CD8 has been discussed previously (see section 1.2.3).
1.3.1. CD28.

CD28 is a 90kDa homodimeric glycoprotein located on the surface of T cells. It has been identified as the major co-receptor in T cell signalling, interacting with the B7/B11 ligand on the surface of APCs (Linsley et al., 1990). Anti-CD28 monoclonal antibodies are known to induce synergic activation of T cells upon co-stimulation of the cells with PHA, phorbol-12-myristate, 13-acetate (PMA) (Martin et al., 1986; Pierres et al., 1988; Weiss et al., 1986b) or CD2 antibodies (van Lier et al., 1988) to produce enhanced amounts of IL-2, TNF-α, IFN-γ and GM-CSF (Thompson et al., 1989). CD28 stimulation appears to provide a secondary signal which is necessary for T cell proliferation, and prevents the induction of anergy (Schwartz, 1992). This is supported by reports that ligation of CD28 can reverse anergy in peripheral T cells and T cell clones (Harding et al., 1992; Sommer et al., 1993).

PI-3-K is a heterodimeric enzyme containing an 85kDa regulatory subunit and 110kDa catalytic subunit. This kinase has been implicated in CD28 signalling, with the observation that D-3 phosphoinositides are formed in T cells on ligation of the CD28 receptor (Rudd et al., 1994; Ward et al., 1993). This was supported by the discovery of a binding motif for PI-3-K in the cytoplasmic tail of CD28 (Prasad et al., 1994). Binding of PI-3-K to CD28 is found to be necessary for T cell signalling (Pages et al., 1994). Interestingly, CD28 co-clustering with CD45 inhibits proliferation (Ledbetter et al., 1988), which may dephosphorylate the phosphotyrosine residue in the cytoplasmic tail of CD28 (Pages et al., 1994), thus hindering the binding of PI-3-K and downstream signalling events.

1.3.2. CD2.

The CD2 antigen is a 50kDa glycoprotein originally defined as the human T-lymphocyte antigen T11/sheep red blood cell receptor. This cell surface molecule appears to have an important role during thymic differentiation. The natural ligand for CD2 has been identified as lymphocyte function-associated
antigen 3 (LFA-3/CD58) (Selvaraj et al., 1987), which is present on thymic epithelial cells (Springer et al., 1987).

Upon crosslinking of CD2 with specific pairs of monoclonal antibodies, it is possible to induce T cell activation, ultimately resulting in the proliferation of lymphocytes: this is known as the 'alternative' pathway of T cell activation, which is independent of TCR ligation although dependent on the CD3 complex (Alcover et al., 1987; Meuer et al., 1984). The activation of this pathway induces the same events as those stimulated by TCR/CD3. These include IL-2 secretion, increased \([\text{Ca}^{2+}]_i\), PIP_2 hydrolysis, p21^{ras} activation (Graves et al., 1991), increases in cyclic AMP production (Carrera et al., 1988; Hahn et al., 1991) and opening of voltage sensitive Ca^{2+} channels (Alcover et al., 1986). However, CD2 does not appear to be essential for T cell activation, where following the ligation of TCR/CD3 complex in the absence of CD2, IL-2 production has been noted (Moingeon et al., 1988a).

CD2 also functions to facilitate cellular adhesion upon interaction between T lymphocytes and its natural ligand LFA-3 on APCs. Upon elevating cyclic AMP levels, there is an increase in avidity between cells, mediated by CD2. This regulation of CD2 avidity is dependent on the presence of an asparagine residue in the cytoplasmic region of CD2, as amino acid substitution of this residue leads to cells that are incapable of avidity regulation. The role of adhesion by CD2 is found to be independent of CD3 surface expression, however CD3 is essential for T cell activation (Hahn et al., 1993; Rabin et al., 1993). This suggests that the signalling and adhesion pathways mediated by CD2 are separate. It is clear that synergy exists between anti-CD2 and anti-CD3 stimulated pathways, as CD2 can have an active signalling role, and also aid in the interaction between the T cell and the APC (Yang et al., 1986). Increases in avidity enhance the T cell activation by a mechanism that does not require the signalling through CD2 (Moingeon et al., 1988b). Interestingly, it has been demonstrated that CD2 is associated with TCR/CD3 complex on the surface of T cells (Brown et al., 1989).
1.4. Signalling during T cell development.

One of the central problems yet to be resolved in T cell signalling is how the TCR/CD3 complex transduces differential signals in a maturation state-dependent manner (Hueber et al., 1994). In mature T cells, ligation of the TCR/CD3 complex can lead to proliferation and differentiation into effector cells, whereas in the thymus, the immature T cell must undergo rigorous selection procedures in order to produce a T cell repertoire that recognises only foreign peptide antigen in the context of self MHC. Auto-reactive clones are thus selected with TCR-derived signals in developing T lymphocytes. This can result in their proliferation (selection) into mature lymphocytes, or apoptosis (deletion) depending on the antigen specificity of the TCR (see section 1.1).

CD2 is one of the earliest cell surface markers to be expressed on the immature T cell, shown to be present on 85-95% of all developing T cells in the thymus (Lanier et al., 1986; Sewell et al., 1986). The interaction of CD2 on thymocytes with LFA-3 on thymic stromal cells is considered to be of major importance in the expansion and differentiation of immature thymocytes which lack TCR/CD3 complex expression, and in providing IL-2-dependent proliferation signals (Alcover et al., 1986; Denning et al., 1987; Reem et al., 1987). This activation can be mimicked with the use of monoclonal antibodies directed against CD2, causing the TCR/CD3 negative subset to express IL-2R, but not to proliferate. This pathway of thymocyte activation is thought to come under the regulation of the CD3-mediated signalling in the latter stages of maturation (Alcover et al., 1988; Blue et al., 1987; Fox et al., 1985; Ramarii et al., 1987).

At the CD4+/CD8− stage of thymocyte development, the β chain has rearranged and expressed on the surface of the immature thymocyte with a surrogate α chain, gp33, and the CD3 complex (see section 1.1). It was thought that signalling through the pre-TCR may reveal some distinct signalling characteristics in this immature subset of cells. Crosslinking of this pre-TCR with either anti-CD3ε or anti-TCRβ antibody results in an increased level of
intracellular calcium, similar to that seen in mature cells (Groettrup and von Boehmer, 1993; von Boehmer, 1992). However, in CD4+/CD8+ cells it has been proposed that the TCR may be partially uncoupled from PTK activation and thus the subsequent activation of PLC-γ1 and calcium mobilisation (Finkel et al., 1991; Sancho et al., 1992). Ultimately this will lead to immunological unresponsiveness due to a lack of IL-2 receptor (IL-2R) expression, IL-2 secretion and proliferation. The reason for this unresponsiveness has been suggested to be due to differences in the signalling network present in immature T cells. Thus it appears that calcium mobilisation is regulated in a developmental state-dependent manner.

p56^lck appears to be essential for T cell maturation as p56^-lck deficient murine thymocytes are developmentally blocked at the CD4+/CD8- stage (Molina et al., 1992). In these cells TCRβ is rearranged, but not the TCRα chain (Levin et al., 1993b). In contrast, overexpression of p56^lck leads to an inhibition of β- but not α-gene rearrangement (Abraham et al., 1991; Anderson et al., 1992). These studies strongly suggest that the p56^lck signal transduction pathway plays a crucial role in thymocyte development regulating, either directly or indirectly, allelic exclusion of the TCR-β gene and activation of a TCR-α gene rearrangement. This property is specific to p56^lck, as p59^B-family cannot be utilised as a substitute (Cooke et al., 1991).

Interestingly, p56^lck signalling at the CD4+/CD8- stage of development occurs in the absence of the co-receptors CD4 or CD8 and therefore via a different mechanism to that ascribed to p56^lck in mature cells (Anderson and Perlmutter, 1995; Levin et al., 1993a; Palmer et al., 1993) and section 1.2.2). Indeed, if the cysteine motif (which is essential for p56^lck association to CD4 or CD8) is removed in mature T cells (Turner et al., 1990), the activity of the kinase is the same as that observed in wild type p56^lck, indicating that this region is of no importance at this developmental stage.
As development progresses, pS610k is found to be associated with CD4 and CD8 (Nakayama et al., 1993) and the expression of the non-receptor PTK pS906k increases. pS906k does not appear to have a central role in T cell development. This is observed in mice lacking pS906k which display normal ratios of all thymocyte and peripheral T cell populations. Nevertheless, TCR-induced thymocyte proliferation is compromised, suggesting a role for this kinase (Appleby et al., 1992). Indeed, hyper-responsiveness to TCR activation was induced in models that overexpressed pS906k (Cooke et al., 1991). The involvement of the ζ chain is also apparent (Nakayama et al., 1989; Vivier et al., 1991). Indeed it is noted that TCR-ζ chain is hyperphosphorylated in vivo in CD4+/CD8+ thymocytes undergoing differentiation and selection (Nakayama et al., 1989).

Interestingly, p7044q is found to be constitutively associated with the tyrosine phosphorylated TCR-ζ in CD4+/CD8+ thymocytes, and TCR ligation induces tyrosine phosphorylation of p7044q (van Oers et al., 1994). This indicates that this PTK may play a central role in T cell signalling during development. This is supported by evidence that a rare human severe combined immunodeficiency (SCID) model is associated with genetic mutations in the p7044q gene (Arpaia et al., 1994). In these p7044q deficient models, there is a lack of CD4+/CD8+ lymphocytes in the circulation, suggesting that this PTK is involved in the regulation of positive selection of CD8+ cells (Arpaia et al., 1994). Indeed, a recent study, where it has been shown that mice lacking p7044q lack CD4 and CD8 single positive populations (Negishi et al., 1995), with T cell development being blocked at the CD4+/CD8+ thymocyte stage. This implies that p7044q is essential in determining the fate of thymocytes for both CD4 and CD8 lineages.

Therefore it appears that although the same pathways exist in immature as mature T cells, there are some subtle differences in the network that allows the differing signals to prevail in either environment. These include: (i) the
expression of a pre-T cell receptor which has a different α chain (gp33) from that seen on mature T lymphocytes; (ii) the signalling of p56^lck in the absence of CD4/CD8 association; (iii) the possibility of partial uncoupling of the TCR from PTK activation at the CD4^+/CD8^+ stage of development; and (iv) the constitutively hyperphosphorylated TCR-ζ chain which recruits p70^S6K to the TCR.

1.4.1. Signalling pathways involved in apoptosis.

Cell death occurs by two processes, necrosis and apoptosis. Necrosis leads to rapid damage to the plasma membrane, as cells tend to swell and lyse releasing the cell contents into the extracellular space and inducing an inflammatory response (Kerr et al., 1972). Programmed cell death or apoptosis is a process by which cells actively commit suicide (Wyllie, 1980). During apoptosis cells shrink, the chromatin becomes condensed and then degrades to oligonucleosome-length, giving a characteristic DNA laddering pattern, before loss of plasma membrane integrity (Wyllie, 1980; Wyllie et al., 1984). The remnants of the dead cells are recognised and rapidly phagocytosed by neighbouring phagocytic cells in the tissue, thus avoiding any inflammatory response. This process inflicts minimal damage on the surrounding tissue and, during T cell development, is an important option as it enables auto-reactive clones to be disposed of without injury to neighbouring cells (Wyllie, 1980). Programmed cell death occurs on a massive scale within the thymus during negative selection with only 5% of the cells being exported to the periphery (Murphy et al., 1990; Swat et al., 1991).

Apoptosis can be induced in thymocytes by ligation of the TCR (McConkey et al., 1989b; Shi et al., 1991; Smith et al., 1989), Fas/Apo 1 activation (Itoh et al., 1991; Rouvier et al., 1993), or neglect (Collins and Lopez Rivas, 1993). The mechanism by which programmed cell death manifests itself has been studied, and it is apparent that there are multiple routes towards
apoptosis. These include calcium-, glucocorticoid-dependent or cyclic AMP-induced apoptosis (McConkey et al., 1989a; Wyllie, 1980). Glucocorticoids have long been recognised as activators of cell death, as seen by their activation of a calcium- and magnesium-dependent endonuclease (Cohen and Duke, 1984; Wyllie, 1980). Endonuclease activity is responsible for cleaving DNA in linker regions between nucleosomes. Such endonuclease activity leads to the DNA fragmentation patterns associated with apoptosis. Mature lymphocytes also contain this endonuclease activity, but its activation is not coupled to glucocorticoid stimulation, implying that its activity may be developmentally regulated. The activation of endonuclease by glucocorticoid, and thus cell death, has been shown to be dependent on a sustained elevation in [Ca]i (McConkey et al., 1989a). As mentioned above, apoptosis occurs following crosslinking of TCR/CD3 complex in CD4+/CD8+ thymocytes in organ culture (Smith et al., 1989) and in vivo (Shi et al., 1991). Endonuclease activation and cell killing are dependent upon the sustained increase in [Ca]i, mostly from extracellular origin (McConkey et al., 1989b). Interestingly, activation of PKC inhibits this calcium-mediated mode of apoptosis.

Agents that elevate cyclic AMP such as E-series prostaglandins or cyclic AMP analogs have also been shown to induce apoptosis in murine thymocytes (Kaye and Ellenberger, 1992; McConkey et al., 1990a). As cyclic AMP is known to exert its effects through cyclic AMP-dependent protein kinase (PKA) (see section 1.5.4) (Taylor et al., 1990), it is interesting to note that agents that suppress TCR-mediated apoptosis via PKC, such as phorbol ester and IL-1 (McConkey et al., 1990b) also block DNA fragmentation in response to cyclic AMP, indicating that 'cross-talk' exists between these two signalling pathways.

The proto-oncogene bcl-2 was the first gene studied in the context of apoptosis regulation during lymphocyte development (Bakhshi et al., 1985). It has been found that when bcl-2 is overexpressed, it has the ability to block apoptotic pathways which are triggered by, for example, ionising radiation or
glucocorticoids (Strasser et al., 1991). When overexpressed in lymphoid tissue, bcl-2 can cause a disruption in the normal lymphocyte cell development process (Hockenberg et al., 1990). However, bcl-2 largely fails to inhibit TCR-mediated apoptosis signals, suggesting the existence of bcl-2-resistant and -sensitive pathways of apoptosis. Recently, it has been noted that bcl-x, a gene which is homologous to bcl-2, is elevated in CD4+/CD8+ thymocytes, and negligible in CD4+/CD8- thymocytes and single positive cells (Boise et al., 1993). This is in direct contrast to the expression pattern of bcl-2 (Ma et al., 1995). Bcl-x deletion appears to shorten the survival of CD4+/CD8+ thymocytes, whilst not affecting the survival of single positive thymocytes or peripheral T cells, indicating that bcl-x plays a critical role in regulating the survival time of thymocytes in the absence of selection (Ma et al., 1995).

1.5. Cyclic nucleotide signalling.

Since their discovery, the cyclic nucleotides, adenosine-3',5'-cyclic monophosphate (cyclic AMP) and guanosine-3',5'-cyclic monophosphate (cyclic GMP) have been found to play a central role in signal transduction (Sutherland, 1972; Sutherland and Rail, 1958). These intracellular second messengers are generated by adenylyl cyclase and guanylyl cyclase, respectively (Goldberg and Haddox, 1977; Krebs et al., 1987; Sutherland et al., 1968), in response to external stimuli, such as hormones or neurotransmitters. Cellular responses of such agents are mediated through the activation of protein kinases, causing the phosphorylation of target proteins within the cell, leading to a physiological effect (Kuo and Greengard, 1969; Taylor, 1989). In comparison to cyclic AMP, substantially less is known about cyclic GMP and the way that it mediates its actions within the cell. However, cyclic GMP is considered to be of great importance within the field of signal transduction especially with the finding that it can be elevated in response to nitric oxide (NO) (Moncada et al., 1991). Cyclic GMP appears to interact with several intracellular receptor proteins, including
protein kinases (Francis et al., 1988; Lincoln et al., 1988), cyclic nucleotide phosphodiesterases (Charbonneau et al., 1986) and cyclic GMP gated ion channels (Kaupp et al., 1989; Lincoln and Cornwell, 1993).

Adenylyl cyclase is coupled to cell surface receptors via GTP binding proteins (G proteins) that either stimulate (G_s) or inhibit (G_i) adenylyl cyclase activity (Gilman, 1987) (fig. 1.7). Cyclic AMP has been shown to bind with high affinity to the regulatory subunits of a protein kinase, thereby activating the enzyme (Taylor, 1989). This enzyme has since been named cyclic AMP-dependent protein kinase (PKA). Binding of cyclic AMP to the PKA-holoenzyme causes the dissociation of the two regulatory subunits from the two active catalytic subunits (Krebs and Beavo, 1979; Taylor, 1989). The catalytic subunits are now free to phosphorylate serine/threonine residues of target proteins, either in the nucleus where they are believed to bind to DNA and alter gene transcription and in the cytoplasm where the subunits may alter post-transcriptional events (Kammer, 1988; Krebs and Beavo, 1979). The modulation of transcription factor activities, such as cyclic AMP responsive element binding protein (CREB) or activation transcription factor (ATF) families, is thought to occur via phosphorylation, under the control of protein kinases such as PKA (Meek and Street, 1992) (fig.1.7). This regulation controls the stimulation or repression of specific gene expression (Comb et al., 1986; Montminy et al., 1986). Cellular cyclic AMP homeostasis is maintained not only by regulating its synthesis by adenylyl cyclase (Cooper et al., 1994; Taussig and Gilman, 1995), but also by control of its degradation, through the action of the cyclic nucleotide phosphodiesterases (PDEs) (Beavo et al., 1994; Conti et al., 1995b).

1.5.1. Receptor-G-protein coupling.

The receptor-adenylyl cyclase complex consists of three components: the receptor, G proteins and the catalytic subunit of the cyclase (fig. 1.7).

Heterotrimeric GTP-binding proteins (G proteins) are a subset of a superfamily of
Figure 1.7. Diagrammatic representation of cyclic AMP metabolism.

Cyclic AMP levels can be regulated at the level of: (i) adenylyl cyclase, via receptor coupling through inhibitory G proteins (G_i) or stimulatory G proteins (G_s); (ii) cyclic AMP phosphodiesterase, by the regulating the level of breakdown of cyclic AMP.
proteins which bind GTP with high affinity (Neer, 1995). They consist of three distinct subunits, α, β and γ, and are generally defined by the nature of their α subunits (Gilman, 1987; Kaziro et al., 1991). α is the guanine nucleotide binding component whilst βγ subunits form a tightly, although non-covalently, associated complex. In the inactive state, heterotrimeric G proteins are bound to the inner leaflet of the plasma membrane via fatty acid modifications such as palmitylation and myristylation (Spiegel et al., 1991; Thissen and Casey, 1994). To date, about twenty subtypes of α subunits, five β subunits and seven γ subunits have been isolated (Milligan, 1995). They are recognised to play a central role in signal transduction, as they couple a diverse array of receptors to intracellular effectors.

All G protein-coupled receptors consist of a single polypeptide chain with seven putative transmembrane domains. Upon agonist binding to the receptor, it is thought that the conformation of the receptor changes, allowing the association of the G protein with cytoplasmic regions of the receptor. Receptor activation and subsequent binding of the G protein promotes the exchange of GTP for GDP. Upon binding GTP, βγ subunits are released as a heterodimer, leaving the α subunit to interact with and modulate the activity of effectors such as adenylyl cyclase (Gilman, 1984). It has become increasingly evident that βγ subunits play a role in signal transduction, having the ability to modulate the activity of certain classes of adenylyl cyclase (see below) (Tang and Gilman, 1991; Tang et al., 1991), PLC (Camps et al., 1992) and ion channels. The α subunit:effector interaction is terminated by an intrinsic GTPase activity which hydrolyses the terminal phosphate of GTP, returning the α subunit in its inactive GDP-bound state. In this state, it re-associates with the βγ complex forming the heterotrimeric conformation once more, which is required for receptor interaction.

1.5.2. Adenylyl cyclases.

Adenylyl cyclases are a group of membrane bound proteins with molecular weights ranging from 120-150kDa, responsible for catalysing the
generation of the second messenger cyclic AMP from ATP (Casey and Gilman, 1988; Gilman, 1987) (fig. 1.8). The initial isolation of a cDNA encoding a polypeptide with adenylyl cyclase activity (Krupinski et al., 1989), termed type-I, was followed by the isolation of multiple other forms of adenylyl cyclase (Bakalyar and Reed, 1990; Cali et al., 1994; Feinstein et al., 1991; Fremont et al., 1992a). There are now over eight genetically distinct mammalian adenylyl cyclase isoforms originating from separate genes. Adenylyl cyclase consists of a single polypeptide, which is predicted to have six transmembrane helices, a large cytoplasmic loop, another six transmembrane helices and a long intracellular tail. This structure is similar to that predicted for transporter species and ion channels (Krupinski et al., 1989). The conservation of primary sequence between species is very high (>90%), but dissimilar between subtypes (overall homology amongst the various isoforms is about 50%). Therefore it appears that these adenylyl cyclase isoforms have evolved differently. Alternatively spliced transcripts (Wallach et al., 1994) and partial sequence identity of novel adenylyl cyclases (type-VII) (Krupinski et al., 1992) are evident, however the extent of molecular diversity within this family is unresolved as yet. Although each isoenzyme family displays different properties of regulation by calcium, PKC, Gi (Giα1, Giα2 and Giα3) and βγ subunits of G proteins (Taussig and Gilman, 1995), all the adenylyl cyclase activities are stimulated via G proteins Gi (Pieroni et al., 1993) and by the diterpene forskolin (Seamon et al., 1981).

1.5.2.1 Type-I, -III, and -VIII adenylyl cyclases.

The expression of these isoforms is largely restricted to the nervous system. Changes in intracellular calcium have profound effects on type-I adenylyl cyclase activity, being stimulated by nanomolar concentrations of calcium/calmodulin, thus causing an increase in cyclic AMP concentrations within the cell. Stimulation by calcium/calmodulin is also seen in the type-III and type-VIII isoforms. Type-III adenylyl cyclase is found to predominate in
Figure 1.8. Diagram showing the action of adenylyl cyclase and cyclic AMP phosphodiesterase on ATP and cyclic AMP respectively.
ATP \xrightarrow{\text{adenylyl cyclases}} \text{Cyclic AMP} \xrightarrow{\text{cyclic AMP phosphodiesterases}} 5'\text{AMP}
olfactory neuroepithelium and is stimulated by PKC isoforms α and ζ (Jacobowitz et al., 1993), however, type-I activity is unaffected by PKC-activation (Yoshimura and Cooper, 1993). The type-I iso-enzyme is also directly regulated by βγ subunits of the G protein, if the level of βγ subunits increases, then adenylyl cyclase activity decreases. At present this is the only isoform which is inhibited by βγ subunits (Tang and Gilman, 1991).

1.5.2.2. Type-II and -IV adenylyl cyclases.

Type-II and -IV isoforms are regulated in a very similar manner: in contrast to type-I, βγ subunits stimulate adenylyl cyclase activity in type-II (Tang and Gilman, 1991) and type-IV isoforms (Gao and Gilman, 1991). Adenylyl cyclase activity is conditionally stimulated by these subunits, i.e. βγ subunits by themselves having no effect, but in the presence of Gαx subunits, cyclase activity is enhanced 5-10 fold (Pieroni et al., 1993). Stimulation of these cyclases requires substantially higher concentrations of βγ than of Gαx, and the source of these subunits is thought to be from Gt or Go.

Evidence is emerging to suggest that the activity of these isoforms can be stimulated by PKC-mediated phosphorylation (Yoshimura and Cooper, 1993), although this is not widely accepted. However, the inhibition of type-II adenylyl cyclase activity mediated by Gt, has been reported to be suppressed by phorbol ester treatment (Chen and Iyengar, 1993). This indicates that there is cross-talk between adenylyl cyclase and Gt-linked receptors at the level of cyclic AMP generation. In addition type-II and type-IV isoforms are insensitive to calmodulin regulation.

1.5.2.3. Type-V, -VI and -VII adenylyl cyclases.

The type-V isoform of adenylyl cyclase has been shown in vitro to be stimulated by phosphorylation mediated by PKC (Kawabe et al., 1994). This effect is specific to the PKC isoforms α and δ (Jacobowitz et al., 1993).
Feedback inhibition of type-V and -VI adenylyl cyclase by PKA-mediated phosphorylation has been studied. Type-VI adenylyl cyclase is found to be present in the S49 lymphoma cell line and contains two PKA phosphorylation sites. Phosphorylation of these sites \textit{in vitro} causes inhibition of this isoform (Premont \textit{et al.}, 1992b). A consensus site for phosphorylation by PKA is also present in the type-V enzyme, the major isoform in the heart. It has recently been shown that this isozyme is inhibited by PKA-dependent phosphorylation (Iwami \textit{et al.}, 1995).

Although these isoforms are both insensitive to micromolar concentrations of calmodulin, they can be inhibited by calcium at low micromolar concentrations (Ishikawa \textit{et al.}, 1992; Yoshimura and Cooper, 1992). Indeed inhibition of cyclic AMP production resulting from elevations in calcium has been correlated with the expression of type-V or type-VI adenylyl cyclases in some cell types (Ishikawa \textit{et al.}, 1992; Yoshimura and Cooper, 1992).

A type-VII adenylyl cyclase activity has been identified in the membranes of S49 lymphoma cell line, but has only been isolated as a partial sequence (Krupinski \textit{et al.}, 1992). However it has been shown that its activity is modestly enhanced by $\beta\gamma$ subunits.

\subsection*{1.5.3. Guanylyl cyclases.}

It is now known that the regulation of synthesis of cyclic GMP can occur via two routes. Some agonists cause calcium-dependent synthesis of membrane-permeant free radicals such as NO (see section 4.3.4), which are able to stimulate the cytoplasmic guanylyl cyclase (Furchgott and Zawadski, 1980; Melion \textit{et al.}, 1981; Rapoport and Murad, 1983). Other agonists interact directly with an extracellular domain of a membrane-spanning guanylyl cyclase, hence increasing its activity (Garbers, 1993). Three mammalian isoforms of plasma membrane guanylyl cyclases have been cloned. They have been designated GC-A, GC-B and GC-C (Lowe \textit{et al.}, 1989; Schulz \textit{et al.}, 1989). Further analysis has revealed
that two of these cyclases are natriuretic peptide receptors GC-A and GC-B, found expressed in the brain (Chinkers et al., 1989).

A cytoplasmic form of guanylyl cyclase has been known to exist for some years as a heterodimer (Kamisaki et al., 1986). The activity of this guanylyl cyclase is dependent on the co-expression of the α (70kDa) and β (82kDa) subunits, as no detectable cyclase activity is found associated with the monomers (Buechler et al., 1991). However from cloning experiments, it appears that each subunit possesses a catalytic domain (Nakane et al., 1990). Soluble guanylyl cyclase in vascular smooth muscle is the target for nitric oxide (NO), resulting in the vasodilation of this muscle (Furchgott and Zawadski, 1980).

1.5.4. Cyclic nucleotide dependent protein kinases.

Cyclic GMP- and cyclic AMP-dependent protein kinases were the first protein kinases that were purported to be related proteins (Lincoln and Corbin, 1977). These kinases are members of a much larger group of protein kinases which phosphorylate serine/threonine residues (reviewed in (Edelman et al., 1987)). Since its discovery, cyclic AMP-dependent protein kinase (PKA) has been found to be the only protein (apart from cyclic AMP phosphodiesterases) that binds to cyclic AMP with such high affinity, in eukaryotic cells. This has led to the suggestion that all the downstream effects of cyclic AMP are mediated through protein phosphorylation via PKA (Taylor et al., 1990).

PKA exists as two major isoforms, type-I and type-II, that differ with respect to their regulatory (R) subunits, as identified by their differing molecular weights, antigenicity, amino acid sequence and affinity for cyclic AMP analogues. Isotypes of each of these regulatory domains have also been identified, RI (α and β) and RII (α and β) subunits, as well as the catalytic (C) (α, β and γ) subunit. These vary between tissues, with type-II PKA being present in all cells, whereas the tissue distribution of type-I PKA is more restricted (Corbin et al., 1977; Rubin et al., 1972). Intracellular localisation also varies, with RI isoforms found
primarily in the cytosolic compartment of the cell, although it has been located to the membrane of erythrocytes (Rubin et al., 1972), and found associated with the T-cell receptor of activated lymphocytes (Skalhegg et al., 1994). The RII isoforms are generally particulate, with up to 75% of the cellular RII pool being associated with the plasma membrane and cytoskeletal compartments (Nigg et al., 1985; Salavatori et al., 1990). In all cases, the inactive holoenzyme form of PKA is a tetrameric conformation, consisting of two R subunits and two C subunits (R₂C₂) (Taylor, 1989) (fig. 1.7). However upon binding of four cyclic AMP to the regulatory subunits (two per R monomer), the active catalytic subunits are released and can migrate through the cytoplasm and to the nucleus (Meinkoth et al., 1990). These subunits are able to phosphorylate their physiological substrates (serine in the context X-R-R-X-S-X) within a number of cytoplasmic and nuclear proteins. Both catalytic and regulatory subunits have been postulated to have a role in the transcriptional regulation of cyclic AMP-responsive genes, however it appears that the catalytic subunits are both necessary and sufficient for this response.

In a similar manner to PKA, there are two classes of cyclic GMP-dependent protein kinase (PKG) present (type-I and type-II forms). PKG is found predominately in cerebellar Purkinje cells, heart and lung tissue (Lincoln and Keely, 1980; Lohmann et al., 1981). Interestingly, PKG is absent from many regions of the brain where cyclic GMP is produced, indicating that it is not the sole modulator of cyclic GMP effects (Lincoln and Cornwell, 1993). Type-I PKG is a homodimer consisting of subunits of approximately 78kDa, whereas type-II PKG is a monomer with a mass of 86kDa. The type-II enzyme is not widespread, being found only in intestinal epithelial cells (deJonge, 1981). However, the type-I class is more widely distributed, consisting of two closely related isoforms type Iα and Iβ, (Lincoln et al., 1988). These differ in their N-terminal region, indicating that they may be alternatively spliced (Francis et al., 1989). Both isoforms are activated by cyclic AMP and cyclic GMP, although a greater
concentration of cyclic nucleotide is needed to activate the β isoform (Landgraf et al., 1992). Binding of cyclic GMP to PKG results in enzyme activation. This enzyme appears to be predominantly cytosolic, although platelets and type-II PKG from intestinal brush border appear to be membrane bound. The consensus sequence which is phosphorylated appears to be, R-L-R-S-R-L-G. This site is very similar to that of PKA and, not surprisingly, these protein kinases can phosphorylate the same sites (Lincoln and Corbin, 1977), although specificity determinants may exist for cyclic GMP kinase. This has been demonstrated, where phenylalanine residues are placed on the C-terminal end of the target serine residues, enhancing the selectivity for PKG compared with PKA (Colbran et al., 1992).

1.5.5. A-kinase anchoring proteins.

In order to perform specific phosphorylations that are necessary in the tight regulation of cellular metabolism one might expect that the activation of a kinase would be compartmentalised, in some way, to ensure targeting and specificity of phosphorylation of the desired substrate. The proposal of selective activation of PKA-pools, which are co-localised with key substrates, may provide the opportunity for this to occur. The type-I PKA isoenzyme has shown to be associated with the TCR/CD3 complex upon TCR ligation (Skalhegg et al., 1994). Interestingly, type-II PKA holoenzyme has been found to be tethered to specific subcellular localisations via an interaction with specific A-Kinase Anchoring Proteins (AKAPs) (Scott and Carr, 1992). The binding to these adaptor proteins is maintained via high affinity interactions. RII dimerisation appears to be required for these interactions to take place (Luo et al., 1990; Scott et al., 1990).

A number of different AKAPs specific for type-II PKA have been identified in the literature by virtue of their molecular weights, subcellular localisation and differential hormone induction (Carr et al., 1993; Carr et al.,
Interestingly, these AKAPs do not appear to anchor PKA II alone, but have recently been reported to bind the protein phosphatase calcineurin (Coghlan et al., 1995). It has been noted that the first 5 amino acids of each RII subunit are critical for AKAP interaction (Hausken et al., 1994). However, it appears that AKAPs contain additional binding sites possibly responsible for targeting them to cellular structures, such as DNA binding domains (Coghlan et al., 1994) and cytoskeletal binding domains (Glantz et al., 1993). This added control to a dynamic system, may allow the redistribution of PKA II to occur in response to external stimuli. The ability of signalling molecules to grouped around AKAPs (Coghlan et al., 1995) may allow tight regulation of the phosphorylation state and thus activity of key substrates.

1.5.6. Nuclear signalling of PKA.

As well as the rapid effects that are associated with PKA in phosphorylation of key signalling molecules, PKA activation also has long-term effects, resulting in the regulation of transcription of specific genes (Lalli and Sassone-Corsi, 1994). The nuclear targets for PKA are transcription factors which bind to palindromic DNA sequences called cyclic AMP-responsive elements (CREs) (TGACGTCA), (Comb et al., 1986; Montminy et al., 1986). The transcription activation factors involved include CRE-binding protein (CREB), and different members of the activation transcription (ATF) family (Weng et al., 1993; Yamamoto et al., 1988). CREB/ATF regulatory proteins are characterised by a leucine-zipper domain and a basic DNA binding domain that recognise the CRE site. These proteins have an important role in regulating basal gene transcription or induced gene transcription in response to cyclic AMP elevation. This family of transcription factors also share a certain degree of homology with members of the activator protein-1 (AP-1) family, Fos/Jun proteins (see section 5.1.2). Indeed, hetero-dimerisation can occur between the families as well as homo-dimerisation, which creates enormous complexity,
leading to a higher order of regulation, involving cross-talk between lipid and cyclic nucleotide signalling at the level of gene transcription (Altschul et al., 1990; Hai and Curran, 1991; Landschulz et al., 1988). CREB/ATF proteins are the targets for several protein kinases including PKC and PKA, with phosphorylation of these factors stimulating or repressing transcription (de Groot et al., 1993; Yamamoto et al., 1988). Different forms of CREB/ATF proteins bind to different CRE sites, or even the same sites, with differing affinities following phosphorylation by such kinases. This control allows a convergence of signals to occur from different signalling pathways, at the level of gene transcription. Attenuation or inhibition of cyclic AMP-stimulated gene transcription requires dephosphorylation of transcription factors to occur, representing a key mechanism in the negative regulation of CREB activity. It appears that protein phosphatase 2A (PP2A) and PP1 oppose the action of PKA (Hagawara et al., 1992; Wadzinski et al., 1993). The PPTs involved may be dependent on the cells being studied.

Another class of transcription factors involved in the transduction of cyclic AMP effects on transcriptional regulation are CRE-modulators (CREM) (Meyer and Habener, 1993). The complexity of the CREM protein repertoire is enhanced by alternative splicing mechanism giving rise to various forms of CREM which can be activators or repressors of transcription (Poulkes and Sassone-Corsi, 1992).

Cyclic AMP inducible transcription factors (ICERs) have now also been identified, which are involved in the repression of transcription (Molina et al., 1993). The kinetic expression of these transcription factors is characteristic of an early response gene, such as c-fos, c-jun and c-rel (Verma and Sassone-Corsi, 1987), with induction being rapid and transient. These are the first transcriptional repressor in the cyclic AMP signalling pathway whose functions are regulated by the modulation of its own intracellular levels and not by phosphorylation. It is thought to be important in the phenomenon of down-regulation of gene activity after a first burst of activation by cyclic AMP.
1.5.7. Cyclic nucleotide phosphodiesterases.

The degradation of the second messengers cyclic AMP and cyclic GMP is achieved solely by cyclic nucleotide phosphodiesterases (PDEs). These elicit the hydrolysis of the 3′ phosphate ester bond to give the corresponding 5′ nucleoside monophosphate, thus rendering the signal inactive (Butcher et al., 1961; Butcher and Sutherland, 1962) (fig. 1.8). This activity is provided by a diverse group of enzymes with complexity resulting from the presence of both multiple genes together with alternative splicing (reviewed in (Beavo et al., 1994; Bolger, 1994; Conti et al., 1995b; Houslay and Kilgour, 1990; Reeves and England, 1990)).

Cyclic nucleotide hydrolysing PDEs exhibit distinct biochemical characteristics and can be divided into at least seven functional classes (table 1.1): (i) PDE1, which can hydrolyse both cyclic GMP and cyclic AMP and whose activity is stimulated by calcium/calmodulin (Ca^{2+}/CaM); (ii) PDE2 hydrolyses of cyclic AMP and cyclic GMP, with the activity being stimulated by micromolar concentrations of cyclic GMP; (iii) PDE3 specifically hydrolyses cyclic AMP in a manner which is inhibited by micromolar concentrations of cyclic GMP; (iv) PDE4 enzymes are cyclic AMP specific PDEs which are insensitive to cyclic GMP, and are selectively inhibited by the selective inhibitor rolipram; (v) PDE5 and PDE6 enzymes specifically hydrolyse cyclic GMP, but differ in their structure and tissue distribution; (vi) PDE7 specifically hydrolyses cyclic AMP and is insensitive not only to cyclic GMP but also to the non-selective PDE inhibitor isobutylmethylxanthine (IBMX) and the PDE4 selective inhibitor rolipram.

The PDE enzyme family shows a diverse range of properties that are exemplified by their differential sensitivity to phosphorylation by various kinases (Beltman et al., 1993; Degerman et al., 1990; Houslay and Kilgour, 1990; Kilgour et al., 1989; Manganiello et al., 1990a; Pyne et al., 1989; Swinnen et al., 1989b), their inhibitor specificities and cofactor requirements (Degerman et al., 1990; Hall
Table 1.1. Summary of cyclic nucleotide phosphodiesterase isoforms.
<table>
<thead>
<tr>
<th>Isoenzyme Type</th>
<th>Effectors</th>
<th>Substrate Specificity</th>
<th>Selective Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE1 formerly Type-I, Ca(^{2+})/CaM-stimulated PDE</td>
<td>Stimulated by calcium/calmodulin</td>
<td>Differing (K_m) for cAMP and cGMP depends on the tissue</td>
<td>vinpocetine</td>
</tr>
<tr>
<td>PDE2 formerly Type-II, cGMP-stimulated PDE</td>
<td>cAMP activity is stimulated by low ((\mu)M) [cGMP]</td>
<td>High (K_m) for both cAMP and cGMP</td>
<td>EHNA</td>
</tr>
<tr>
<td>PDE3 formerly Type-III, cGMP-inhibited PDE</td>
<td>cAMP activity is inhibited by low ((\mu)M) [cGMP]</td>
<td>Low (K_m) for both cAMP and cGMP</td>
<td>milrinone, cilostamide, amrinone</td>
</tr>
<tr>
<td>PDE4 formerly Type-IV, cAMP-specific PDE</td>
<td>Specific for cAMP</td>
<td>Low (K_m) for cAMP only</td>
<td>rolipram, Ro20-1724</td>
</tr>
<tr>
<td>PDE5 formerly Type-V, cGMP-binding cGMP-specific PDE</td>
<td>Specific for cGMP located in the periphery eg. heart or lung</td>
<td>Isoforms with high and low (K_m) for cGMP only</td>
<td>zaprinast, dipyridamole</td>
</tr>
<tr>
<td>PDE6 formerly Type-V, Photoreceptor PDE</td>
<td>Specific for cGMP Located in rods and cones of visual system</td>
<td>Micromolar (K_m) for cGMP</td>
<td>dipyridamole, M&amp;B22 948</td>
</tr>
<tr>
<td>PDE7 formerly Type-VII, cAMP-specific rolipram-insensitive PDE</td>
<td>Specific for cAMP</td>
<td>Very low (K_m) for cAMP only</td>
<td>None reported Insensitive to rolipram and IBMX</td>
</tr>
</tbody>
</table>
1.5 7.1. Structure of cyclic nucleotide PDEs.

1.5.7.1. Catalytic domain of cyclic nucleotide PDEs.

It has become apparent that PDEs are multidomain proteins, with distinct catalytic and regulatory regions. The comparison of the two mammalian PDEs, Ca\(^{2+}\)/CaM-stimulated cyclic nucleotide PDE from bovine brain and cyclic GMP-stimulated PDE genes from bovine heart indicated that these were unique gene products (Charbonneau et al., 1986). However as amino acid sequences have been determined, it has become clear that there is a conserved region of about 270 amino acids (35kDa) present in nearly all PDE isoenzymes, displaying as much as 90% within and about 30% sequence identity between PDE families (Charbonneau et al., 1986) (fig. 1.9). The homology of this region appears to have been conserved through evolution as deduced by comparing mammalian PDE sequences with sequences of low \(K_m\) yeast PDE2 (Sass et al., 1986) and *Drosophila dance(dnc)* genes (Chen et al., 1986) (fig. 1.9). However, this domain is not present in the higher \(K_m\) PDE enzyme of yeast or in the *Dictyostelium discoideum* PDE (Lacombe et al., 1986; Sass et al., 1986), although these enzymes do show some homology to each other, indicating that there at least two major PDE families from an evolutionary stand-point. Interestingly, yeast PDE2 enzyme is functional in mammalian cells, indicating that this enzyme family is very highly conserved (van Lookeren Campagne et al., 1990).
Figure 1.9. Schematic representation of the catalytic and regulatory domains of cyclic nucleotide phosphodiesterases.

The diagram compares (from top to bottom), Ca\(^{2+}\)/CaM-stimulated, PDE1; cyclic GMP-stimulated, PDE2; cyclic GMP-inhibited, PDE3; cyclic AMP-specific, PDE4 and cyclic GMP-specific, PDE5. The black area indicates the conserved catalytic domain of about 270 amino acid residues between almost all PDEs. PDE1 contains a CaM binding domain in the N-terminal region of the PDE (horizontal lines). PDE2 and PDE5 contain a homologous non-catalytic cyclic GMP binding domain (wavy-lined area). PDE3 has a 44 amino acid insert within its catalytic domain, unique to this class of PDEs. PDE4 is shown to contain upstream conserved regions (UCR-1 and UCR-2) upstream of the conserved catalytic domain unique to this class of PDEs.
The conservation of this 270 amino acid region led to the proposal that this was the catalytic domain, which has been supported by a number of studies. The region was shown to contain a motif (Glu-Leu-Ala-Leu-Met-Tyr-Asn) which is also found in the regulatory subunits of cyclic AMP-dependent protein kinases. This was suggested to be a cyclic AMP binding sequence motif (Scott, 1987). Limited proteolysis of different PDE isoenzymes such as PDE1 and PDE2 has revealed that the catalytic domains of these enzymes are separate from the regulatory domains (Kincaid et al., 1985; Stroop et al., 1989). A 35-40kDa fragment was produced in this way, which had a cyclic AMP hydrolysing activity, but could no longer be regulated by cyclic GMP of Ca\(^{2+}/CaM\) respectively. Site-directed mutagenesis of residues within the conserved domain has revealed that selected amino acids are essential for catalysis (Jin et al., 1992). Among these are a number of invariant histidine residues which are highly conserved in the catalytic region of PDEs obtained from divergent sources (Charbonneau, 1990; Charbonneau et al., 1986; Francis et al., 1994). These amino acids appear to be important in sustaining enzymatic activity, as mutation of these residues abolishes PDE activity (Jin et al., 1992).

1.5.7.1.2. Regulatory domains of cyclic nucleotide PDEs.

The primary sequence towards the N- and C-terminal area of PDEs appear to be more divergent between groups, sharing little homology. However, within groups there still appears to be a similarity of 60-80% extending throughout the entire coding region. This has led to the suggestion that these regions of PDEs may be involved in modulating the function of PDEs. Non-catalytic conserved regions have been reported in all PDE groups, some of which are involved in the modulation of catalytic activity, for example CaM binding site on PDE1. Meanwhile, other conserved sequences are of unknown function such as upstream conserved regions (UCR) identified in PDE4 enzymes.
The binding of CaM to PDE1 causes at least a 4 fold stimulation in PDE activity (Cheung, 1967). Peptides derived from PDE1 were tested for their ability to bind CaM and it was revealed that the CaM binding site is in N-terminal region of these PDEs (Charbonneau et al., 1991) (fig. 1.9). Limited proteolysis of PDE1 produced a 36kDa fragment which is constitutively active and independent of regulation by CaM (Charbonneau et al., 1991). In support of this, PDE1A2 (61kDa) and PDE1A1 (59kDa) have been shown to have similar substrate specificities, but differ in molecular weight and affinity for CaM (Hansen and Beavo, 1986). These PDEs appear to have identical primary sequences except for the N-terminal portion of the enzyme which incorporates the CaM binding site (Novack et al., 1991).

PDE2, PDE5 and PDE6 all possess a conserved domain which is separate from the catalytic domain (Charbonneau et al., 1989) (fig. 1.9). The degree of homology in this region is high (30% between PDE2 and PDE6) (Gillespie and Beavo, 1989b; Stroop and Beavo, 1991). Studies using limited proteolysis on PDE2 have shown that cyclic GMP binding can be separated from the catalytic activity and is localised to a 60kDa chymotryptic fragment (Stroop and Beavo, 1991). PDE2 binds cyclic GMP with high affinity (Martins et al., 1982) via an allosteric domain and a catalytic domain (Charbonneau et al., 1986; Erneux et al., 1985; Yamamoto et al., 1983b). This results in the display of complex kinetics due to actions mediated through the cyclic GMP-binding regulatory site (Erneux et al., 1982; Manganiello et al., 1990b; Pyne et al., 1986; Stroop et al., 1989).

Indeed, hydrolysis of cyclic AMP by PDE2 isolated from hepatocytes shows positive cooperativity, with a Hill coefficient greater than unity observed (about 1.9), indicative of positive homotropic interactions occurring between the regulatory and catalytic sites (Pyne et al., 1986). However, removal of the regulatory site by limited proteolysis has been shown to abolish the cooperativity of catalysis of cyclic AMP (Stroop and Beavo, 1992). This implies that the regulatory and catalytic domains are separate.
Conserved regions has recently been identified in the N-terminal of PDE4 (Bolger et al., 1993). These domains, UCR-1 and UCR-2 appear to be distinct features of the PDE4 family and are strongly conserved between organisms as evolutionarily divergent as *Drosophila melanogaster* and mammals. However, no other close homologs appear to be present in other sequences in GenBank or EMBL databases (Bolger et al., 1993). UCR-1 and UCR-2 are distinct from each other and are separated by a region of low homology. It has been found that UCR-1, but not UCR-2, undergoes alternative splicing (Bolger, 1994). The strong evolutionary conservation of these domains suggests that they may have particular functions.

There is increasing evidence that N-terminal domains of PDEs can have important effects on the localisation and activity of the enzyme. Indeed, on removal of the N-terminal domain of PDE4A (RD1) an increase in PDE activity was observed (McPhee et al., 1995; Shakur et al., 1995). This elevation in PDE activity has also been observed in PDE4B (DPD) and PDE4D upon cleavage of the N-terminus (Jin et al., 1992; Pillai et al., 1993). Studies carried out on PDE4A also revealed that the extreme N-terminus of this PDE is important in conferring membrane association on the PDE (Shakur et al., 1993). Removal of this domain caused the protein to become soluble. A membrane binding domain has also been found in PDE3 enzymes which appears to be isolated in the N-terminal domain (Meacci et al., 1992; Taira et al., 1993).

### 1.5.7.2. PDE1, calcium/calmodulin-stimulated PDE.

PDE1 was first described in bovine brain and rat brain, as a cyclic AMP hydrolysing activity which was elevated in the presence of CaM and Ca²⁺ (Cheung, 1967; Kakiuchi and Yamazaki, 1970). This PDE activity was later isolated from the heart (Teo and Wang, 1973). PDE1 appears to be expressed in a wide range of tissues with the exception of, for example human peripheral blood lymphocytes (Thompson et al., 1976) and monocytes (Thompson et al., 1980).
Ca\textsuperscript{2+}/CaM stimulated PDEs have been found at high concentrations in Purkinje cells and pyrimidal cells of the cerebral cortex (Kincaid et al., 1987), heart (Hansen and Beavo, 1982), lung (Sharma and Wang, 1986b) and testis (Rossi et al., 1988).

The isozymes of this group of PDEs are the product of at least three genes PDE1A (59/61kDa), PDE1B (63kDa) and PDE1C (70kDa). They differ regarding their kinetics, structure, tissue distribution and modes of regulation (Hansen and Beavo, 1986; Sharma and Wang, 1986a; Sharma and Wang, 1986b). Different isoforms of this group were distinguished in the brain and heart by the use of monoclonal antibodies (Hansen and Beavo, 1986; Sharma et al., 1984).

Indeed the amino acid sequence of the PDE1A2 (61kDa) and PDE1B1A (63kDa) from bovine brain tissue have since been determined by direct sequencing of the protein (Charbonneau et al., 1991) and sequencing of cDNA clones (Bentley et al., 1992; Repaske et al., 1992). A comparison of these isoform sequences suggested that although they were homologous they were the products of different genes. PDE1B1A also differs with respect to kinetic properties, having a higher $V_{\text{max}}$ for cyclic GMP than cyclic AMP. Comparing the sequences from the PDE1A2 protein and a PDE1A1 (59kDa) Ca\textsuperscript{2+}/CaM-stimulated PDE from bovine heart tissue revealed them to be identical except for the N-terminus (thought to provide the CaM binding domain (Charbonneau et al., 1986)), implying that these two isozymes are generated from alternatively spliced genes (Novack et al., 1991; Sonnenburg et al., 1993). Moreover, these isozymes appear to differ in their ability to hydrolyse cyclic AMP and cyclic GMP, and their affinity for CaM (Hansen and Beavo, 1986; Sharma and Wang, 1986b). This is of interest, as the brain splice variant is less sensitive to CaM than the enzyme from heart in vitro (Mutus et al., 1985) although the concentrations of CaM in the brain are elevated when compared to the heart. This may be due to the regulatory effect the N-terminal tail modulating the catalytic activity of the enzyme.
The other PDE identified in this group is the larger isoenzyme, PDE1C (70kDa) present in the lung (Sharma and Wang, 1986b), which has a distinct mode of interaction with CaM, associating with CaM independently of calcium concentrations. Interestingly a novel enzyme in this group which is a Ro20-1724 sensitive, Ca²⁺/CaM-stimulated PDE which exhibits a high affinity for cyclic AMP. This enzyme is poised to have a major effect on cyclic AMP concentrations within the cell in the event of increasing [Ca²⁺]ᵢ (Rossi et al., 1988). The PDE has been found to be expressed in monocytes of atopic dermatitis sufferers. Cyclic AMP PDE activity is elevated in leukocytes from patients with atopic disease (such as atopic dermatitis or asthma) (Grewe et al., 1982). It has been proposed that this PDE activity may be the cause of defective cyclic AMP metabolism in these cells (Chan et al., 1993).

The initial work carried out on this group of enzymes was misleading, as these proteins are highly susceptible to proteolysis, thus producing a PDE activity which is constitutively active, and independent of regulation by CaM (Cheung, 1971; Moss et al., 1978). Purification of this enzyme activity in the past gave contaminated samples upon DEAE anion exchange chromatography, as it eluted with the cyclic GMP-specific, PDE5 activity, hence giving misleading kinetics and inhibitor preferences. PDE1 isoforms appears to exist as homodimers, which have the ability to hydrolyse cyclic GMP and cyclic AMP with varying affinities depending on the isoform within the group which is being studied. PDE1 activity can be selectively inhibited by nicardipine (Matsushima et al., 1988) and vinpocetine (Hidaka and Endo, 1984) at micromolar concentrations.

1.5.7.2.1. Regulation of PDE1.

It has become apparent that this group of PDEs is regulated by phosphorylation in vitro. The PDE1A2 and the PDE1B1A isoforms in brain can be phosphorylated by PKA and CaM-dependent protein kinase (CaMPK) (Hashimoto et al., 1989; Sharma and Wang, 1986a) respectively in the N-
terminal domain, in the vicinity of the CaM binding domain (Florio et al., 1994; Sharma and Wang, 1985). These phosphorylation events cause a decrease in the sensitivity of the PDE to CaM and require an elevation in calcium for optimal stimulation of activity. Dephosphorylation of these isoforms by calcineurin restores normal CaM affinity and Ca^{2+} sensitivity (Sharma and Wang, 1986a; Sharma and Wang, 1985). As yet, phosphorylation events in these enzymes have not been reported in intact cells.

A rapid and transient induction of PDE1 activity has recently been reported in CHO cells via a PKC-mediated process (Spence et al., 1995). It is possible to envisage this group of PDEs playing a linking role in the regulation of calcium/phospholipid pathways and cyclic nucleotide signalling.

1.5.7.3. PDE2, cyclic GMP-stimulated PDE.

PDE2 was isolated initially from liver cytosolic fractions (Beavo et al., 1970) and particulate fractions of other rat tissues such as thymocytes (Franks and MacManus, 1971) and brain (Beavo et al., 1971). This isozyme was first purified to homogeneity from bovine adrenal glands and heart tissues (Martins et al., 1982), and has now been identified in a number of other tissues (Erneux et al., 1981; Pyne et al., 1986; Yamamoto et al., 1983a). PDE2 has now cloned from bovine brain and adrenal glands (Sonnenberg et al., 1991; Tanaka et al., 1991), rat brain libraries (Yang et al., 1994) and from rat liver mRNA (Repaske et al., 1993). Two distinct subfamilies of this enzyme exist, PDE2A1 the heart / adrenal isoform (Martins et al., 1982), PDE2A2 the membrane bound form (Yamamoto et al., 1983a) and the PDE2B1 liver form (Pyne et al., 1986) which may be a proteolytic product of PDE2A due to its smaller molecular size. Although the PDE2 enzyme has thus far only been identified as the product of one gene, there is evidence of two potential splice products. These appear to be different at the 5' end of the protein (Yang et al., 1994).
The native enzyme is a homodimer of about 220KDa (Le Trong et al., 1990). Both cyclic AMP and cyclic GMP are hydrolysed by PDE2, although at physiological concentrations of cyclic nucleotides its preferred substrate is cyclic AMP (Moss et al., 1977). PDE2 differs profoundly from all other known cyclic AMP PDE families in that as well as having a catalytic site, they also have a distinct regulatory site (Charbonneau et al., 1986; Charbonneau et al., 1989) (see section 1.5.7.1.2). Cyclic GMP binds to PDE2 with high affinity (Martins et al., 1982) via an allosteric domain and a catalytic domain (Charbonneau et al., 1986; Erneux et al., 1985; Yamamoto et al., 1983b). PDE activity of PDE2 can be stimulated up to 50 fold by micromolar cyclic GMP concentrations. This may have physiological relevance as cyclic GMP may reach these levels in cells following stimulation by atrial natriuretic factor (ANF) or treatment of the cells with sodium nitroprusside (Hamet et al., 1984; Trembly et al., 1986).

Both cyclic AMP and cyclic GMP can be hydrolysed by PDE2 isoenzymes, although cyclic AMP is the preferred substrate (Moss et al., 1977). Members of this enzyme family display complex kinetics due to actions mediated through the cyclic GMP-binding regulatory site (Erneux et al., 1982; Manganiello et al., 1990b; Pyne et al., 1986; Stroop et al., 1989). Thus, in the case of the soluble type-II enzyme from hepatocytes, hydrolysis of cyclic AMP shows positive cooperativity, with a Hill coefficient greater than unity observed (about 1.9). This is indicative of positive homotropic interactions occurring between the regulatory and catalytic sites (Pyne et al., 1986). Indeed removal of the regulatory site by limited proteolysis has been shown to abolish the cooperativity of catalysis of cyclic AMP (Stroop and Beavo, 1992). Cyclic AMP can also bind to the regulatory site and stimulate activity, although much more weakly, leading to a complex kinetic behaviour (Erneux et al., 1982; Pyne et al., 1986). For these reasons, it has been difficult to find effective inhibitors against this enzyme activity, as inhibitors capable of interacting at both sites might stimulate as well as inhibit cyclic GMP-stimulated PDE activity (Erneux et al., 1982; Pyne et al.,
To date, only one inhibitor has been found to abrogate PDE2 enzymatic activity, the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) (Mery et al., 1995; Pages et al., 1994; Podzuweit et al., 1995). This is discussed in section 3.1.

Studies focusing on PDE2 regulation by phosphorylation have shown that the particulate brain isozyme is phosphorylated by PKA in vitro. However, this modification does not alter the kinetic properties of the enzyme (Whalin et al., 1988) and, to date, in vivo phosphorylation of this enzyme activity has not been shown.

Thus, members of this enzyme family are placed in the unique position of being able to tailor cyclic AMP metabolism to the specific requirements of different cell types. This PDE family may also has the ability to integrate regulatory signals from NO signalling systems, as well as modulating the effects of cyclic nucleotides in both heart and adrenal tissues (Hartzell and Fischmeister, 1986). This PDE may be the molecular basis for the opposing actions of cyclic AMP and cyclic GMP seen in some biological systems, such as lymphocytes, as an elevation in cyclic GMP levels reduces cyclic AMP (Hadden, 1988).

1.5.7.4. PDE3, cyclic GMP-inhibited PDE.

This group of PDEs was identified, along with PDE4 (see below) as the low $K_m$ PDEs due to their ability to hydrolyse cyclic AMP with high affinity (Weber and Appleman, 1982). However it was soon realised that this group consisted of two separate activities, one which was inhibited by cyclic GMP and cilostimide, and the other which was sensitive to Ro20-1724. This was the basis of the separation of the low $K_m$ PDE group into two separate PDEs, cyclic GMP-inhibited, PDE3, and cyclic AMP-specific, PDE4 (Yamamoto et al., 1984).

PDE3 isoforms have been shown to occur as both membrane bound (liver and adipose enzyme) (Heyworth et al., 1983; Pyne et al., 1987a) as well as cytosolic (platelet isoform) (MacPhee et al., 1988) species. They are expressed in
a wide array of tissues, such as human platelets (Grant and Coleman, 1984), bovine heart (Harrison et al., 1986), rat liver (Pyne et al., 1987a) and bovine and rat adipocyte tissue (Degerman et al., 1987; Degerman et al., 1988). Some variation has been found upon analysis of the molecular weights of PDE3 isoforms. Generally it has been shown that PDE3 are polypeptides in the 110-135kDa range, although endogenous proteolysis produces fragments in the 44-90kDa range in adipocytes, platelets and cardiac tissue (Degerman et al., 1987; Degerman et al., 1988; Harrison et al., 1986; MacPhee et al., 1988). However, the ‘dense-vesicle’ rat liver cyclic GMP-inhibited PDE has a smaller molecular weight (62 kDa) (Marchmont et al., 1981; Pyne et al., 1987a) and this is proposed to be the native molecular weight of this PDE. Evidence also suggests that this PDE may exist as a 110kDa dimer in situ (Pyne et al., 1987a).

Differences in molecular weights may also be accounted for because PDE3 enzymes are found to consist of separate gene products or alternative splice variants. To date, molecular cloning studies have revealed that the PDE3 family is derived from at least two distinct gene families isolated from human cardiac (PDE3A) and rat adipocyte (PDE3B) cDNA libraries (Meacci et al., 1992; Taira et al., 1993). These two groups are structurally very similar, including the 44 amino acid insert in the catalytic domain, which is a feature of this group of PDEs (fig. 1.9). However, the N-terminal regions are very divergent, with a possible membrane binding role for this region (Meacci et al., 1992; Taira et al., 1993).

Activities in this group of isoenzymes are inhibited by micromolar cyclic GMP. PDE3 have a high affinity (low \( K_m \)) for both cyclic AMP and cyclic GMP (0.1-0.8\(\mu\)M range) (Degerman et al., 1987; Grant and Coleman, 1984; Pyne et al., 1987a). However, cyclic AMP is much more readily hydrolysed (\( V_{\text{max}} \) ratio cyclic AMP/cyclic GMP < 10). As a result, cyclic GMP serves as a potent competitive inhibitor of cyclic AMP hydrolysis (\( IC_{50} > 1\mu\)M for PDE3 from human and bovine platelets (Grant and Coleman, 1984; MacPhee et al., 1986)).
This enzyme is believed to play an important role in allowing 'cross-talk' between cyclic AMP and cyclic GMP signalling systems.

1.5.7.4.1. Regulation of PDE3.

Hormonal regulation of PDE3 has been investigated and well characterised in rat adipocytes (Anderson et al., 1989), in 3T3-L1 adipocyte cell line (Manganiello et al., 1983) and in hepatocytes (Heyworth et al., 1983). Treatment of intact rat or human platelets with prostaglandin E₁ or cyclic AMP elevating agents, such as forskolin stimulates cyclic AMP phosphodiesterase activity (Alvarez et al., 1981). In human platelets, PKA is reported to mediate a phosphorylation event which leads to an increase in this cyclic GMP-inhibited PDE activity (MacPhee et al., 1988). The heart isozyme has been found to be a very good substrate for PKA in vitro (Harrison et al., 1986). This phosphorylation event may mediate a negative feedback mechanism which aids in the restoration of resting cyclic AMP levels within the system (Manganiello et al., 1990a). The increase in cyclic AMP phosphodiesterase activity may play an important role in the desensitisation of platelets to further exposure to adenylyl cyclase agonists.

It has been shown by several investigators that many of the metabolic effects of insulin on carbohydrate and lipid metabolism are mediated at least in part by a cyclic GMP-inhibited PDE activity. Pretreatment of adipocytic and hepatocytes with insulin, or agents that elevate cyclic AMP, stimulates the phosphorylation of the membrane bound (or dense-vesicle) PDE3B (Gettys et al., 1987; Heyworth et al., 1983; Loten and Snyed, 1970; Smith et al., 1991). This has lead to the theory that this isoform is an integral part of these two signalling pathways, and the regulation of glycogenolysis and gluconeogenesis. In hepatocytes it has been suggested that PKA is responsible for this phosphorylation event (Kilgour et al., 1989). The phosphorylation of, and subsequent increase in, PDE3 activity mediates a depression of cyclic AMP levels in hepatocytes. This
study also suggested that an unidentified kinase phosphorylated PDE3, without causing a change in PDE3 activity, but preventing activation and phosphorylation of the PDE by PKA (Kilgour et al., 1989). A possible candidate for this 'silent' kinase was 5'AMP kinase, as it has been previously shown that this kinase prevents PKA from phosphorylating acetyl-CoA carboxylase (Munday et al., 1988).

A similar scenario is seen in adipocytes, where lipolysis is modulated by agents that increase cyclic AMP concentrations such as adrenaline (Anderson et al., 1989) and antagonised by agents that decrease cyclic AMP levels, such as insulin (Manganiello et al., 1992; Smith et al., 1991). Cyclic AMP levels appear to be modulated by the regulation of PDE3, which is found exclusively associated with the endoplasmic reticulum subfraction (Anderson et al., 1989). It appears that phosphorylation in this case occurs at two distinct sites on PDE3 allowing a greater degree of control of PDE activity, and promoting synergy between the two phosphorylation events (Degerman et al., 1990; Smith et al., 1991).

Isoproterenol-induced phosphorylation occurs via a PKA-dependent mechanism promoting lipolysis, but it is thought that a different kinase is responsible for the insulin-induced phosphorylation. Indeed, this phosphorylation occurs mainly on serine/threonine residues, therefore implying that the phosphorylation event is not a direct result of insulin receptor phosphorylation mediated events. Incubation of adipocytes with okadaic acid (a potent inhibitor of protein phosphatase-1 and -2A) reproduced the hormone action (Shibata et al., 1991).

The identity of this ‘insulin-activated kinase’ is unknown, however it acts in a similar manner in adipocytes and liver (Shibata and Kuno, 1990a; Shibata and Kuno, 1990b). The cyclic GMP-inhibited PDE activity in platelets (PDE3A) is also activated upon phosphorylation with the ‘insulin-activated kinase’ (Lopez et al., 1992; Lopez-Aparicio et al., 1993). Interestingly, it has been suggested that there may be a link between insulin activation of the PDE3 activity through PI-3-K, as wortmannin abolishes the phosphorylation event. However, this is not
thought to be direct, suggesting that the action of PI-3-K is upstream of PDE3 (Rahn et al., 1994).

Due to their well documented hormonal regulation, PDE3 isoforms are important targets for therapeutic agents in disease models. There are several inhibitors of this class of PDEs, including the cardiotonic agents amrinone, milrinone and cilostimide, which are described in more detail in section 3.1 (Reeves and England, 1990).

1.5.7.5. PDE4, cyclic AMP-specific PDE.

This group of PDEs is the probably the most intensely studied of the PDE enzymes, encompassing a diverse group of proteins that are important regulators of intracellular signalling. In the past they have been often referred to (along with PDE3) as low-\(K_m\) PDEs due to their ability to hydrolyse cyclic AMP specifically and with a high affinity (\(K_m\) in the micromolar range). Purification of this PDE was initially achieved from dog kidney, and revealed a PDE enzyme of between 48-60kDa which was specific for cyclic AMP, insensitive to inhibition to cyclic GMP (Thompson et al., 1979a). Prior to molecular cloning technology the isolation of PDE4 isoforms proved to be difficult because of various factors, including the PDE4 isoforms being present only in trace amounts, they can be compartmentalised within the cell, and like other PDEs are unstable and easily proteolysed. Despite this, PDE4 isoforms have been isolated from a variety of tissues and species including human heart (Reeves et al., 1987) and rat liver and hepatocytes (Lavan et al., 1989; Marchmont et al., 1981). However, as with PDE3 isoforms there was a difference of opinion on the size of this PDE, ranging from 52kDa for the peripheral, membrane bound rat liver PDE4 (Marchmont et al., 1981), to 60kDa species isolated from human lung (Moore and Schroedter, 1982). This may be due to proteolysis, however it has become evident that the PDE4 family encompasses different gene products (table 1.2).
Table 1.2. Summary of the properties of the PDE4 isoenzyme family.
<table>
<thead>
<tr>
<th>PDE4 Isoform</th>
<th>Splice Variant (Former name)</th>
<th>Molecular Weight (kDa)</th>
<th>Tissue Localisation</th>
<th>Characteristics</th>
</tr>
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<tbody>
<tr>
<td>PDE4A</td>
<td>RNPDE4A1A (RD1)</td>
<td>79</td>
<td>liver heart</td>
<td>N-terminal tail associated with membrane localisation</td>
</tr>
<tr>
<td></td>
<td>RNPDE4A5 (RPDE6)</td>
<td>109</td>
<td>lung brain</td>
<td></td>
</tr>
<tr>
<td>PDE4B</td>
<td>RNPDE4B1 (DPD)</td>
<td>60</td>
<td>liver heart</td>
<td>N-terminal tail associated with subcellular localisation</td>
</tr>
<tr>
<td></td>
<td>RNPDE4B2A (RPDE4)</td>
<td>64</td>
<td>lung brain</td>
<td></td>
</tr>
<tr>
<td>PDE4C</td>
<td>RNPDE4C1A (ratPDE1)</td>
<td>40</td>
<td>liver kidney</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(possibly a partial sequence)</td>
<td></td>
<td>heart</td>
<td></td>
</tr>
<tr>
<td>PDE4D</td>
<td>RNPDE4D2A (ratPDE3.1)</td>
<td>67</td>
<td>brain heart</td>
<td>Regulated by PKA, by phosphorylation and transcription</td>
</tr>
<tr>
<td></td>
<td>RNPDE4D3 (ratPDE3.2)</td>
<td>67</td>
<td>kidney lung</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RNPDE4D4B (ratPDE3.3)</td>
<td>93</td>
<td>blood</td>
<td></td>
</tr>
</tbody>
</table>
PDE4 enzymes were found to have a high sensitivity to what is now widely accepted to be PDE4 selective inhibitors, such as 4-{3-(cyclopentoxy)-4-methoxyphenyl}-2-pyrrolidone (rolipram) and [4-(Butoxy-4-methoxybenzyl)-2-imidazolidinone] Ro20-1724 (Nemoz et al., 1985; Watchel, 1983a) (see section 3.1). They differ from other cyclic AMP PDEs in that their activities are unaffected by Ca²⁺/CaM, or cyclic GMP (Beavo and Reifsnnyder, 1990; Weishaar et al., 1985).

1.5.7.5.1. Molecular cloning of PDE4 isoenzymes.

Studies in Drosophila melanogaster led to the isolation of a region in the X chromosome which encodes the dnc gene (Davis and Kauvar, 1984). It was established that mutations within this locus led to a variety of behavioural defects, disrupted cyclic AMP metabolism and caused sterility in adult females. The dnc mutants showed a phenotypic learning defect, revealing shortened memory of conditioned behaviour (Tulley and Quinn, 1986). The abnormal cyclic AMP metabolism was due to a mutation in a PDE gene, leading to an absence or greatly reduced PDE activity and an elevated cyclic AMP concentration within the cells (Byers et al., 1981; Davis and Kiger, 1981), indicating that dnc was the structural gene for cyclic AMP PDE (Davis and Kauvar, 1984). The connection between cyclic AMP metabolism and learning was supported by the fact that such features were characteristic of a number of lesions in the cyclic AMP pathway in dnc mutants (Chen et al., 1986). The dnc gene was isolated by the chromosome walking technique which generated six RNAs subsequently reported as dnc gene products (Davis and Davidson, 1984). The dnc locus of Drosophila was cloned and sequence analysis of revealed a 45kDa protein exhibiting cyclic AMP-specific PDE activity. Comparison of its sequence with partial protein sequences from other PDEs revealed a conserved domain believed to contain the active site (Charbonneau et al., 1986; Chen et al., 1986). A dnc gene cDNA probe was used to obtain mammalian counterparts by low stringency cross hybridisation screening.
rat brain cDNA libraries (Davis et al., 1989). This procedure identified a series of cDNAs termed rat dnc-like-1 (RD1), RD2, and RD3, which corresponded to a single gene, containing 75% identity in the conserved domain. RD1 was found to be full length, and RD2 and RD3 thought possibly to be splice variants, with alterations in their N-termini (Davis et al., 1989).

The dnc gene cDNA was also used to screen rat testicular cDNA libraries, in order to identify PDE4 enzymes which may be important in gametogenesis (Conti and Swinnen, 1990; Conti et al., 1992; Swinnen et al., 1989a). This resulted in the isolation of two groups of cDNA clones, differing in nucleotide sequences, ratPDE1 and ratPDE2. Further screening of a Sertoli cell cDNA library, using rat PDE2 probe uncovered ratPDE3 and ratPDE4 (Swinnen et al., 1989a) (table 1.2). There was a highly homologous coding region in the middle of the proteins, but the sequence diverged at the N- and C-termini. Sequence analysis showed that ratPDE2 was the same as the RD1 gene. When cDNA clones from the four different groups were used to probe specific tissues, multiple transcripts were found of different sizes, some of which were organ specific (Swinnen et al., 1989a; Swinnen et al., 1989b), indicating that alternate RNA processing or the differential use of transcriptional start sites leads to the expression of multiple proteins from a single gene (Qui et al., 1991).

The genes for two major PDEs of Saccharomyces cerevisiae had also been cloned, a high affinity and a low affinity form (Nikawa et al., 1987; Sass et al., 1986), on the basis of the gene products ability to suppress phenotypes associated with a ras2val19 mutation. Ras proteins are regulators of adenylyl cyclase and consequently cyclic AMP production in yeast. The ras2val19 mutation lead to an elevation of cyclic AMP levels as a consequence of acute sensitivity to heat shock. Selection was performed on the basis of rescue of heat-shock sensitivity, by a lowering of cyclic AMP levels. A rat brain cDNA clone was isolated by virtue of these cross-hybridisation studies, by expressing a rat brain cDNA in yeast containing a ras2val19 mutation (Colicelli et al., 1989). This led to the
isolation of a further PDE gene, DPD (dunce-like phosphodiesterase), which was characterised as a PDE4 and shows high homology to dnc, with 90% homology to RD1 in the catalytic domain (Colicelli et al., 1989). *Dictyostelium discoideum* was also found to contain a PDE4 activity, which has been sequenced by Kessin and colleagues (Lacombe et al., 1986).

Sequence analysis of the mammalian cyclic AMP-specific PDEs shows that they are encoded by four different genes. These have been designated as four separate subclasses, PDE4A, B, C and D. Figure 1.10 shows the peptide sequence alignments of rat PDE4 isoforms. The sequences are of PDE4A1 (RD1), PDE4B2 (ratPDE4), PDE4C1 (RPDE36) and PDE4D1 (rat PDE3.1). A conserved domain is evident between residues 220-500, which is thought to be the catalytic domain (see section 1.5.7.1.1) (Charbonneau et al., 1986; Jin et al., 1992). Using the rat brain cDNA probes, two human homologs of the cyclic AMP PDE were isolated from a human monocyte cDNA library (Davis et al., 1989). The sequence homology resulted in one of these being designated a PDE4B isoform (hPDE4B) and the other a PDE4A (hPDE4A) (Livi et al., 1990; McLaughlin et al., 1993). There was a conserved region of about 270 residues in hPDE4A and hPDE4B, but the N- and C-termini diverged considerably. cDNAs for four human genes have recently identified (DPDE1 to DPDE4) (Bolger et al., 1993). The deduced amino acid sequences are homologous to the *Drosophila* dnc proteins, containing not only the conserved catalytic domain, but also two aminoterminal regions extending upstream of the catalytic domain, upstream conserved regions (UCR-1 and UCR-2), which lack homology with each other (see section 1.5.7.1.2). The UCR domains appear to be unique to the PDE4 and are highly conserved throughout the evolution of this family from *Drosophila* to mammals (Bolger et al., 1993). Although UCR motifs are of unknown function, it has been suggested that they may have critical roles in the regulation of this family, as these regions are conserved through evolution between splice variants (Bolger, 1994).
Figure 1.10. Comparison of primary amino acid sequences of rat PDE4A, B, C, and D.

The peptide sequences of rat PDE4 isoforms have been aligned. The PDE sequences are as follows from top to bottom:
PDE4A1 (RD1) (Accession Nos: M26715, J04554); PDE4B2 (ratPDE4) (Accession Nos: M25350, M28413); PDE4C1 (RPDE36) (Accession No: L27061); and PDE4D1 (ratPDE3.1) (Accession Nos: M25349, M28412). • denoted an identical amino acid residue in both sequences. Note the area of conservation in the middle of the proteins, the catalytic domain, (residues 220-500) and the two conserved regions N-terminal to this UCR2 (residues 176-200) and UCR1 (residues 94-130).
Figure 1.10. Comparison of primary amino acid sequences of rat PDE4A, B, C, and D.

(continued)
The complexity of this family is now being appreciated, with it becoming apparent that four dnc gene homologs have been uncovered in human and rat (Bolger et al., 1993; Davis et al., 1989; Swinnen et al., 1989a). All mammalian homologs of the dnc gene that have been tested have biochemical and pharmacological properties typical of the PDE4 class of PDEs (Beavo and Reifsnnyder, 1990). This includes high specificity for cyclic AMP and inhibition by a specific class of compounds, such as the antidepressant rolipram (Colicelli et al., 1989; Henkel-Tigges and Davis, 1989; Swinnen et al., 1989b). The rat PDE4B1 (DPD) cDNA was shown to encode a protein with a high degree of amino acid sequence identity (~80%) with the cognate Drosophila dnc species within the conserved region. The homologies within the conserved domain between the isoforms are very high, for example PDE4B2 (ratPDE4) shows 90% homology with PDE4A1 (RD1), but diverges considerably at the N- and C-termini (fig. 1.10). However, up to 50% of identical amino acid residues within the conserved domain utilise alternative codons, which suggest that the two cDNA clones are encoded by distinct but related genes (Swinnen et al., 1989a).

The conservation of PDE4 isoforms between species is remarkable, with a greater homology being observed in group members (>98% in PDE4D members) between rat and human, than between groups from the same species (Bolger et al., 1994). This correspondence of sequences of PDE4 genes between different mammals has been further supported by mapping of human and mouse genes (Bolger et al., 1994; Milatovitch et al., 1994). The similarity between PDE4A sequences derived from rat and human is shown in figure 1.11. These sequences appear to be approx. 80% homologous throughout the entire sequence. It may be tempting to suggest the existence of four dnce gene homologs in all mammals.

1.5.7.5.2. Distribution of PDE4 isoenzymes.

It has been demonstrated that PDE4A, B, C and D genes are expressed in specific tissues such as liver, kidney, lung, lymphoid cells (Engels et al., 1994),
Figure 1.11. Comparison of primary amino acid sequence between PDE4A (RD1) and PDE4B (h6.1).

The sequences have been aligned, and h6.1 is above the RD1 amino acid sequence. • denoted an identical amino acid residue in both sequences. Note the area of conservation throughout almost the entire protein.
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<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
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| MCPFPVTTVPLCGPTFVCKATLSRETCQQMEETLLEDWCLEQLSLMTQYR *
| MPLVDFFCETCSK |

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<td>60</td>
<td>70</td>
<td>80</td>
<td>90</td>
<td>100</td>
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| SVSEMAHKTFRMLMRELTHLESMRSNGNQVSEYISTTFDLQKNEVEIPSPST
|   |   |   |   |   |
| 110 | 120 | 130 | 140 | 150 |
| MKERQKQAPPFRPSQPFPVPVHPMQSITGLKLMRNSDNSNNIPRFG
|   |   |   |   |   |
| 160 | 170 | 180 | 190 | 200 |
| VKTDQEEELLASELENLNKWGLNIFCVSDYAGRSSLTCIIMIFQERDILKKF
|   |   |   |   |   |
| 210 | 220 | 230 | 240 | 250 | 260 |
| RIEVTDTMVTYMTLEDHYHAVYHNLSHADVQSTHVLALTAPALDAVFTD
|   |   |   |   |   |
| 270 | 280 | 290 | 300 | 310 |
| LEILAALFAAAIHDVHDHPGSNQFLINTNSELALMNGESVLENHHLAVGFK
|   |   |   |   |   |
| 320 | 330 | 340 | 350 | 360 |
| LLQEDNCIFQNLKSRQRQLRKMVIDMVLATDMSKHMTLLADLKTVMETKK
|   |   |   |   |   |
| 270 | 280 | 290 | 300 | 310 |
Figure 1.11. Comparison of primary amino acid sequence between PDE4A (RD1) and PDE4B (h6.1).
(continued)
and in selected regions of the brain such as the olfactory system, hippocampus and cerebellum (Engels et al., 1995). PDE4C appears to have a more restricted expression, compared to the other isoforms, being absent from human or rat lymphoid cells (Engels et al., 1994). The leukemic T cell line Jurkat was found to express only PDE4A in the resting state, but upon long-term stimulation of the cells with dibutyryl cyclic AMP, the expression of PDE4A and PDE4D was up-regulated (Engels et al., 1994).

The complexity of the PDE4 family has been further complicated by the existence of over twenty splice variants (Boiger, 1994). The importance of such a large family could be to enable cells to maintain a high degree of regulation on cyclic AMP degradation. Each PDE4 locus appears to have distinct tissue distribution, suggesting that these alternatively spliced transcripts have specific and subtle differences in regulation and sub-cellular targeting which enable the tailoring of these PDEs to a specific job. In support of this, investigations carried out on rat PDE4A (RD1), have shown that the N-terminal domain targets RD1 for plasma membrane association (Shakur et al., 1993). This association may be with a specific anchor protein, as opposed to a nonspecific binding with the lipophilic bilayer, due to the nature of the extreme N-terminus (lacks hydrophobic amino acids), and its punctate distribution around the inner leaflet of the membrane (Shakur et al., 1995). Indeed this N-terminal sequence has been shown to confer membrane association on a normally cytosolic protein chloramphenicol acetyltransferase (Scotland and Houslay, 1995) indicating that the N-terminal domain of RD1 contains structural information that targets proteins to the membrane. Membrane association ceases upon cleavage of this region of PDE4A (McPhee et al., 1995; Shakur et al., 1995).

Splice variants of the PDE4B locus are targeted to different regions of rat brain (Lobban et al., 1994), indicating that differential splicing may contribute to tissue specific- as well as subcellular-localisation of these enzymes (Houslay et al., 1995). PDE4B1 (DPD) was found to be exclusively cytosolic and was
selectively expressed in hippocampal, cortex, hypothalamus and striatum regions, whereas PDE4B2 (ratPDE4) was located at the membrane of all brain regions with the exception of the midbrain (Lobban et al., 1994). The restriction of these activities to specific regions of the brain may have selective effects upon brain function. Indeed, it has been found recently that many of the alternatively spliced mRNAs transcribed from rat *dnc* homologs have different patterns of expression in the brain (Bolger et al., 1994). Moreover, mRNAs from the *dnc* loci can be initiated from independent transcriptional start sites (Qiu and Davis, 1993), each of which may be regulated independently (Monaco et al., 1994).

### 1.5.7.5.3. Regulation of PDE4.

PDE4 isoforms have been shown to be regulated via phosphorylation. In rat liver, there is a peripheral membrane bound PDE4 which is subject to phosphorylation in response to insulin (Marchmont et al., 1981; Marchmont and Houslay, 1980a). Phosphorylation causes PDE activation in intact hepatocytes (Heyworth et al., 1983) as well as in isolated plasma membranes (Marchmont and Houslay, 1980b) and has been demonstrated to occur through a cyclic AMP-dependent pathway *in vitro* (Marchmont and Houslay, 1980a). Upon phospho-amino analysis of this enzyme, it was shown that phosphate was incorporated on the serine and tyrosine residues of the enzyme depending on whether membranes or intact cells were studied, respectively (Marchmont and Houslay, 1980a; Pyne et al., 1989).

Cyclic AMP has also been shown to play a role in the regulation of PDE4D splice variants (PDE4D3.1, -D3.2 and -D3.3), via PKA. In the FRTL-5 thyroid cell line PDE4 activity is elevated in response to thyroid stimulating hormone, which is known to elevate intracellular cyclic AMP (Sette et al., 1994a). This hormone dependent cyclic AMP regulation in FRTL-5 cells involves an initial rise in cyclic AMP which returns to basal level within minutes. This return to basal occurs despite the constant presence of hormone due to an activation of
PKA and subsequent phosphorylation and activation of PDE4D. This investigation demonstrated that splice variants from the same locus could be differentially regulated on stimulation of the cell by a particular hormone (Sette et al., 1994b). Differential regulation led to short-term activation of PDE4D3.3, via changes in phosphorylation status and long-term regulation by stimulating de novo protein synthesis of two other splice variants PDE4D3.1 and PDE4D3.2. In addition an elevation of cyclic AMP has also been demonstrated to cause to an increase in ratPDE3 (PDE4D) expression in immature Sertoli cells on stimulation with follicle stimulating hormone (FSH) (Monaco et al., 1994; Sette et al., 1994b; Swinnen et al., 1991) and in cardiac myoblasts (Kovala et al., 1994).

Clearly PDE4 enzymes are extremely important for the regulation of cyclic AMP signalling pathway, with close safeguards on their regulation evolving to ensure that the action of PDE4D is controlled and specific.

1.5.7.6. PDE5, cyclic GMP-binding, cyclic GMP-specific PDE.

Recently the cyclic GMP-specific PDEs were divided into two distinct groups PDE5 and PDE6, as it became clear that the photoreceptor PDEs (PDE6) are quite different from cyclic GMP hydrolysing PDE activities expressed, for example, in the lung (Beavo et al., 1994).

PDE5 was first noted in the supernatants of lung extracts, as a cyclic GMP binding activity (Lincoln et al., 1976). Primary amino acid sequence differences have aided in the identification of splicing or post-translational modifications of the lung PDE5 activity (McAllister-Lucas et al., 1988). As well as its presence in the lung (Coquil et al., 1985), type-V PDE is also found to be present in platelets (Coquil et al., 1980), having similar native molecular weights (177kDa).

Studies of this group of PDEs have been hampered through co-elution with Ca²⁺/CaM-stimulated PDE, therefore giving some misleading results (Beavo, 1988). PDE5 enzymes have an almost complete inability to hydrolyse cyclic AMP. This group of PDEs possess an allosteric, high affinity cyclic GMP
binding site associated with the enzyme, similar to that seen on the cyclic GMP-stimulated PDE although a function has not yet been clearly attributed to this region (Charbonneau et al., 1989; Coquil et al., 1980; Francis et al., 1980). Interestingly, the binding capacity of this site may be increased by IBMX (Coquil et al., 1985). Phosphorylation may also contribute to this effect (Tremblay et al., 1985). PDE5 isoenzymes have been observed to resemble zinc hydrolases, as they contain conserved histidine residues which bind zinc (Francis et al., 1994) and the removal of zinc greatly decreases the PDE activity.

The lung PDE5 is a substrate for PKA and PKG (Thomas et al., 1990) and, in vivo the phosphorylation event appears to be dependent on cyclic GMP being bound to the non-catalytic binding site present on the PDE. This may be important in determining which kinase phosphorylates it, as an increase in cyclic GMP leads to the occupation of the non-catalytic site, and the PDE is then preferentially phosphorylated by PKG (Thomas et al., 1990). Exposure of rat vascular smooth muscle to ANF leads to the phosphorylation of cyclic GMP PDE within one minute (Wyatt et al., 1994).

This group of PDEs is selectively inhibited by dipyridamole and M&B 22948, with IC_{50} values in the region of 1μM (Asano et al., 1977). In a similar way to PDE2 activity, competitive inhibitors such as IBMX, dipyridamole or papaverine enhance cyclic GMP binding to the PDE (Coquil et al., 1985). An important role for this PDE in modulating cyclic GMP effects has been supported by the fact that significant levels of this protein is found in many of the tissues where it is expressed, relative to PKG (Francis et al., 1980).

1.5.7.7. PDE6, photoreceptor PDE.

The mammalian visual pathway is modulated by cyclic GMP specific PDE6 isoenzyme family, which hydrolyses cyclic GMP in response to light in both rod and cone photoreceptors. PDE6 isoforms are found in the outer segments of the rod and cone photoreceptors (Hurwitz et al., 1984; Hurwitz et al.,
Three distinct genes encode this subfamily of PDEs, one cone and two rod PDE genes. The structure of this group of PDEs varies enormously when compared to other cyclic nucleotide PDEs. The membrane bound molecule is a tetramer, that has two larger subunits (α (88kDa) and β (84kDa)), and two smaller subunits (γ) (11kDa) which have an inhibitory function (Baehr et al., 1979; Miki et al., 1975). The soluble rod PDE differs from the membrane bound form as it also has a 15kDa δ subunit associated with it (Gillespie et al., 1989). Upon cloning of the α subunit, it was noted that it contained the conserved region found in the major subclass of PDEs (Charbonneau et al., 1986). As mentioned previously, this group of PDEs has also been to possess a high affinity non-catalytic binding site for cyclic GMP, similar to that seen in cyclic GMP-stimulated, PDE2 (Charbonneau et al., 1989; Gillespie and Beavo, 1989b; Gillespie and Beavo, 1989c; Le Trong et al., 1990; Li et al., 1990). The conservation of this domain implies that the members of this group of enzymes evolved from common ancestry, with functions ranging from visual transduction to memory.

Retinal cyclic GMP-specific PDEs are effector proteins in photoreceptor signal transduction. The interaction of photo-isomerised opsin with molecules of the retinal G protein, transducin, allows GTP to bind, leading to the activation of transducin. α-GTP interaction with and activation of an inactive form of PDE6 causes the hydrolysis of cyclic GMP and a subsequent decrease in intracellular cyclic GMP concentrations. The large local change in cyclic GMP concentrations is sensed by the cyclic GMP binding site on, or closely associated with the cation channel in the plasma membrane (Fesenko et al., 1984). This channel requires bound cyclic GMP in order to remain open, therefore the decrease in cyclic GMP causes the closure of the cyclic GMP-gated cationic channels of the plasma membrane photoreceptor. This results in the hyperpolarisation of a light response in photoreceptors (Hurley, 1992).
Phosphorylation of this isozyme has been reported within the catalytic (Udovichenko et al., 1993), and inhibitory subunits (Tsuboi et al., 1994). These PDEs can be inhibited by IBMX in a similar way to all other PDE isozymes (except PDE7), with a $K_i$ in the region of 10$\mu$M for bovine rod PDE (Baehr et al., 1979; Gillespie and Beavo, 1989a). However, selective inhibitors also exist for this subclass of PDEs, dipyridamole (Coquil et al., 1980), and M&B 22,948 (Weishaar et al., 1986) inhibiting rod and cone PDEs with $K_i$ values of 125 and 400nM respectively (Cerveto et al., 1988).

1.5.7.8. PDE7, cyclic AMP specific PDE.

PDE7 is a low $K_m$ cyclic AMP specific PDE, which is the least characterised of all the isoforms. PDE7 was originally cloned from a human glioblastoma cell line (Michaeli et al., 1993). The novel gene was discovered by complementation strategy based on the rescue of S. cerevisiae mutants as described previously (Colicelli et al., 1989) which was utilised to isolate DPD (see section 1.5.7.5.1). The human gene isolated, HCP1, encoded a PDE with significant homology to other cyclic AMP PDEs including the ~270 amino acid region proposed to contain the catalytic site (Charbonneau et al 1986, Chen et al 1986).

This PDE activity was found to behave like the PDE4 isoenzyme regarding its high affinity for cyclic AMP specifically ($K_m$ 0.2$\mu$M). However, PDE7 differed, as it is insensitive to inhibition by the classical PDE4 inhibitors rolipram and Ro20-1724 and the general phosphodiesterase inhibitor IBMX. Northern blot analysis showed that PDE7 was predominantly expressed in skeletal muscle.

This activity was previously identified in the soluble fractions of rat hepatocyte preparations (Lavan et al., 1989) as an IBMX-insensitive PDE, which shows an aberrant response to Mg$^{2+}$. This is in contrast to PDE4 activities which are activated in a dose dependent fashion by Mg$^{2+}$ and Mn$^{2+}$ (Lavan et al., 1989).
This activity has also been found present in mammalian T cell lines (Bloom, 1994; Ichimura and Kase, 1993).

1.6. Cyclic nucleotides and the immune system.

The function of cyclic nucleotides in the immune response has been under investigation now for many years. Early research suggested that cyclic AMP exerted negative modulations on proliferation of lymphocytes, and cyclic GMP had opposing effects to this. However, it has become increasingly evident that this picture is a little oversimplified. Thus, whilst cyclic AMP-mediated negative modulation of the proliferative response has been reported (Kammer, 1988), there is also a body of research that supports a positive role for cyclic AMP in mitogenesis (Rochette-Egly and Kempf, 1981; Wang et al., 1978).

1.6.1. PKA inhibition of TCR-mediated signalling pathways.

The vast majority of evidence supports the view that cyclic AMP is an inhibitory signal to lymphocyte mitogenesis (Kammer, 1988). However, cyclic GMP has not been studied a great deal. Intracellular levels of cyclic AMP have been manipulated using forskolin (activator of adenylyl cyclase), cell permeable cyclic AMP analogues and cyclic AMP phosphodiesterase inhibitors (Alava et al., 1992; Lerner et al., 1988; O'Shea et al., 1987). Agonists to receptors which are coupled to G<sub>q</sub> proteins such as prostaglandins E<sub>2</sub> or isoproterenol, or through covalent modification (ADP-ribosylation), and activation of G<sub>q</sub> subunits with cholera toxin have also been utilised to elevate cyclic AMP levels. The results of cyclic AMP elevation being an inhibition of proliferation of lymphocytes. Sustained elevation of cyclic AMP levels has also been shown to induce programmed cell death (apoptosis) in T cell hybridomas and thymocytes (Dowd and Miesfeld, 1992; McConkey et al., 1990a) (see section 1.4.1). In addition, evidence has also been collected from disease states, such as leukemic cell lines.
and sarcoidosis patients (Epstein and Hachisu, 1984; Nemoz et al., 1993). Taken together, the general conclusion is that cyclic AMP has an anti-proliferative role.

Cyclic AMP has been shown to be inhibitory at a number of sites on signalling pathways leading to proliferation. Addition of cyclic AMP elevating agents have revealed an uncoupling of the TCR/CD3 complex from its downstream signalling mediators. These events include the inhibition of tyrosine phosphorylation (Anastassiou et al., 1992; Klausner et al., 1987; Park et al., 1992), PIP2 breakdown (Alava et al., 1992; Lerner et al., 1988; O'Shea et al., 1987; Park et al., 1992; Takayama et al., 1988; Tamir and Isakov, 1991), [Ca^{2+}]; elevations and PKC activation (Chouaib et al., 1987; Gray et al., 1988; Paliogianni et al., 1993; Rothman et al., 1993), raf-1 activation (Whitehurst et al., 1995) and K+ conductance (Bastin et al., 1990; Payet and Dupuis, 1992).

The mechanisms underlying such modulation of T cell signalling are as yet poorly understood, but there are some interesting indications of cyclic AMP and TCR-coupled mitogenic signalling pathways which provide some insight into the role(s) of cyclic AMP pathways in lymphocyte signalling. Cyclic AMP appears to exert negative effects on lymphocytes proliferation via PKAI (Skalhegg et al., 1992). Following phosphorylation by PKA, PLCy1 (Ser^{1248}) becomes inactivated thus preventing PIP2 hydrolysis and the activation of PKC (Alava et al., 1992; Park et al., 1992; Takayama et al., 1988; Tamir and Isakov, 1991). Indeed, recent work (Skalhegg et al., 1994) has demonstrated that in activated T lymphocytes, the PKAIα isoform can be isolated with the TCR/CD3 complex.

Moreover, cyclic AMP elevation suppresses raf-1 activity, enabling cyclic AMP to regulate the MAPK signalling cascade. Raf-1 can be activated via p21^{ras} (ras-dependent mechanism) or via PKC (ras-independent mechanism) (see section 1.2.5). Recent studies have shown that cyclic AMP modulates the ras-independent mechanism of raf-1 activation (Whitehurst et al., 1995). However, in
fibroblasts, cyclic AMP is able to inhibit raf-1 activation via both routes (Cook and McCormick, 1993; Lange Carter and Johnson, 1994).

In the later events of proliferation, the main target of T cell inhibition by cyclic AMP appears to be at the level of IL-2 production (Anastassiou et al., 1992; Aussel et al., 1988; Averill et al., 1988; Mary et al., 1987; Novogrodsky et al., 1983; Paliogianni et al., 1993). The regulatory region of the IL-2 gene consists of compact, clustered binding sites for a range of transcription factors that are differentially regulated. It is clear that some factors are more critical than others in the regulation of IL-2 gene transcription: AP-1 and NF-AT appear to be rate limiting for IL-2 expression. IL-2 promoter activity is found to be reduced by elevations in cyclic AMP (Paliogianni et al., 1993). The mechanism is at present poorly understood, but seems to involve the phosphorylation of substrates as the addition of PKA inhibitor restores IL-2 production (Anastassiou et al., 1992; Averill et al., 1988). Post-transcriptionally PKA activation reduces the half-life of IL-2 mRNA transcripts by >50% (Anastassiou et al., 1992), blocks induction of c-jun encoding Jun/AP1 transcription factors (Tamir and Isakov, 1991) and modulates DNA binding activities of nuclear factor κB (NF-κB) and a TGGGC binding factor (Chen and Rothenberg, 1994). This has the effect of leaving no stable protein/DNA contacts for entire IL-2 enhancer region. The inhibition by cyclic AMP appears to target specific genes, i.e. not inhibiting global gene transcription, for example IL-2R, c-fos, c-myc and c-myb genes are unaffected (Chouaib et al., 1985; Farrar et al., 1987; Namamiya et al., 1987; Ramarli et al., 1987; Scholz and Altman, 1989).

Although cyclic AMP exerts inhibitory effects on thymocytes in a similar manner to that observed in mature T cells, some interesting studies have been carried out to show that different subsets of T cells have differing sensitivities for cyclic AMP. The clearest work has been carried out in T helper cells, analysing the two subsets T_{H1} and T_{H2}. T helper cells are separated into two groups on account of the cytokines which are produced upon proliferation. T_{H1} cells
produce IL-2, Th2 cells secrete IL-4, IL-5. Increases in cyclic AMP appears to inhibit IL-2 production, but does not effect IL-4 production (Alava et al., 1992; Galjewski et al., 1990; Novak and Rothenburg, 1990).

Leukemic T cells have been shown to have an elevated phosphodiesterase activity compared with that of normal T cells (Epstein and Hachisu, 1984). Interestingly increases in cyclic GMP PDE activities as well as cyclic AMP PDE activities have been reported in leukemic T cell lines (Epstein et al., 1977; Hait and Weiss, 1977; Hait and Weiss, 1976). Cyclic GMP has been documented to have a regulatory role in the hydrolysis of cyclic AMP in resting lymphocytes, inhibiting cyclic AMP PDE by 80% at micromolar concentrations (Takemoto et al., 1979; Takemoto et al., 1978). However this inhibition is diminished in transformed human B and T cell lines and patients with lymphocytic leukemia (Takemoto et al., 1978). In a further study to analyse this loss of regulation by cyclic GMP, Takemoto et al. (1979) activated human peripheral blood lymphocytes using mitogens. These were found to lack the cyclic GMP-inhibited PDE isoform within four hours of mitogenic stimulation. It would appear that the removal of cyclic GMP regulation resulted in the elevation of cyclic AMP PDE activity. This led to a loss of growth control, possibly due to the lower cyclic AMP levels present in the cells. PDE4 has been shown to be important in controlling the levels of cyclic AMP, as the use of rolipram in mitogenically-stimulated thymocytes leads to an inhibition of proliferation by 60% (Marcoz et al., 1993). Interestingly, in this study, it was found that elevating cyclic GMP via the stimulation of guanylyl cyclase synergised with the inhibition of PDE4 activity. This implies that cyclic GMP has an inhibitory role in rat thymocyte proliferation (Marcoz et al., 1993).

A rather different disease state is illustrated by the study of sarcoidosis. Sarcoidosis is a chronic granulomatous disorder which is characterised by increased immune response at sites of disease activity. One of the features that characterises this disease is the low proliferative response of peripheral blood
lymphocytes to mitogenic lectins. Interestingly, when these cells were studied, it was apparent that cyclic AMP and cyclic GMP PDE activities were decreased by 26% compared with controls (Nemoz et al., 1993). This could be attributed to a sustained elevation in cyclic nucleotide levels which causes anti-proliferative effects.

The studies described above have shown how cyclic AMP may exert a negative effect on proliferation. However, in all of these cases, the elevation of cyclic AMP was achieved either by using pharmacological agents, which mimic rather unphysiological levels of cyclic AMP within normal cells, or by the studies using leukemic cells and proliferating cell lines. This research nevertheless, clearly highlights the importance of a regulated cyclic AMP response and illustrates the deleterious effects that a dysfunction in this system can have on the immune system.

1.6.2. Positive roles of cyclic nucleotides in lymphocyte proliferation.

It was shown over fifteen years ago that lymphocyte proliferation could not proceed without fluctuations in the levels of cyclic nucleotides. Cyclic AMP was shown to be transiently elevated upon stimulation of lymphocytes with mitogenic lectins. Treatment of the cells with indomethacin reversibly prevented the increase in cyclic AMP and the onset of S phase (Wang et al., 1975). This study also highlighted that sustained elevation of cyclic AMP levels was inhibitory to proliferation. Another study demonstrated that the cyclic nucleotide fluctuations and resultant proliferation were dependent on the presence of calcium (Rochette-Egly and Kempf, 1981). Thus showing that cyclic AMP and calcium work in concert to achieve cellular proliferation. Cyclic GMP has also been shown to be important for the proliferation of lymphocytes. Upon stimulation with PHA, cyclic GMP becomes elevated in a calcium dependent manner (Coffey et al., 1977; Coffey et al., 1981; Rochette-Egly and Kempf, 1981).
A positive role of low levels of cyclic AMP in lymphocyte proliferation was supported by the demonstration that upon inhibition of adenylyl cyclase with 2,3-dideoxyadenosine, there was a marked inhibition of proliferative responses upon stimulation of cells with phorbol ester and ionomycin. Moreover, this inhibition could be reversed with the addition of micromolar concentrations of dibutyryl cyclic AMP, enhancing phorbol ester/calci um ionophore induced lymphocyte proliferation by 25-50% (Koh et al., 1995). Transient increases in cyclic AMP have also been observed following ligation of the TCR/CD3 complex (Ledbetter et al., 1986), resulting in a rapid activation of the PKAI isoform (Laxminarayana et al., 1993).

It was shown that CD3, CD4 and CD8 crosslinking activates a cyclic AMP-dependent pathway that regulates the mobility and directional polarisation of these molecules (Kammer et al., 1988). This has been shown previously where crosslinking of Thy-1 resulted in increases in cyclic AMP and a rise in receptor mobility (Butman et al., 1981). Thy-1 expression appears to be under the control of cyclic AMP at CD4+/CD8- stage of thymocyte development. Indeed cells are induced to enter accelerated differentiation in response to prostaglandin E2 or cyclic AMP analogs, protecting them from apoptosis (Bach et al., 1975; Goetzl et al., 1995; Scheid et al., 1975; Singh and Owen, 1975). This implies that cyclic AMP may have a role in the early differentiation of thymocytes and also highlights that altered signalling occurs in at different developmental stages.

Interesting evidence exists for the positive role of cyclic AMP in lymphocyte proliferation (Sugiyama et al., 1992). However, there are some studies in B and T cells which imply that cyclic AMP may have a dual role in regulating the proliferation state of the cell. The first of these involves the effects of treating cytotoxic T lymphocytes (CTLs) with antisense mRNA for the catalytic subunit of PKA (Cα), to identify which processes required PKA activity, without increasing the cyclic AMP levels (Sugiyama et al., 1992). It was found that TCR-triggered protein-synthesis independent responses were down-regulated,
such as cytotoxicity and exocytosis, thereby counteracting TCR-triggered activation. However, cyclic AMP (or the Cα activity, appears to be required for nuclear activity and/or cytoplasmic events in CTL activation, such as cytokine synthesis and secretion. This represents a dual regulation model by cyclic AMP (Pollock \textit{et al.}, 1991).

1.7. Aims and objectives.

The aim of this research project was to gain an understanding of cyclic AMP catabolism in T cells, the cellular arm of the adaptive immune system. The research presented will study the isoforms of cyclic AMP PDEs present in T cells from different species and distinct developmental stages. The research concentrates on immature murine thymocytes, to investigate the differential regulation of these enzymes upon ligation of the T cell antigen receptor complex with monoclonal antibodies or mitogenic lectins.
CHAPTER 2.

MATERIALS AND METHODS.
2.1. Methods.

2.1.1. Isolation of thymocytes/lymphocytes.

2.1.1.1. Preparation of reagents.

In general all reagents were prepared as explictly, as this prolonged their shelf-life and was necessary for some experiments.

(i) RPMI-1640 was stored at 4°C and warmed to 37°C for the experiment.

(ii) Percoll was aliquoted in universals (18ml) and stored at 4°C. Freshly prepared 100% percoll was obtained by addition of 2ml of 10 x strength phosphate buffered saline, pH7.4 (PBS). Gradient strength percoll was produced by dilution of 100% percoll with 1 x PBS, to the desired concentration.

(iii) 10mM Hepes-supplemented RPMI-1640 media: Appropriate volume of 1M Hepes was added to RPMI-1640 media to achieve a final concentration of 10mM. This was stored at 4°C until required, then it was warmed to 37°C. 10 x PBS was prepared to give final concentrations of 1.4M NaCl / 27mM KCl / 15mM KH2PO4 / 81mM Na2HPO4. This was diluted 1 in 10 in double distilled water (ddH2O) to obtain 1 x PBS.

(iv) 2-aminoethylisothiouronium (AET) (102mg) was dissolved in ddH2O and adjusted to pH9.0 with NaOH and then filter sterilised using Millipore Filters (0.22μM). This was freshly prepared for each treatment.

(v) Sheep erythrocytes (SRBCs) were coated with AET. SRBCs (5ml) were centrifuged and washed twice in RPMI-1640 medium (120gav for 10 minutes). AET (4ml) was added to the SRBCs and the suspension was incubated at 37°C for 20 minutes. After this time, the cells were washed 5 times with RPMI-1640 (120gav for 10 minutes) and RPMI-1640 (9ml) was added. AET treated-SRBCs were stored at 4°C for up to a week.

(vi) Anti-immunoglobulin (anti-Ig) coated plates were prepared by coating a petri dish (90mm uncoated for tissue culture) with 10-50μg/ml goat anti-Ig antibody solution. The antibody, in 130mM NaCl / 0.05M Tris/HCl, pH9.0, was sterile filtered using Millipore filters (0.22μM) and incubated on the plates for an hour at
37°C, then overnight at 4°C. After this time, the antibody solution was poured off and the plates were washed with 5% BSA solution containing 1μg/ml anti-Ig antibody. The plates were washed five times in PBS and then stored until required for use at -20°C.

2.1.1.2. Isolation of murine thymocytes/lymphocytes.

The cells were isolated as described previously (Sancho et al., 1992). Briefly, 4 or 12 week old Balb/c mice were killed by an overdose of Sagatal, and the spleens/thymuses were removed and pressed through a steel mesh screen using a 5ml syringe plunger to obtain a single cell suspension. The suspension was then placed into a 50ml centrifuge tube and the debris was allowed to settle out under gravity for 5 minutes. A mononuclear cell suspension was obtained by Ficoll-paque density centrifugation, by layering the cells onto a Ficoll-paque cushion (3ml) and spinning in a swing-out rotor at 440gav for 15 minutes. The red blood cells pelleted, and the thymocytes/lymphocytes sedimented at the interface. The cells were then washed twice with RPMI-1640 media (first 630gav for 10 minutes and then 440gav for 10 minutes). At this stage lymphocyte populations were B cell depleted (see section 2.1.1.3). In vivo-activated and resting populations were separated by differential percoll density gradient (85%-65%-50%) centrifugation. Alternatively, ‘mixed’ populations of cells were obtained by separating the cells on 85%-50% gradients, thus removing red blood cells and dead cells. Cells were loaded per gradient (allowing approx. 3 spleens/thymuses per gradient) and the cells were spun at 950gav for 15 minutes in the swing-out rotor at 4°C. The cells were harvested from the gradients (resting cells from the 85%-65% interface, in vivo-activated cells from the 65%-50% interface and mixed cells at the 85%-50% interface) and washed twice more, as described previously in 10mM Hepes-supplemented RPMI-1640. Cell number and viability was determined by a haemocytometer during the final wash, using Trypan Blue exclusion (see section 2.1.1.5). The cells were resuspended to the desired concentration.
2.1.1.3. B cell depletion of murine lymphocytes.

B lymphocytes were removed from lymphocyte preparations by panning the cells on anti-Ig coated plates. The cells from approx. 3 spleens were panned per plate. The cell suspension was poured on to the plate and left for half an hour at room temperature, during which time the B cells bound to the anti-Ig. The cells were then carefully removed and poured on to a fresh anti-Ig coated plate for a further 30 minutes. After this time, these cells were separated on Percoll gradients as described previously (see section 2.1.1.2).

2.1.1.4. Preparation of human tonsillar T lymphocytes.

Tonsillar T cells were prepared by a modification of a method described previously (Cheung et al., 1982). Tonsils were rinsed twice in pre-warmed RPMI-1640 media, and then diced in a petri dish containing 15ml media. The resulting lumps of tissue were then pressed through a steel mesh screen using a syringe plunger to obtain a single cell suspension. The spill was transferred to a 50ml centrifuge tube (one tube per tonsil) and the debris was allowed to settle under gravity for 5 minutes. Ficoll-paque density centrifugation of the 'supernatant' was carried out (440gav for 15mins at room temperature) to obtain a mononuclear cell suspension. The lymphocytes were removed from the interface and pooled. The suspension was then washed twice in RPMI-1640 media (first 630gav for 10 minutes and then 440gav for 10 minutes) before the cells were resuspended in media (9ml) and AET-SRBCs (1ml). The suspension was pelleted at 120gav for 15 minutes, then foetal bovine serum (FBS) (1ml) was layered just above the 'interface' of the pellet, and the tube was incubated on ice for 30mins. The pellet was resuspended by gently rocking the tube and the entire suspension was transferred to a cushion of Ficoll-paque (10ml). This was spun at 440gav for 20 minutes at room temperature in a swing-out rotor. T lymphocytes, which separated with the AET-SRBCs, were obtained by lysing the erythrocytes with 0.144M NH₄Cl (5ml), allowing to stand for 2 minutes at room temperature, and then adding
RPMI-1640 media and centrifuging at 440g av for 10 minutes. The cells were then washed twice as described previously. The T lymphocytes were then layered on to a three-step percoll gradient (85%-65%-50%), and centrifuged at 950g av for 20 minutes to obtain in vivo-activated and resting populations of cells (resting cells from the 85%-65% interface, and in vivo-activated cells from the 65%-50% interface). The cells were washed twice in RPMI-1640 media containing 10mM Hepes and resuspended at the desired concentration. The cell number and viability was determined by Trypan Blue exclusion during the last wash (see section 2.1.1.5).

2.1.1.5. Determination of cell number and viability.

An aliquot of cells, which had been removed from the suspension of cells during the final wash after differential density centrifugation, was mixed with an equal volume of Trypan Blue solution (0.4%) and the number and viability of the cells was determined. This was achieved by viewing the cells under a light microscope in a hemocytometer. The viability of newly isolated cells was established as the percentage of cells that excluded the Trypan Blue dye; that is the proportion of cells whose membranes remained intact, thus remaining white under the microscope (>95%). The cell number was calculated by using the grid on the slide, which ensured that the same area was counted for each determination. 100 cells in 32 squares denoted 1 x 10^6 cells per ml, thus the cell number could be calculated. Only viable cells were counted, thus obtaining the number of viable cells isolated.

2.1.2. Cell Culture.

2.1.2.1. Jurkat - Human T cell leukemic cell line.

The human leukemic T cell line - Jurkat, is a proliferating mature T cell line, which was isolated from a 14 year old boy suffering from acute lymphoblastic leukemia. This cell line grows in suspension, with a doubling time of 24 hours.
The cells required the following:

- 500ml RPMI-1640 media with glutamine
- 5% (v/v) foetal bovine serum (heat inactivated)
- 1% (v/v) penicillin / streptomycin solution
- 1% (v/v) glutamine solution

On removing cells from liquid nitrogen, the medium was further supplemented with an additional 5% (v/v) FBS, 0.1% (v/v) gentimycin and 0.1% (v/v) ciproxin for the first week of culture.

Jurkat cells were maintained in the media detailed above, under a humidified atmosphere of 95% air and 5% CO₂, at 37°C. Twice weekly, the cells were split, diluting cells into fresh, pre-warmed medium in new 260ml tissue culture flasks (1 in 5 dilution), under sterile conditions.

When freezing cells, they were first pelleted by centrifugation in 50ml tubes, spinning at 440g for 10 minutes, before being resuspended in the freezing medium at a density of 10⁷ cells/ml. This medium consisted of 90% FBS (v/v) and 10% (v/v) dimethyl sulphoxide (DMSO). After aliquoting into cryotubes, the cells were frozen slowly overnight in a -80°C freezer, before being transferred to liquid nitrogen the following day, and stored there until required.

2.1.2.2. T3.2 murine thymoma cell line.

The murine thymoma cell line - T3.2 is a proliferating immature T cell line derived from double positive (CD4⁺/CD8⁺) thymocytes which were taken from transgenic mice expressing the myc under the Thy-1 promoter. The cell line grows in suspension with a doubling time of about 24 hours.

The cells required the following:

- 500ml RPMI-1640 medium with glutamine
- 8% (v/v) foetal bovine serum (heat inactivated)
- 1% (v/v) penicillin / streptomycin solution
- 1% (v/v) glutamine solution
On removing cells from liquid nitrogen, the medium was further supplemented with an additional 2% (v/v) FBS, 0.1% (v/v) gentimycin and 0.1% (v/v) ciproxin for the first week of culture.

T3.2 cells were maintained in the medium detailed above, under a humidified atmosphere of 95% air and 5% CO₂, at 37°C. The cells were able to grow to a density of 2 x 10^6 cells/ml, so twice weekly the cells were passaged, diluting cells into fresh, pre-warmed medium in new 260ml tissue culture flasks (1 in 10 dilution), under sterile conditions.

When freezing cells, they were first pelleted by centrifugation in 50ml tubes, spinning at 440gav for 10 minutes, before being resuspended in the freezing medium at a density of 10^7 cells/ml. This medium consisted of 90% FBS (v/v) and 10% (v/v) DMSO. After aliquoting into cryotubes, the cells were frozen slowly overnight in a -80°C freezer, before being transferred to liquid nitrogen the following day and stored there until required.

2.1.3. Preparation of the monoclonal antibody, 145-2C11.

145-2C11 is a monoclonal antibody (IgG) in hamster directed against the ε chain of the murine T cell receptor/CD3 complex (Leo et al., 1987).

2.1.3.1. 145-2C11 cell line.

The antibody was produced as a hybridoma culture supernatant. The cells required the following:

- 500ml RPMI-1640 media with glutamine
- 5% (v/v) foetal bovine serum (heat inactivated)
- 1% (v/v) penicillin / streptomycin solution
- 1% (v/v) glutamine solution

On removing cells from liquid nitrogen, the medium was further supplemented with an additional 5% (v/v) FBS, 0.1% (v/v) gentimycin and 0.1% (v/v) ciproxin for the first week of culture.
145-2C11 cells were maintained in the medium detailed above, under a humidified atmosphere of 95% air and 5% CO$_2$, at 37°C. Twice weekly, the cells were passaged and the antibody was collected. This was achieved by pelleting the cells by centrifuging them at 440g$_{av}$ for 10 minutes, and freezing the antibody-containing supernatant until it could be further purified (see section 2.1.3.2.). The cells were then diluted 1 in 5 with fresh, pre-warmed medium in new 260ml tissue culture flasks, under sterile conditions.

When freezing cells, they were first pelleted by centrifugation in 50ml tubes, spinning at 1500rpm for 10 minutes, before being resuspended in the freezing medium at a density of 10$^7$ cells/ml. This medium consisted of 90% FBS (v/v) and 10% (v/v) DMSO. After aliquoting into cryotubes, the cells were frozen slowly overnight in a -80°C freezer, before being transferred to liquid nitrogen the following day, and stored there until required.

2.1.3.2. Purification of 145-2C11 antibody.

The hamster anti-mouse CD3-ε mAb was purified as outlined previously (Phillips et al., 1991).

2.1.3.2.1. Ammonium persulphate precipitation of the antibody.

Saturated ammonium solution prepared by dissolving approx. 77g into 100ml distilled water. Heating was required in order to achieve this. The pH of the solution was then adjusted to pH 7.0 using 10M NaOH. The solution could then be stored at 4°C, and was heated in order to dissolve the needle-like crystals when required for use. The supernatant obtained from pelleting the cells (see section 2.1.3.1) was defrosted and an equal volume of saturated ammonium sulphate solution was added slowly to the tissue culture supernatant, stirring at all times. A cloudy precipitate formed. The solution was allowed to stand overnight at 4°C in order to complete the precipitation. The following day, the precipitate was pelleted at 15300g$_{av}$ for 30 minutes at 4°C, and the supernatant was discarded.
(10ml) was added to resuspend the pellet, and the resulting solution was dialysed against PBS as described in section 2.1.3.2.2.

2.1.3.2.2. Dialysis of the antibody precipitate.

The solution was dialysed in order to lower the concentration of ammonium sulphate solution in the antibody containing solution. The dialysis membrane was washed with distilled water, before being boiled for 30 minutes in 2mM EDTA/5mM NaHCO₃. The tubing was then rinsed thoroughly with distilled water, and stored in 70% ethanol. The tubing was rinsed in distilled water before sealing the membrane using two clips at one end, and then the antibody containing solution was added to the membrane. The residual air was eased out of the tubing, leaving an equal amount of the membrane empty, before clipping the other end. Note, the volume of the solution increases about 2 fold upon dialysis. The solution was dialysed overnight (frequent changes of buffer) against 1.5-2.1 PBS or for 48 hours with less frequent buffer changes. After this time, the antibody solution was purified further using a protein A-sepharose column.

2.1.3.2.3. Protein A-sepharose antibody purification.

The column was prepared in a 2ml syringe, by putting a small amount of glass wool into the syringe and then adding Protein A-sepharose (1ml) to the syringe. The sepharose was washed with five volumes of PBS, and then dialysed antibody solution was run through the column. The supernatant contained 0.1-10μg/ml of monoclonal antibody and the binding capacity of the column is 6mg/ml, so the ‘run-through’ was saved for further purification. The column was then washed with PBS, until unbound proteins had been washed off. This was determined by reading the absorbance of the fractions at 280nm (A₂₈₀) (see section 2.1.9.2). The antibody was eluted using five volumes of 0.2M glycine/HCl (pH2.8). The eluate was immediately diluted into 1M Tris buffer to prevent denaturation of IgG at pH2.8. An A₂₈₀ was carried out to determine the antibody
concentration of each fraction, in order to obtain an elution profile, before pooling the fractions. If the protein content was less than 500 µg/ml, the solution was concentrated using Centricon tubes, adding the solution to the tubes and centrifuging at 613 gav for 15 minutes. The solution was then filter-sterilised using Millipore filters (0.22 µM) before being aliquoted and stored at -20°C.

2.1.3.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

The purity of the antibody solution was checked by separating the proteins in the mixture by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples of the antibody containing 100 µg protein (see section 2.1.9.1) were concentrated in a 0.3% (w/v) acrylamide/0.08% (w/v) N,N'-methylenebisacrylamide stacking gel followed by resolution in a 10% (w/v) acrylamide gel, in the presence or absence of β-mercaptoethanol (i.e. reduced or non-reduced) and controls of γ-globulin and BSA (in reduced and non-reduced conditions) were run alongside these samples. Under non-reducing conditions the antibody was observed at ~150 kDa. This should run in parallel with purified γ-globulin. Upon addition of β-mercaptoethanol, the protein is reduced (i.e. the disulphide bonds in the antibody molecule are cleaved), the antibody is seen on the gel as two lower molecular weight forms (heavy chain 50 kDa, light chain 25 kDa). A similar situation is seen with γ-globulin.

2.1.3.3.1. Preparation of buffers for SDS-PAGE.

Resolving gels were prepared by mixing stock acrylamide solution (30% (w/v) acrylamide, 0.8% (w/v) N, N'-methylene bisacrylamide), buffer A (1.5 M Tris/HCl (pH 8.8), 0.4% (w/v) SDS) and ddH2O to give final concentrations of 10% (w/v) acrylamide, 0.24% (w/v) N, N'-methylene bisacrylamide, 0.375 M Tris/HCl (pH 8.8), 0.1% (w/v) SDS, 3% (v/v) glycerol. Polymerisation of the gel
was achieved by the addition of 12μl of TEMED and 80μl of freshly prepared 10% (w/v) ammonium persulphate.

The upper stacking gel was prepared by mixing stock acrylamide solution (30% (w/v) acrylamide, 0.8% (w/v) N, N'-methylene bisacrylamide), buffer B (0.5M Tris/HCl (pH 6.8), 0.4% (w/v) SDS) and ddH₂O to give final concentrations of 3% (w/v) acrylamide, 0.08% (w/v) N, N'-methylene bisacrylamide, 0.125M Tris/HCl (pH6.8), and 0.1% (w/v) SDS. This was polymerised by adding 15μl of TEMED and 45μl of freshly prepared 10% (w/v) ammonium persulphate.

The sample buffer (2x) was prepared by mixing 1M Tris/HCl (pH6.8), glycerol, 10% (w/v) SDS and 0.007% (w/v) bromophenol blue and ddH₂O to give final concentrations of 0.13M Tris/HCl (pH6.8), and 4% (w/v) SDS. For the samples which required reducing, 0.1ml β-mercaptoethanol was added to 1.9ml sample buffer just prior to use.

2.1.3.3.2. Preparation of samples.

The samples containing 100μg protein were added to an equal volume of 2 x sample (Laemmli) buffer with or without β-mercaptoethanol. These samples were boiled for 5 minutes and then analysed in a discontinuous gel system (Laemmli, 1970).

2.1.3.3.3. Protein molecular weight markers.

The pre-stained protein molecular weight standards were myosin H-chain (200kDa), phosphorylase B (97.4kDa), BSA (68kDa), ovalbumin (43kDa), carbonic anhydrase (29kDa), β-lactoglobulin (18.4kDa) and lysozyme (14.3kDa). These standards were made up as described by the manufacturers. Briefly, 500μl 1mM dithiothreitol (DTT) was added to the vial of standards. The contents were boiled for 5 minutes and then vortexed to ensure the solution was homogeneous. The standards were then aliquoted into 20μl aliquots and thawed when required. Once thawed an
equal volume of sample buffer was added to the standard and the vial was boiled for 5 minutes with the rest of the samples, then 20μl was applied to the gel.

2.1.3.3.4. Preparation and running of slab gels.

The gel plates measured 160mm x 180mm, and the gels cast were 1.5mm thick. The gel apparatus was assembled, ensuring that the plates, spacers and comb had been cleaned with acetone prior to use. After ensuring that there were no leaks at the bottom of the gel plates, the resolving gel was cast. Distilled water was then carefully layered over the top to exclude air, and enhance polymerisation. Once set, the water was poured off, and the stacking gel solution was layered on. A gel comb was immediately inserted so that wells would form in the gel, which was removed once the stacking gel had set. The gel was removed from the casting stand, and placed into a gel tank containing 4.5 l running buffer (25mM Tris / 191mM Glycine / 3.5mM SDS). The cathode and anode were immersed in the remaining buffer forming a reservoir above the gel. Samples were loaded onto the gels using a Hamilton microsyringe (Hamilton Co., Reno, Nevada, USA.). Electrophoresis was toward the anode at 40V, 25mA per gel until the bromophenol blue dye front was 0.5cm from the bottom.

2.1.3.3.5. Staining/destaining/drying.

This procedure was carried out as described previously (Sambrook et al., 1989). Following electrophoresis, gels were soaked, with gentle shaking on a rotary shaker, for 1hr in 45% (v/v) methanol, 10% (v/v) acetic acid containing 0.25% (w/v) Coomassie Blue R-250. Gels were destained by washing with 45% (v/v) methanol, 10 % (v/v) acetic acid with frequent changes of the wash solution. Gels were dried down onto Whatman 3MM paper under vacuum at 60°C for two hours and then scanned (fig. 2.1). From this gel, it is possible to see that no BSA is present in the antibody sample. The antibody sample and γ-globulin run...
alongside each other in reducing and non-reducing conditions. Thus indicating that the antibody is uncontaminated with other proteins.

2.1.4. Flow cytometry.

In order to ascertain the lineage/maturation of lymphocytes, defined by cell receptors, fluorescence-activated cell scanning (FACS) was carried out. The principle being that on addition of fluorescence-labelled monoclonal antibodies to a population of cells, they will bind to specific cell surface receptors. On analysis of the fluorescence patterns produced, in the FACS, one can gather information about the level of development of T cells, and the composition of the heterogeneous cell population isolated from murine or human sources, as judged by the distribution of three cell surface markers. In this case FACS was utilised to gain information regarding the murine thymocyte populations.

2.1.4.1. Procedure.

Cells were washed twice with ice cold PBS in plastic FACS scanning tubes, spinning each time at $270g_{av}$, and resuspended at a concentration of $10^6$ cells/100µl. Biotinylated anti-CD3 antibody (1µg/ml), PE-labelled anti-CD8α antibody (1µg/ml) and/or FITC-labelled anti-CD4 antibody (1µg/ml) were added to cells (100µl). The tubes were covered in foil (to stop bleaching of the fluorescent dyes) and incubated on ice for 30 minutes. Blanks were carried out for each condition, preparing cells for incubation with PBS alone. After the appropriate incubation time, cells were washed twice with PBS as before, and Quantum Red Streptavidin (10µl) was added to those tubes that contained biotinylated anti-CD3 antibody. The tubes were again incubated on ice for 30 minutes as described previously. The cells were then washed twice in PBS, suspended in PBS (0.5ml), and fluorescence data was acquired in a Becton-Dickenson Facscan. Analysis of the data was then carried out.
2.1.4.2. Analysis.

The type of analysis carried out on the data was dependent on the number of antibodies used in the labelling process. 'Paint-a-Gate' was used if three fluorescent antibodies were used, as this programme allows the viewing of 3-colour stained, triple positive cells. However, if only two antibodies were used, then two colour 'Dot-plots' were used to analyse the cell populations.

With the use of the 'Paint-a Gate' programme on the Becton-Dickenson Facscan, it was possible to analyse the proportions of single, double and most importantly triple populations present in cells isolated. The fluorescence channels were assigned to a given axes, thus green fluorescence (CD4) was plotted along the x axis; red fluorescence (CD3) was plotted along the z axis; orange fluorescence (CD8) was plotted along the y axis. The blanks were read in the FACS, which gave an indication of the background fluorescence in the absence of antibody, then the samples were read, eliminating the blanks in each case. In this way, the percentages of different populations were obtained.

In order to study dot-plots, gates were set up to eliminate the background fluorescence. After these gates had been created, the data collected previously could be analysed, once again giving an indication of the thymocyte populations present in the cell preparation. FACS analysis showed that the resulting cell populations were approx. 70% CD4+/CD8+ double positive cells of which 15% CD3+/CD4+/CD8+ triple positive, 10% CD3+/CD4+ and 7% CD3+/CD8+ positive.

2.1.5. Cell Lysis.

The cells were disrupted by the procedure described previously (Harnett et al., 1991). This method of cell disruption was employed rather than either homogenisation or sonication procedures, as they not only failed to disrupt all the cells as indicated by Trypan Blue exclusion but caused disruption in a highly variable fashion.
2.1.5.1. Protocol.

Briefly, the cells were pelleted, and disrupted on ice for 5 minutes in homogenisation buffer of 10mM Tris/HCl, pH7.4, 1mM EDTA, 0.25M sucrose, 0.1mM DTT and a protease inhibitor cocktail (1.7mM antipain, 0.15mM aprotinin, 1mM benzamidine hydrochloride, 230µM PMSF, 1.5mM pepstatin A, 1µg/ml leupeptin) together with 0.5mg/ml digitonin. The mixture was then centrifuged for 5 minutes at 12,000g_{av} and the supernatant taken for PDE activity analysis (see section 2.1.7).

In order to extract the membrane proteins, the pellet obtained from soluble extraction was further extracted. This was carried out on ice for 45 minutes in homogenisation buffer and the protease inhibitor cocktail together with 5mg/ml digitonin. As before the mixture was centrifuged for 5 minutes at 12,000g_{av} and the supernatant taken for PDE activity analysis (see section 2.1.7). These concentrations of digitonin did not affect PDE activity (fig. 2.2).

2.1.6. Separation of cyclic nucleotide phosphodiesterase activities using Mono-Q ion-exchange chromatography.

This was performed using the Pharmacia Fast Performance Liquid Chromatography system (FPLC).

2.1.6.1. Preparation of reagents.

Low salt and high salt buffers (A and B respectively) were prepared fresh with buffer A containing; 10mM Tris/HCl, pH7.4, 1mM EDTA, 0.1mM DTT and protease inhibitor cocktail (see section 2.1.5.1). Buffer B contained; 10mM Tris/HCl, pH7.4, 1mM EDTA, 0.75M NaCl, 0.1mM DTT and protease inhibitor cocktail (see section 2.1.5.1).

In order to prepare the sample, thymocytes were prepared as described in section 2.1.1, and were lysed using digitonin as described in section 2.1.5.1. All buffers were filtered through a 0.2 µM filter, and kept at 4°C.
2.1.6.2. Equilibration and maintenance.

When the FPLC system was not in use it was stored in 24% ethanol. In order to prepare the equipment for use, it was rinsed with the buffers. The superloop and the injection valve were washed with buffer A, and the pumps were washed with the respective buffers (low salt buffer for pump A, and high salt buffer for pump B). The column was equilibrated before use by washing with buffer A for 10 minutes with a flow-rate of 1 ml/minute, then washing with buffer B for the same time, and finally again with buffer A. All operations were carried out at 4°C. After use the column was washed in buffer A as before, and then stored in 24% ethanol. The pumps and superloop were washed with 24% ethanol and stored this way until required for further use.

2.1.6.3. Separation of cyclic nucleotide phosphodiesterases.

This was carried out as a modification of the procedure outlined previously (Lavan et al., 1989). The sample (10 ml) was loaded onto the Mono Q column at a flow rate of 1 ml/minute (3-4 mg protein). Fractions were eluted at 1 ml/min using a 120 ml, 3-step gradient of NaCl (0-0.75M) in buffer A as described in Table 2.1. Fractions of 1 ml were collected and 25 μl aliquots assayed for PDE activity as described in section 2.1.7.3.

2.1.7. Cyclic AMP phosphodiesterase assay.

Cyclic AMP phosphodiesterase activity was assayed by a modification of the two-step procedure of Thompson and Appleman (1971) and Rutten et al. (1973) as previously described by Marchmont and Houslay (1980b). The principle of the assay is outlined in fig. 2.3. Briefly, [3H]-cyclic nucleotide (8 position of the adenine ring) is hydrolysed to form labelled nucleotide mono-phosphate. The nucleotide mono-phosphate ring is then converted to the corresponding labelled nucleoside by incubation with snake venom which has 5'-nucleotidase activity. The conditions are such that complete conversion takes place within the incubation
time. Unhydrolysed cyclic nucleotide is separated from the nucleoside by batch binding of the mixture to Dowex-1-chloride. This binds the charged nucleotides, but not the uncharged nucleosides.

2.1.7.1. Stimulation of cells for the assay.

Cells (4 x 10^7 cells/ml) were incubated in RPMI-1640 medium, for up to 30 minutes in the presence of either PHA or anti-CD3 mAb (at concentrations specified in the relevant figure legends) at 37°C. In the experiments where kinase inhibitors are used, the conditions are specified in the appropriate figure legend. At the indicated time points the cells were harvested by a brief centrifugation for 5 seconds at 12 000g. The cells were then disrupted by the procedure described above (see section 2.1.5.1). The supernatant obtained was ‘snap’ frozen in liquid nitrogen and stored in the -80°C freezer until required.

2.1.7.2. Preparation of reagents.

- 3',5'-cyclic AMP: Prepared at a concentration of 1mM in 20mM Tris/HCl, pH7.4 containing 5mM MgCl₂ and frozen as stock. The concentration required for the assay was obtained by diluting the stock on the day of each assay. Note, Mg²⁺ ions are required as a cofactor for cyclic AMP phosphodiesterase activity.

- Rolipram, milrinone, 3-isobutyl 1-methylxanthine (IBMX): These inhibitors were routinely prepared as stock solutions in DMSO and frozen down. On the day of the assay, they were subsequently diluted in 20mM Tris/HCl / 5mM MgCl₂ buffer, pH7.4 to the desired concentration with a maximum concentration of 0.1% DMSO being employed in assays. Such levels of DMSO did not affect the PDE activities (fig. 2.4).

- Hannah ophiophagus snake venom: A 10 mg/ml stock solution was dissolved in distilled water and frozen down in aliquots. This was diluted to 1 mg/ml in distilled water for use in the assay.
Dowex 1X8-400-chloride: This was prepared as described previously (Thompson et al., 1979b). Briefly, at 4°C, 400g Dowex 1-chloride was washed with 1M NaOH (4l) for 15 minutes. After this time, the resin was washed extensively with distilled water, until the pH of the eluate fell to 7.0. The resin was then washed with 1M HCl (4l) for 15 minutes, followed by more washing, in distilled water until the eluate pH rose to 3.0. The resin was stored at 4°C with ddH2O (1:1). For use in the assay, the Dowex:water mix was further diluted with ethanol, to a final ratio of Dowex:water:ethanol (1:1:1). Ethanol is used in order to prevent non-specific binding of adenosine to Dowex and to inhibit adenosine deaminase converting adenosine to inosine, which can bind to Dowex.

2.1.7.3. Assay procedure.

Enzyme containing sample (10^6 cells) (25μl) was added to 20mM Tris/HCl, pH7.4, 5mM MgCl₂ (25μl) containing the required phosphodiesterase effectors at four times the desired concentration, at 4°C. Blanks were included with every experiment done, replacing the enzyme sample with homogenisation buffer (25μl) (see section 2.1.5.1). The reaction was started with the addition of 2μM cAMP containing 0.15μCi cAMP (50μl), mixing the tubes by vortexing, and incubating for 10 minutes at 30°C. After this time the tubes with placed in a boiling water bath for 2 minutes to terminate the phosphodiesterase activity. The samples were then allowed to cool on ice. 25μg snake venom was added to the tubes and they were incubated for 10mins in a 30°C waterbath. The tubes were then placed on ice, and freshly prepared slurry of Dowex:ethanol:water (1:1:1) (400μl) was added. During this addition, the Dowex was stirred gently, in order to allow a homogeneous suspension was being added. The tubes were vortexed twice over a 15 minute period, and then spun in a benchtop centrifuge (Jouan) at 12 000g, to sediment out the Dowex resin. An aliquot of 150μl supernatant was removed and mixed with 2ml Ecoscint, before being counted in a liquid scintillation counter for 3 minutes per sample.
The effector concentration used is described in the appropriate figure legends. All assays were carried out in triplicate unless otherwise stated.

2.1.7.4. Calculation of specific activities.

The corrected cpm per sample was obtained by subtracting the blank value in each case. Determining the protein content (see section 2.1.9.1) of each sample allowed the results to be expressed in terms of specific activity. The results were then calculated as pmols cAMP hydrolysed/min/mg +/- SEM.

2.1.8. Cyclic AMP binding assay.

The measurement of cyclic AMP concentrations was carried out as outlined previously (Kishihara et al., 1993). The binding protein utilised in the assay is cyclic AMP-dependent protein kinase (PKA) which has binding sites for cyclic AMP. A crude preparation of adrenal glands was made (Brown et al., 1972), which is a rich source for this enzyme. Upon stimulation of the cells in the presence of the desired effectors for a fixed time, the cells were lysed, thus releasing the cytosolic cyclic nucleotides. After neutralising, the cells were incubated with \(^{3}\text{H}\)-cyclic AMP (5- and 8- position of the adenine ring) and cyclic AMP binding protein, allowing competition of labelled and unlabelled cyclic AMP for a limited number of binding sites on the binding protein. A standard curve was obtained by incubating known concentrations of unlabelled cyclic AMP with fixed amounts of binding protein and radioactive cyclic AMP, an example of which is shown in fig. 2.5. Thus the unknown cyclic AMP concentrations can be determined in samples by comparison with the standard curve. After the incubation, activated charcoal was added to the sample to absorb unbound cyclic nucleotide from the solution. The charcoal with the bound cyclic nucleotide was removed by a brief centrifuging step, and a proportion of radioactivity in the sample was ascertained.
2.1.8.1. Preparation of cyclic AMP binding protein.

Bovine adrenal glands (20-30), obtained from the abattoir were transported to the laboratory on ice. At 4°C, the fat was removed from the outside of glands, and they were then hemisected revealing the pale coloured inner medulla and the darker outer cortex. The medulla was removed and discarded, retaining the cortex. One volume of tissue was homogenised in one and a half volumes of buffer (250mM sucrose / 25mM KCl / 5mM MgSO₄ / 50mM Tris/HCl, pH7.4) in a Waring blender. The homogenate was filtered through muslin and then centrifuged at 15 000rpm on MSE 18 for 15 minutes at 4°C. The supernatant was decanted through filter paper, and the eluate was aliquoted into 1ml fractions and stored at -20°C for up to 3 months. Separate samples were thawed and diluted for each assay.

2.1.8.2. Stimulation of cells.

Cells were prepared as described previously (see section 2.1.1), and suspended at 2 x 10⁷ cells/ml. An aliquot of 100μl was used for each time point (2 x 10⁶ cells/assay), with each time point being carried out in triplicate. The agonist was added to the cells and incubated at 37°C. After the desired period an addition of 100μl 4% perchloric acid (PCA) was made to terminate the reaction. The cells were incubated on ice for 2-3 minutes to allow the lysing of the membrane, and the extraction of the cyclic nucleotides. After this time, the cell debris was removed by centrifuging in a bench-top centrifuge for 2 minutes.

2.1.8.3. Neutralising the sample.

Universal indicator was added to the samples, on addition the solution turned pink. The solution was then neutralised by addition of 2M KOH / 0.5M triethanolamine (TEA) dropwise until the solution turned green. Any precipitate that formed was pelleted in a microfuge for 3 minutes, and 50μl of the supernatant was removed for assay.

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2.1.8.4. Preparation of reagents for assay.

(i) [5',8-^3H]-cyclic AMP: 130µl radioactive cyclic AMP was added to 200ml Tris/EDTA buffer, and stored at 4°C until required for use.

(ii) Binding protein: the binding protein was prepared as described in section 2.1.8.1, then diluting the aliquoted sample to the desired concentration.

(iii) Charcoal solution: The desired amount of charcoal solution was made up of 2% activated charcoal and 1% bovine serum albumin in buffer. This was stirred on ice for at least 45 minutes before it was required.

2.1.8.5. Assay procedure.

As mentioned previously, a standard curve was carried out with each assay, for cyclic AMP concentrations between 0.0625 and 16 pmols. This part of the assay was carried out in duplicate, whilst the unknown samples were assayed in triplicate, and was set up as follows:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Standards</th>
<th>Buffer (µl)</th>
<th>[^3H]-cAMP (µl)</th>
<th>Binding Protein (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>-</td>
<td>200</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>3-4</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5-22</td>
<td>0.0625-16</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>23-25</td>
<td>unknown x3</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Tube 1 and 2 were blanks, calculating the amount of cyclic AMP not bound by charcoal, tubes 3 and 4 represented the total binding of cyclic AMP, and tubes 5-22 represent the standard curve, giving counts for fixed concentrations of cyclic AMP, allowing unknown cyclic AMP values to be calculated.

The tubes were set up as described above on ice, adding binding protein to the tubes last, (note, this allows a fair competition for binding sites between the labelled and unlabelled cyclic AMP). The tubes were vortexed and incubated at 4°C for 2-3 hours. After this time, 250µl charcoal solution was added, and tubes were
immediately vortexed and spun in a microfuge at 12,000 g for 5 minutes. (Note, if
the charcoal solution is not spun out promptly, cyclic nucleotides will be stripped
from the binding protein). An aliquot of 300 µl was removed from the resulting
supernatant and added to 4 ml Ecoscint, and counted using a curve fitting program.

2.1.8.6. Calculation of cyclic AMP levels.

The results are expressed as an average of the triplicates assayed, and
expressed as pmols cyclic AMP produced/10^6 cells +/-SEM

2.1.9. Protein assays.

2.1.9.1. Bradford method.

This method of protein determination is based on the Bradford method
(Bradford, 1976). Briefly, a standard curve was constructed using 0-10 µg BSA as
the protein standard, carrying out the standards in duplicate. These concentrations
of protein were dissolved in distilled water (800 µl). Bio-Rad reagent (200 µl) was
added and the tubes were vortexed and the absorbance was read against a blank
cuvette containing no protein, at an absorbance of 595 nm. Protein concentrations
of the samples were determined in a similar way, diluting 10 µl of the sample into
800 µl distilled water, adding Bio-Rad reagent and reading the absorbance in the
spectrophotometer as before. The unknown protein samples were also assayed in
duplicate. Protein concentrations were determined by plotting the standard curve (x
axis = protein concentration (mg/ml), y axis = A595), gaining the equation of the
line, and using the absorbance readings gained as ‘y’, calculating ‘x’ i.e. 10 x
protein concentration (fig. 2.6).

2.1.9.2. A280 protein determination.

A quicker determination of protein concentration when measuring the
concentration of antibodies can be achieved by taking the absorbance of a solution
at 280nm, in a quartz cuvette, against the appropriate blank. An \( A_{280} \) of 1.0 is equivalent to 1.4mg/ml.

2.1.10. Proliferation assay.

In order to determine the effect of certain stimuli on the proliferation of the cells, \[^{3}H\]-thymidine incorporation as a measure of DNA synthesis were carried out. The principle of this assay is that after incubating the cells with the stimuli for a period of time, 0.5μCi \[^{3}H\]-thymidine is added to the cells. This becomes incorporated into the DNA of cells whilst they are dividing, and the amount of incorporation can then be measured. The level of DNA synthesis observed is a direct indicator of the proliferative response of these cells.

2.1.10.1. Preparation of reagents.

The cells required the following media:

- 500ml RPMI-1640 media
- 50ml foetal bovine serum (heat inactivated)
- 5ml penicillin / streptomycin solution
- 5ml glutamine solution
- 5ml pyruvate solution
- 5ml non-essential amino acid solution
- 0.5ml \( \beta \)-mercaptoethanol

\[^{3}H\]-thymidine: 100μl labelled thymidine was diluted in 2ml RPMI-1640 media on the day of use. 0.5μCi (10μl) of \[^{3}H\]-thymidine was used/well.

2.1.10.2. Assay procedure.

The cells were prepared as described previously (see section 2.1.1) with 2 x \( 10^5 \) cells being used/well (200μl). The cell number was determined before the cells were centrifuged for 5 minutes at \( 270g_{av} \), and resuspended in the proliferation
assay buffer (see section 2.1.10.1). The cells were aliquoted into flat-bottomed 96 well plates with desired effectors under sterile conditions, and incubated for 48 hours under a humidified atmosphere of 95% air and 5% CO₂, at 37°C. The conditions were carried out in triplicate. After the required time, 0.5 μCi [³H]-thymidine was added to each well, and the plates were incubated for a further 4 hours, then the cells was harvested.

2.1.10.3. Harvesting of cells.

The cells were harvested using a Skatron cell harvester. The filter paper was pre-wet with distilled water, before the cells were aspirated on to the paper, causing the cells to lyse, and the DNA to stick to the paper. Once the cells in the plate were harvested, the filter paper was dried in the oven, and the individual discs were dissolved in 2ml Ecoscint and counted in a liquid scintillation counter for 3 minutes per tube. The results were calculated as the average cpm +/- SEM.


All statistical analysis was carried out by the Students t test, unless otherwise stated.
2.2. Materials.

2.2.1. Chemicals and reagents.

BDH.,
Poole, U.K.

β-Mercaptoethanol

Coomassie Blue (Brilliant Blue R250)

DMSO

Triethanolamine

Universal indicator

Bio-Rad Laboratories Ltd.,
Hertfordshire, U.K.

Bradford reagent

TEMED

Boeringer (U.K.), Ltd.,
Lewes, U.K.

Dithiothreitol

Calbiochem,

Chelerythrine chloride

H89 dichloride

Tyrophostin 1

Tyrophostin 25

Fisons,
Loughborough, U.K.

Ammonium sulphate

Glycerol

Gibco. BRL.,
Paisley, U.K.

Protein molecular weight markers

Hepes

Peptide Research Foundation, Leupeptin
distributed by Scientific
Associates Marketing,
London, U.K.
Pfizer Central Research, Sandbach, Kent, U.K.

Pharmingen, Cambridge Bioscience, Cambridge, UK.

Packard, Groningen, N.L.

Rhone Merieux, France.

Schering Aktiengesellschaft, Rolipram
Postfach 650311, D-1000 Berlin 65, Germany.

Sigma Chemical Co., Poole, UK.

AE Intervening chemical compounds:
- Actinomycin
- Antipain
- Aprotinin
- Benzamidine hydrochloride
- Biotinylated anti-CD3 antibody
- BSA
- Calmodulin (bovine brain)
- Charcoal (Norit A)

CIostimide

Anti-TCR mAb, H57-597
FITC-labelled anti-CD4 antibody
PE-labelled anti-CD8α antibody

Ecoscint, Scintillation fluid

Sagatal
Cyclic AMP (disodium salt)
Cyclic GMP
Cycloheximide
Digitonin
Dowex 1X8-400 (chloride form, 200-400 mesh)
Ficoll-paque
Genistein
IBMX
Ionomycin
Pepstatin A
Percoll
Phytohaemagglutinin
PMA/TPA
PMSF
Protein A-agarose beads
Quantum Red Streptavidin
Snake venom (*Hannah ophiophagus*)
Trypan Blue (0.4%)

Sterling Winthrop,
Milrinone
Guildford,
Surrey, U.K.

All other chemicals were of AR grade and obtained from BDH (Poole, U.K.) and Fisons (Loughborough, U.K.)

2.2.2. Equipment.

Medicell Int. Ltd.,
Dialysis tubing
London, U.K.
Nunc, Cryotubes
InterMed 260ml tissue culture flasks
         96 well plates

Pharmacia Fine Chemicals, FPLC
Sweden

Sigma Chemical Co., 50 mesh steel screens
Poole, UK.

Whatman Ltd., Whatman 3MM Paper
Maidstone, U.K. Whatman 0.2μM nitrocellulose paper

2.2.3. Radiochemicals.

Amersham, [8-3H] - Adenosine-3',5'- cyclic monophosphate
Bucks, UK. [5',8-3H] - Adenosine-3',5'- cyclic monophosphate
[6',3H]- Thymidine

2.2.4. Animals.

Male 4 week and 12 week old Balb/c mice were used as the source of
primary thymocytes and lymphocytes from Biochemistry Department, University of
Glasgow and Immunology Department, University of Strathclyde, Glasgow.

2.2.5. Cell lines.

The Jurkat T cell line was obtained from European Collection of Cell
Cultures (ECACC), Centre for Applied Microbiology and Research, Salisbury,
UK. The monoclonal antibody cell line (145-2C11) was brought to the Department
of Biochemistry and Molecular Biology by Dr. M.M. Harnett, from the National
Institute of Medical Research, Mill Hill, London, U.K. The T3.2 thymoma cell
line was a very kind gift from Dr. R. Zamoyska, National Institute of Medical Research, Mill Hill, London, U.K. All materials used for cell culture were purchased from Gibco, BRL, Paisley, Scotland, unless otherwise stated.

2.2.6. Miscellaneous.

Tonsils were obtained from the Royal Hospital for Sick Children, Glasgow. They were stored at 4°C after the operations, and the lymphocytes were prepared as soon as possible. Sheep red blood cells and donkey horse radish peroxidase-labelled anti-rabbit antibody were obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland. Bovine adrenal glands were obtained from Duke St. abattoir, Glasgow, immediately after slaughter, transported at 4°C and processed without delay.
Table 2.1. FPLC programme format.

This table represents the parameters that were set to obtain the NaCl gradient used for the separation of phosphodiesterase activity from murine thymocytes.

Conc\%B represents the percentage of buffer B (see section 2.1.6.1).

ml/min represents the speed at which the column is run.

cm/ml represents the chart speed.

Port set 6.1 and 6.0 are the instructions for turning the fraction collector on and off respectively.
<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>Conc^n % B</td>
<td>0.0</td>
</tr>
<tr>
<td>0.0</td>
<td>ml/min</td>
<td>1.0</td>
</tr>
<tr>
<td>0.0</td>
<td>cm/ml</td>
<td>0.5</td>
</tr>
<tr>
<td>0.0</td>
<td>Port set</td>
<td>6.1</td>
</tr>
<tr>
<td>20.0</td>
<td>Conc^n % B</td>
<td>20.0</td>
</tr>
<tr>
<td>60.0</td>
<td>Conc^n % B</td>
<td>40.0</td>
</tr>
<tr>
<td>120.0</td>
<td>Conc^n % B</td>
<td>100</td>
</tr>
<tr>
<td>125.0</td>
<td>Conc^n % B</td>
<td>100</td>
</tr>
<tr>
<td>125.0</td>
<td>Port set</td>
<td>6.0</td>
</tr>
</tbody>
</table>
Figure 2.1. **Confirmation of the purity of the monoclonal antibody, 145-2C11.**

The antibody was purified as described previously in section 2.1.3.2 and the purity of the antibody was checked using SDS-PAGE gel chromatography as described in section 2.1.3.3.1. The protein samples which were run on the gel were prepared in the presence and absence of β-mercaptoethanol (ie. in reducing and non-reducing conditions, respectively). Purified BSA and γ-globulin run alongside the antibody sample (2C11-145) as indicated.
Figure 2.2. Effect of digitonin on rat kidney homogenate PDE activity

Kidney cells were lysed, by homogenisation, in an isotonic solution at 4°C. The resulting homogenate was assayed in the presence or absence of digitonin (0.5 or 5μg/ml as indicated). PDE assays were performed in the presence of 1μM cyclic AMP as substrate, as described in section 2.1.7. The data presented are from single experiments representative of three separate experiments. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein).
Figure 2.3. The principle of the cyclic AMP PDE assay.
Cyclic AMP

\[ \text{cyclic AMP phosphodiesterase} \]

5'AMP

\[ \text{snake venom} \]

Adenosine
Figure 2.4. Dose response of thymocyte PDE activity to DMSO.

PDE assays were performed in the presence of 1μM cyclic AMP as substrate, as described in section 2.1.7. The dose dependent action on cyclic AMP hydrolysis by DMSO is shown. The data presented are from single experiments representative of three separate experiments. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.)
Figure 2.5. Standard curve for a cyclic AMP binding assay.

The samples for the standard curve were prepared as described in section 2.1.8.5. Cyclic AMP concentrations of 0.0625-16 pmols were assayed as indicated. The assays were carried out in duplicate. This is a representative experiment which has been carried out at least six times.
Figure 2.6. Standard curve for a Bradford protein assay.

The samples for the standard curve were prepared as described in section 2.1.9.1. BSA concentrations of 0-10µg were used as indicated, and the absorbance was read at 595nm. The tube without protein added was used to zero the spectrophotometer. The assays were carried out in duplicate. This is a representative experiment which has been carried out at least fifteen times.
CHAPTER 3.

CHARACTERISATION OF CYCLIC AMP PDE ACTIVITIES IN T LYMPHOCYTES.
3.1. Introduction.

Cyclic nucleotide phosphodiesterases regulate various physiological responses in many different cells and tissues. These include, for example, platelet aggregation (Hidaka et al., 1979; Simpson et al., 1988), vascular relaxation (Kauffman et al., 1987; Tanaka et al., 1988), cardiac muscle contraction (Farah et al., 1984; Weishaar et al., 1987b) and inflammation (Plaut et al., 1980; Torphy et al., 1992). The differential distribution of cyclic AMP PDE isoforms in specific cells (see section 1.5.7), as well as their regulation of physiological responses, have made members of this enzyme family attractive targets for the development of selective inhibitors as potential therapeutic agents (Nicholson et al., 1991; Reeves and England, 1990; Weishaar et al., 1985).

With the detection of PDE activities within cells, came the discovery of a group of compounds, methylxanthines, which inhibited this enzyme activity (Butcher and Sutherland, 1962). This group includes theophylline, papaverine and IBMX. The structure of IBMX is outlined in figure 3.1. Theophylline has been used as a bronchodilator for many years in the treatment of asthma (Weiss et al., 1974) and has also been shown to exert a positive inotropic response in the heart (Korth, 1978). However, it is a weak inhibitor of cyclic AMP hydrolysis, reducing PDE activity in pulmonary tissue by only 6% when administered at therapeutic concentrations (Polson et al., 1978). This lack of sensitivity means that a higher concentration of inhibitor must be used in order to get a beneficial effect. However, at higher levels these PDE inhibitors are limited by their other pharmacological activities, including adenosine receptor antagonism (Fredholm, 1980), altering intracellular calcium mobilisation (Kolbeck et al., 1979) and the ability to inhibit the guanine nucleotide binding protein, G; (Parsons et al., 1988).

From the IC$_{50}$ values summarised in table 3.1, it is possible to see that IBMX has inhibitory effects on all the types of cyclic AMP PDEs, hence the attribution as a non-selective PDE inhibitor.
Selective PDE inhibitors exhibit at least a twenty fold specificity as inhibitors of their target isozyme family, and they lack many side effects produced by non-selective inhibition (Alousi et al., 1978). Confusion has arisen over initial reports of selective inhibition, because of investigators studying samples which were contaminated with other PDE activities (see section 1.5.7). However these problems have largely been overcome now, with improved purification techniques and more sensitive detection methods.

Inhibitors can be generated for the PDE1 Ca\(^{2+}\)/CaM-stimulated PDE by taking advantage of three specific facets of this enzyme; (i) the ability to block Ca\(^{2+}\)/CaM interaction; (ii) the ability to inhibit CaM stimulation by blocking CaM binding to the PDE; (iii) and the ability to inhibit the PDE activity directly. In biochemical assays, the most common way to inhibit PDE1 activity is use Ca\(^{2+}\) chelators, thus interfering with the binding of Ca\(^{2+}\) to CaM. The addition of EGTA to cell extracts stops the stimulation of PDE activity by Ca\(^{2+}\)/CaM. Also possible is the use of CaM antagonists, such as trifluoperazine and mastporan (Malencik and Anderson, 1983; Weiss et al., 1974). However these two methods have the drawback of being non-selective with respect to other CaM-dependent enzymes, such as myosin light chain kinase and CaM-dependent protein kinases within the cell, and therefore have shortcomings in the therapeutic arena and for in vivo studies (Kanamori et al., 1981). More selective inhibitors have been developed however, by altering the structure of the non-selective PDE inhibitor IBMX. This has produced the inhibitor 8-methoxymethyl-IBMX, that is 30-50 times more selective for CaM-dependent PDE activities compared with other PDE isoforms (Lorenz and Wells, 1983). Another inhibitor described is vinpocetine (Hagiwata et al., 1984) which has an IC\(_{50}\) of 21\(\mu\)M for PDE1, whilst the IC\(_{50}\) of this inhibitor for other PDEs is in the millimolar range.

Cyclic GMP-stimulated, PDE2 has been shown to be inhibited by a number of compounds known to act non-selectively on various PDE species, including IBMX, papaverine and dipyridamole (Moit et al., 1985) as well as unsaturated fatty
acids, such as palmitic acid and arachidonic acid (Pyne et al., 1986). Until recently, it was found that most selective inhibitors of the catalytic site of this PDE also bound with high affinity to the allosteric site of this class of enzyme, leading to activation at low concentrations (Erneux et al., 1981; Yamamoto et al., 1983b). It has been reported, however, that the erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA; previously known as MEP-1) is an inhibitor of PDE2 activity (Podzuweit et al., 1992). This compound is a well established inhibitor of adenosine deaminase (Bessodes et al., 1982; Cristalli et al., 1988). However, an investigation carried out on frog heart PDE2 suggests an IC$_{50}$ of approximately ~4μM EHNA which is considerably lower than the concentrations required for EHNA to inhibit adenosine deaminase in cardiac tissues (Mery et al., 1995).

EHNA has been shown to be selective for PDE2 activities present in human and porcine cardiac tissue with IC$_{50}$ values of 0.8 or 2μM respectively, as opposed to >100μM for other soluble PDE isoforms (see table 3.1) (Podzuweit et al., 1995).

PDE3, cyclic GMP-inhibited PDE inhibitors are the largest group of selective PDE inhibitors. Amrinone was the first cardiotonic and antithrombotic drug which was found to have positive inotropic effects (Alousi et al., 1979; Silver, 1989) (fig. 3.2). This group now consists of a number of drugs such as milrinone, cilostimide and siguazodan. When administered, these drugs have the ability to increase the force of contraction of the heart, presumably by elevating [Ca$^{2+}$], secondary to an increase in the phosphorylation status of a cardiac calcium channel (Olson et al., 1987). These effects appear to be mediated through the inhibition of membrane-bound, PDE3, cyclic GMP inhibited PDE and the subsequent increases in cyclic AMP levels (Ahn et al., 1986; Weishaar et al., 1985). PDE3 inhibitors have also been shown to be effective as smooth muscle relaxants and platelet aggregation inhibitors.

Although there are tissue-dependent differences in the sensitivity of PDE3 inhibitors, it is clear that this group of inhibitors is selective for cyclic GMP-inhibited PDEs above other PDEs (Hoey and Houslay, 1990; Torphy and
The IC$_{50}$ values for milrinone, cilostimide and amrinone are summarised in table 3.1. It is possible to see that they are all selective for PDE3 activities, however their potencies of inhibition differ (cilostimide > milrinone > amrinone).

The evidence for a functional role of PDE4 isoforms in mammalian CNS initially came from the development of selective inhibitors of this class of PDEs. Rolipram is a neurotropic drug that can induce a profile of behavioural modifications in animals (Watchel, 1983b) such as hypothermia, hypoactivity and head twitches. It and other similar drugs, such as its structural analogue Ro20-1724 (fig. 3.3), have activities involved with regulation of central nervous system functions (Horowshi and Stastre, 1985; Watchel, 1983a). As well as the established anti-depressant properties of rolipram (Watchel, 1983b), this inhibitor also been shown to exert a relaxant effect on bovine trachea (Hall and Hill, 1992) and anti-inflammatory actions (Chan and Hanifin, 1993; Torphy et al., 1992).

These drugs have been shown to act as specific cyclic AMP PDE inhibitors in mammalian tissues (Davis and Davidson, 1984; Fredholm et al., 1979; Nemoz et al., 1985; Schwabe et al., 1976). More recently with the cloning of mammalian homologues of the Drosophila dnc locus, it has been established that the PDE4 isoforms are selectively inhibited (Henkel-Tigges and Davis, 1989; Swinnen et al., 1989b; Swinnen et al., 1991b) by rolipram and Ro20-1724. All mammalian PDE4 isoforms tested exhibit inhibition by rolipram but interestingly, the Drosophila dnc PDE is not inhibited by rolipram (Henkel-Tigges and Davis, 1989). This may indicate that drug sensitivity has been established by sequence changes of the vertebrate progenitor PDE4 gene (Chen et al., 1986; Davis et al., 1989) after the separation of vertebrates from invertebrates. When contrasting the sensitivity of rolipram between PDE4 and other PDEs, the sensitivity of this inhibitor for PDE4 activities is striking. IC$_{50}$ values for rolipram and its structural analogue Ro20-1724 are summarised in table 3.1. It is seen from these values, that rolipram is a more potent PDE4 inhibitor than Ro20-1724.
3.1. Aims and Objectives.

The purpose of this study was to identify the cyclic AMP PDE activities expressed in T lymphocytes and to detect possible differences in cyclic AMP catabolism during T cell maturation. The main part of my research was carried out using murine thymocytes isolated from four week old Balb/c mice. Comparisons were made between these thymocytes and mature murine lymphocytes, a murine thymoma cell line (CD4+/CD8+), mature human T lymphocytes derived from tonsillar tissue and a human leukemic cell line, Jurkat.

PDE activities are known to be modulated by effectors such as cyclic GMP and Ca²⁺/CaM (see section 1.5.7). These agents, as well as the isoform selective PDE inhibitors rolipram, milrinone and EHNA were used to identify the PDE activities present in T lymphocytes. Confirmation of the specificity of these effectors was carried out by assessing the PDE activities upon separation of cell extracts using an ion-exchange chromatography system.
3.2. Results.

3.2.1. Cyclic AMP PDE activities in murine thymocytes.

The availability of selective inhibitors and activators of PDE activity (Houslay and Kilgour, 1990; Manganiello et al., 1990a; Nicholson et al., 1991; Reeves and England, 1990) and the separation of PDE activities by FPLC using ion exchange chromatography (Mono-Q) provided a means of determining the relative activities of PDE isoform groups in a murine thymocyte extract.

3.2.1.1. Dose response analysis of thymocyte extracts.

The cells were prepared from four week old Balb/c mice, lysed and assayed for cyclic AMP PDE activity as described in section 2.1.1.1. The extracts were assayed in the presence of highly selective PDE inhibitors in order to ascertain the proportion of PDE activity that existed in unstimulated thymocytes. As discussed previously (see section 3.1 and table 3.1), rolipram is a highly selective inhibitor for PDE4 enzymes with IC$_{50}$ values observed in the 0.1μM-1μM cyclic AMP range (Bolger, 1994; Henkel-Tigges and Davis, 1989; Lobban et al., 1994; McLaughlin et al., 1993; Nicholson et al., 1991; Sullivan et al., 1994). In contrast, other PDE forms are either insensitive to rolipram, or show IC$_{50}$ values at least 50-100 fold higher ((Nicholson et al., 1991) and table 3.1). Thus the inhibition of total PDE activity caused by 10μM rolipram can be taken to represent the magnitude of PDE4 activity, as it can be expected to result in maximal inhibition of this isoform group, with no effect on the other isoform groups present. Indeed, when rolipram inhibition was analysed in thymocyte extracts, there was a dose-dependent decrease in PDE activity (fig. 3.4a), with an IC$_{50}$ of 65 +/- 9nM (n=3; errors are SEM). At 10μM rolipram, the reduction of PDE activity in the extract was 75 +/- 6% (n=3; errors are SEM). A reduction in PDE activity was also apparent when rolipram was added to thymocyte extracts together with 10μM cyclic GMP. The amount of activity inhibited by rolipram was consistent with the proportion of PDE4 present in the sample (fig. 3.4b), as determined from fig. 3.4a.
In the presence of low cyclic GMP concentrations, there was a profound increase in the hydrolysis of cyclic AMP, which occurred in a dose-dependent fashion (fig. 3.5), with an EC\textsubscript{50} of 1.3 +/- 0.3\textmu M cyclic GMP (n=4; errors are SEM). However, at cyclic GMP concentrations above 10\textmu M, an inhibition of PDE activity can be expected to occur as a result of competitive inhibition of the conversion of cyclic AMP to 5'-AMP (Pyne \textit{et al.}, 1986), and as result of cyclic GMP competing with cyclic AMP for the active site (Hidaka and Asano, 1976).

This cyclic GMP-stimulated PDE activity was inhibited by EHNA in a dose-dependent manner (fig. 3.6) (Podzuweit \textit{et al.}, 1992). This yielded an IC\textsubscript{50} of 3.5 +/- 0.6\textmu M (n=3; errors are SEM).

Upon addition of cyclic GMP, one would expect to inhibit PDE3, cyclic GMP-inhibited PDE activity (Manganiello \textit{et al.}, 1990a; Pyne \textit{et al.}, 1987a; Pyne \textit{et al.}, 1987b), leading to a reduction in cyclic AMP hydrolysis. However with the use of the selective PDE3 inhibitor milrinone (Nicholson \textit{et al.}, 1991; Pyne \textit{et al.}, 1987a; Torphy \textit{et al.}, 1992) no inhibitory effect on cyclic AMP hydrolysis (< 5%) was noted at low milrinone concentrations, suggesting this activity was absent from murine thymocytes (fig. 3.7). However at higher concentrations (> 10\textmu M) it appeared to inhibit PDE activity, suggesting that the inhibitor is becoming non-selective.

PDE1, Ca\textsuperscript{2+}/CaM-stimulated PDE activity was assessed by determining the ability of Ca\textsuperscript{2+}/CaM (50\textmu M/20ng/ml) to activate cyclic AMP hydrolysis. This was done in the presence or absence of the calcium chelator EGTA (1mM) in the buffer, to prevent Ca\textsuperscript{2+} arising from cell extracts eliciting full activation of any PDE1 activity present, in the absence of exogenous Ca\textsuperscript{2+}. However no stimulation of PDE activity was observed under any conditions (table 3.2) indicating the absence of this isoform.

The non-selective PDE inhibitor IBMX is considered to inhibit all PDE forms, with IC\textsubscript{50} values in the range of 1-30\textmu M (Beavo, 1990; Hoey and Houslay, 1990; Torphy and Cieslinski, 1990), except for cyclic AMP specific type-VII
isoform (Lavan et al., 1989; Michaeli et al., 1993). In murine thymocytes in the presence of 100μM IBMX, the PDE activity was totally abrogated in the absence (IC\textsubscript{50} of 4.6 +/- 0.8μM; errors are SEM; n=3) (fig. 3.8a.) or presence (IC\textsubscript{50} is 10 +/- 2μM; errors are SEM; n=3)(fig.3.8b.) of stimulating concentrations of cyclic GMP. This indicates that PDE7 is not present in these cells.

The basal PDE activities in murine thymocytes were the same in buoyant density centrifugation-separated ‘in vivo-activated’ and ‘resting’ populations of cells (table 3.3). The activities in activated and resting, and thus in ‘mixed’ population were found to be similar in the case of each isoform. The PDE activities are given in the presence (table 3.3b.) and absence (table 3.3a.) of cyclic GMP.

In order to ascertain whether there was any PDE activity associated with the membrane fraction of murine thymocytes, further extraction of the pellet present after the cell lysis was carried out as outlined in section 2.1.5.1. No further PDE activity was found to be present in murine thymocytes membranes.

3.2.1.2. Separation of thymocyte PDE activities by FPLC.

The presence of PDE2 and PDE4 activities in murine thymocytes was supported by studies carried out using ion exchange chromatography to resolve thymocyte PDEs, as described in section 2.1.6.3. These studies identified two peaks of cyclic AMP PDE activity (fig. 3.9a). The activity of the first peak (Peak ‘1’) was stimulated in the presence of 10μM cyclic GMP (310 +/- 34% stimulation; n=3; errors are SEM) and was therefore identified as PDE2. The second peak of activity (peak ‘2’) was unaffected by cyclic GMP (95 +/- 8%; n=3; errors are SEM) or Ca\textsuperscript{2+}/CaM (96 +/- 14%; n=3; errors are SEM) (fig. 3.9a and fig. 3.9b) and was susceptible to inhibition by rolipram (92 +/- 2% inhibition; n=3; errors are SEM) (fig. 3.10). Upon closer analysis of the PDE profile obtained by this separation, studying fractions 31-80, there was no evidence for a peak of cyclic AMP PDE activity which could be inhibited by either cyclic GMP (fig. 3.9a and fig. 3.11a) or milrinone (fig. 3.11b) (95 +/- 8%; n=3; errors are SEM). Similarly, both peaks of
cyclic AMP PDE activity were completely (>95%) inhibited by 1mM IBMX, indicating the absence of a PDE7 activity (fig. 3.12).

3.2.1.3. Dose response curves of isolated PDE activities.

Dose response curves were carried out on pooled fractions of each peak of PDE activity, in order to demonstrate the specificity of the selective PDE inhibitors used in this study. Cyclic GMP-stimulated PDE activity was inhibited in a dose dependent manner by EHNA only in the presence of cyclic GMP (74-80%; range; n=3) compared with 11-17% (range; n=3) in the absence of cyclic GMP (fig. 3.13). No inhibition of PDE activity was observed upon addition of milrinone up to 50μM (fig. 3.14a). There was no inhibition by rolipram at lower concentrations, although appeared to cause a slight inhibition (15%) of PDE activity at 50μM, indicating that this inhibitor may be non-selective at this high concentration (fig. 3.11b).

In the same way as described for peak '1', peak '2' fractions were pooled and dose response analysis of PDE inhibitors was carried out. PDE activity of peak '2' was shown to be selectively inhibited by rolipram (fig. 3.15), causing a 95% inhibition of activity at 10μM with an IC<sub>50</sub> of 50 +/- 1 nM (error is SEM; n=3). However, PDE activity in these fractions was unaffected by milrinone (fig. 3.16a), EHNA (fig. 3.16b).

3.2.2. Cyclic AMP PDE activities in lymphocytes from 4 week old mice.

Lymphocytes were isolated from spleens of four week old Balb/c mice and separated into resting and in vivo activated cell populations, or T lymphocytes were purified by B cell depletion, as described in section 2.1.1. On studying the activities of cyclic AMP PDEs in these cells, it was noted that activated lymphocyte populations had a higher basal cyclic AMP PDE activity (assayed in the presence of 1μM cyclic AMP) than the resting population (table 3.4). This may suggest a
difference in the regulation of cyclic AMP degradation in activated cells, which may be of importance to the proliferation status of the cell (Epstein and Hachisu, 1984; Rochette-Egly and Kempf, 1981; Smith et al., 1971). However, upon studying a B cell depleted 'mixed' T lymphocyte population, it was interesting to note that the PDE activity was not elevated. This indicated that the elevation of cyclic AMP PDE activity in in vivo-activated lymphocytes may be due to the presence of in vivo-activated B lymphocytes.

Upon addition of a stimulatory concentration of cyclic GMP (10μM) there was a profound increase in cyclic AMP PDE activity which was similar to that observed in thymocytes. However it was noted that the activation of PDE activity by cyclic GMP was at least 6 fold in lymphocytes, compared with about 3.5 fold in thymocytes (table 3.3). As outlined in table 3.4, no PDE1 activity was found to be present on addition of Ca²⁺/CaM. This was similar to that seen in thymocytes (table 3.2 and fig. 3.12).

3.2.3. Cyclic AMP PDE activities in lymphoid cells from 12 week old mice.

Thymocytes and lymphocytes were isolated and PDE activities were determined in the presence and absence of cyclic GMP. Basal cyclic AMP PDE activity in murine thymocytes was found to be lower than that of lymphocytes (table 3.5). However, the specific activities of in vivo-activated and resting populations of thymocytes isolated from immunologically immature and mature mice did not differ (table 3.3 and table 3.5). However, in vivo-activated lymphocytes isolated from 12 week old mice did not have an elevated basal cyclic AMP PDE activity (table 3.5), which was noted in the in vivo-activated population of lymphocytes isolated from immature mice (table 3.4).

Upon addition of cyclic GMP to cell extracts from mature mice, there was a pronounced stimulation of PDE activity in all of these cell populations (approx. 10 fold). This was of interest, as it indicates that the capacity of cyclic GMP to
stimulate cyclic AMP hydrolysis is greater in lymphoid cells isolated from mature mice than in immature mice. This increase in cyclic AMP PDE sensitivity to cyclic GMP is particularly pronounced in thymocytes. PDE1 activity could not be detected in thymocytes isolated from twelve week old mice.

3.2.4. Cyclic AMP PDE activities in T3.2 thymoma cell line.

The T3.2 cell line was studied, as it was derived from a murine thymoma which undergoes apoptosis following ligation of its TCR. This cell line allowed comparisons to be made between primary thymocytes and proliferating cell lines. Also this cell line represented a homogeneous population of cells as it was derived from a defined population of thymocytes (expressing both CD4 and CD8 co-receptors). Table 3.6a and table 3.6b outlines the cyclic AMP hydrolysing activities which were found to be present with the use of selective PDE isoform inhibitors. Of particular interest in these cells was the lack of stimulation of total PDE activity in the presence of cyclic GMP (table 3.6a.). Indeed, addition of cyclic GMP lead to a reduction in PDE activity by about 20%. This does not necessarily indicate the absence of the cyclic GMP stimulated, PDE2 activity, but it does indicate that any cyclic GMP-stimulated activity that may be present was dominated by the presence of a PDE3, cyclic GMP-inhibited PDE activity.

The use of selective inhibitors revealed the presence of PDE2, PDE3 and PDE4 activities (table 3.6b.). The predominant activity present was the rolipram-sensitive PDE4 activity. This constituted over 50% of the activity in the absence in cyclic GMP. However, in direct contrast to primary thymocytes, the percentage of PDE4 activity increased on addition of cyclic GMP. The presence of a PDE3 isoform was surprising, when one considers that this activity appears to be absent from primary thymocytes (fig. 3.11 and fig.3.14a). However, this may suggest that PDE2 and PDE3 activities are developmentally regulated within the thymus.
3.2.5. Cyclic AMP PDE activities in human T lymphocytes.

Upon analysis of the Jurkat leukemic T cell line, it was revealed that these cells possessed a similar cyclic AMP PDE profile to the T3.2 thymoma cell line. Jurkat cells appeared to express PDE1, PDE2, PDE3 and PDE4 activities (table 3.6a. and table 3.6b.). In a similar manner to thymoma cells, following addition of cyclic GMP the total PDE activity was reduced by about 30%. The predominant activity in the Jurkat cell line was found to be a cyclic GMP-inhibited PDE activity. Rolipram inhibition of the Jurkat cell extract was small (approx. 6%) indicating little or no PDE4 activity in unstimulated cells. IBMX (100µM) inhibited about 80% of the total PDE activity, implying that the IBMX-insensitive PDE7 activity may be present. However, due to the competitive nature of IBMX as an inhibitor, the presence of PDE7 activity cannot be confirmed without further analysis. However, this isoform has been reported to be expressed in proliferating T cell lines (Bloom, 1994; Ichimura and Kase, 1993). A Ca²⁺/CaM-stimulated PDE activity was found to be present in the Jurkat cell line when PDE activity was assayed in the presence of stimulatory concentrations of Ca²⁺/CaM (50µM/20ng/ml) elevating PDE activity 1.37 fold (Table 3.6a).

Additional studies in human tonsillar T cells were carried out to establish the effect of cyclic GMP on PDE activities in human cells. In a similar fashion to Jurkat cells, the addition of cyclic GMP caused a reduction in PDE activity of approximately 35% (Table 3.7). This was apparent in resting and in vivo-activated T cells. The absence of stimulated activity upon addition of cyclic GMP does not necessarily indicate the absence of a PDE2 isozyme, but it does strongly suggest the presence of a PDE3. This may indicate a species specific difference between murine and human primary cells. Similarities were apparent between human tonsillar T lymphocytes and the human T cell line, Jurkat (table 3.6 and table 3.7).
3.3. Discussion

It is clear from these studies that the major enzymes which provide cyclic AMP PDE activity in murine thymocytes are a rolipram-sensitive, PDE4 activity and an EHNA-inhibited, cyclic GMP-stimulated, PDE2 activity. The magnitude of the cyclic GMP-stimulated, PDE2 activity increased the cyclic AMP hydrolysis capabilities of the cell, by approximately 3.5 fold, in the presence of stimulatory cyclic GMP concentrations. Thus PDE2 forms the major portion of PDE activity within thymocytes. This stimulation of PDE activity by cyclic GMP was also evident in murine splenic lymphocytes. However the stimulation of PDE activity was nearer 8 fold in these cells, indicating an enormous potential for cyclic GMP to regulate the levels of cyclic AMP within murine lymphoid tissues. The expression of PDE2 activity in lymphocytes may confer maturity on these murine cells. A cyclic GMP-stimulated PDE activity has previously been identified in rat thymic lymphocytes (Franks and MacManus, 1971; Valette et al., 1990). A comparison of the PDE activities present in the cell types studies here is summarised in table 3.8.

These studies revealed that in vivo-activated murine lymphocytes have an elevated basal PDE activity in comparison to resting populations of cells. In the past, studies carried out in leukemic cells have established that these proliferating cells have elevated PDE activities (Epstein et al., 1980; Epstein et al., 1977; Hait and Weiss, 1979; Hait and Weiss, 1976). One possible reason for the proliferation of these cells may be a lesion in cyclic AMP metabolism. Sustained increases in cyclic AMP levels within lymphocytes have been shown to be inhibitory to the induction of proliferation at a number of stages in T cell proliferation (see section 1.6.1). Therefore it may be that upon receiving a proliferative signal, lymphocytes elevate their PDE activities, and once in the proliferative state, the regulation of PDE activities may be uncoupled, thus maintaining a lower level of cyclic AMP.

In murine thymocytes, Ca2+/CaM-stimulated, PDE1 activity, cyclic GMP-inhibited, PDE3 activity and IBMX-insensitive, PDE7 activity did not provide any significant basal activity. This was surprising, as both rat thymocytes and primary
human lymphocytes have been shown to express a PDE3 activity, implying species-specific differences in the distribution of PDE activities in lymphoid tissue (Franks and MacManus, 1971; Marcoz et al., 1993; Meskini et al., 1992; Robicsek et al., 1991; Valette et al., 1990) (table 3.8). Moreover, studies carried out in the leukemic T cell line Jurkat, identified a cyclic GMP-inhibited PDE3 activity, which constituted the majority of basal PDE activity in these cells. This PDE3 activity also appears to be present in human primary T cells isolated from tonsils. Jurkat cells were also shown to contain a cyclic GMP-stimulated PDE2 activity and a rolipram-sensitive PDE4 activity. Therefore, it appears that cyclic GMP plays an opposing role in the regulation of cyclic AMP hydrolysis in lymphoid cells from the two species. This may lead to very divergent biological responses as a consequence of cyclic GMP elevations.

The study of murine T3.2 thymoma cells was therefore interesting, as it was found that upon addition of cyclic GMP there was a decrease in PDE activity, similar to that seen in human T cell models studied. This suggested the presence of PDE3 activity in T3.2 cells. This cell line is a thymoma derived from the CD4+/CD8+ stage of thymocyte development. It is possible that alterations in PDE isoform expression reflect changes due to cell transformation or differences in thymocyte maturation state. Indeed, it has been shown that PDE expression changes upon alteration of the proliferation status of lymphoid cells (Takemoto et al., 1979; Takemoto et al., 1978). However, the situation presented here indicates that PDE3 activity is gained not lost (Takemoto et al., 1979), upon proliferation. However, the resulting aberrant regulation of cyclic AMP may be partially responsible for the uncontrolled proliferation of the cells.

Consistent with our finding that Ca²⁺/CaM-stimulated, PDE1 activity was absent in murine thymocytes and lymphocytes, and human tonsillar T cells, this activity has also been reported to be absent in rat splenic lymphocytes (Hait and Weiss, 1977), rat thymocytes (Valette et al., 1990), human and bovine peripheral blood lymphocytes (Hurwitz et al., 1990; Thompson et al., 1976) and human
monocytes (Thompson et al., 1980). Intriguingly, however, PDE1 activity has been shown to be induced by the mitogenic lectin, PHA after 14 hours of stimulation of these peripheral blood lymphocytes (Hurwitz et al., 1990). Hurwitz et al. showed that PDE1 activity remained elevated for at least 100 hours following stimulation. Rapid induction of PDE1 has also been reported in Chinese hamster ovary (CHO) cells in response to the PKC activator, PMA (Spence et al., 1995). In the light of this, it is interesting to note that PDE1 activity was found to be present in the proliferating T cell line Jurkat and has been found to be present in a B-lymphoblastoid cell line derived from a patient with acute lymphocytic leukaemia (Epstein et al., 1987). However the inability to detect PDE1 activity in primary lymphoid cells might be due either to the loss of Ca^{2+}/CaM-dependent activity through proteolysis or to the binding of endogenous CaM by the PDE, thus preventing detection of a stimulation of PDE activity upon addition of exogenous Ca^{2+}/CaM (Epstein et al., 1980). Indeed, steps were taken to ensure that proteolysis was limited by using a 'protease inhibitor cocktail' upon lysing the cells (see section 2.1.5.1). Experiments were also carried out in the presence of EGTA, a Ca^{2+} chelator, to remove endogenous calcium, ensuring that any Ca^{2+}/CaM stimulated activity could be activated with the addition of exogenous Ca^{2+}/CaM. However, no stimulation of PDE was detected in the presence of EGTA, suggesting that PDE1 activity was absent from these cells.

Cyclic GMP increases cyclic AMP PDE activity in a dose-dependent manner in murine thymocytes extracts, indicative of a cyclic GMP-stimulated activity. The pattern of cyclic AMP hydrolysis in the presence of increasing concentrations of cyclic GMP was similar to that seen in calf liver (Wada et al., 1987) and rat liver (Pyne et al., 1986). Cyclic GMP-stimulated PDE activity has also been observed in rat thymocytes. However, this activity was only observed at higher concentrations of cyclic AMP (10μM), with a maximal stimulation of PDE activity seen at approximately 10μM cyclic GMP (Franks and MacManus, 1971). At 1μM cyclic AMP, rat thymocyte PDE activity was inhibited by increasing
amounts of cyclic GMP due to the presence of a PDE3 activity. This implies that
murine thymocytes lend themselves to the study of cyclic GMP-stimulated PDE
activity, as they do not have the 'interference' of a PDE3 activity complicating the
interpretation of the results. Inhibition of cyclic AMP hydrolysis at higher levels of
cyclic GMP (20μM) was probably due to cyclic GMP competing with cyclic AMP
at the catalytic site (Wada et al., 1987), but may also be due to competitive
inhibition of the conversion of cyclic AMP to 5'-AMP (Pyne et al., 1986).

EHNA inhibited PDE activity in thymocyte extracts and the cyclic GMP-
stimulated PDE activity fractions in a dose-dependent manner, in the presence and
absence of cyclic GMP. The inhibition was however greater in the presence of
cyclic GMP. This suggested that at maximally effective concentrations of EHNA
the stimulation of PDE activity induced by cyclic GMP could be entirely ablated.
Thus indicating that EHNA may serve to prevent the stimulatory action of cyclic
GMP, perhaps by competing with cyclic GMP for binding of the allosteric site.
This is consistent with our observations that EHNA produced a small inhibition of
PDE2 activity in the presence of cyclic AMP alone. At concentrations up to 50μM,
rolipram did not inhibit the PDE2 fraction of activity, indicating that at 10μM
rolipram the only PDE activity being inhibited is the PDE4 portion of activity.
Milrinone did not inhibit PDE2 activity. Milrinone and EHNA did not effect the
cyclic AMP PDE activity in the PDE4 peak of activity in murine thymocytes,
indicating that the PDE inhibitors were selective.

Rolipram elicited a dose-dependent inhibition on cyclic AMP hydrolysis in
murine thymocytes indicating the presence of a rolipram-sensitive PDE activity in
these cells. The IC50 of this enzyme is comparable with PDE4 activities from other
sources (Shakur et al., 1993; Sullivan et al., 1994). The particular isoform of
PDE4 present in thymocytes is currently unknown. However, mRNA of PDE4B
has been isolated in a human lymphocytic B cell line (Oberholter et al., 1993). This
transcript was absent from unstimulated Jurkat cells, where only PDE4A mRNA
was identified when tested for the four isoforms (Engels et al., 1994). This may
illustrate another example of lineage specific PDE expression in lymphoid cells (see above and (Ichimura and Kase, 1993)).

Upon addition of IBMX to Jurkat cell extracts, about 20% of the total PDE activity remained. This observation, coupled with previous reports (Bloom, 1994; Ichimura and Kase, 1993) indicated the presence of the IBMX- and rolipram-insensitive PDE7 activity in Jurkat cells. Due to the competitive nature of this inhibitor, one would not expect to observe a complete inhibition of PDE activity, so whilst PDE7 activity may be present in Jurkat cells, it does not constitute a fifth of the PDE activity in these cells. Interestingly, a rolipram-insensitive PDE activity has also been reported in other human T cell lines. However it was found to be absent from human B cell lines and in human promyelocytic cell line HL-60 (Ichimura and Kase, 1993). This finding contrasted with murine thymocytes where IBMX fully inhibited cyclic AMP PDE activity. This indicates a lineage-specific difference in the expression of cyclic AMP PDE activities.

The findings presented here identify the predominance of PDE4 and PDE2 isoforms in murine thymocytes and lymphocytes. This may suggest the existence of a molecular ‘switch’, which is responsive to intracellular cyclic GMP levels and could be expected to have a profound effect on the control of cyclic AMP metabolism within these cells. At concentrations of cyclic GMP which are too low to stimulate PDE2 activity, the predominant PDE species involved in the hydrolysis of cyclic AMP in thymocytes/lymphocytes would be the PDE4 activity. However, under conditions where cyclic GMP levels become elevated, such as through the stimulation of soluble guanylyl cyclase via NO (Moncada et al., 1991), then a dramatic increase in PDE2 activity may become apparent. This may be relevant to the control of apoptosis within thymocytes, and is discussed further in section 4.3.4.

These studies indicate that cyclic AMP PDE activities in primary murine lymphocytes and thymocytes appear to be highly sensitised to changes in intracellular cyclic GMP levels. These studies also highlight the differences that
exist in different populations of T cells with respect to cyclic AMP hydrolysis and may have a direct effect on the role which cyclic GMP and calcium has in the regulation of cyclic AMP hydrolysis.
Table 3.1. Summary of IC_{50} values of cyclic AMP PDE inhibitors for cyclic AMP PDE activities.

This table summarises information about cyclic AMP PDE inhibitors, concerning their selectivity for particular isoforms of activity. The values given represent IC_{50} values (μM) (the concentration at which 50% inhibition of PDE activity has ensued). The values have been gathered from different tissue and species sources. The references cited are as follows:

a Hoey and Houslay, 1990, b Weishaar et al., 1986,
ço Harrison et al., 1986, d Kithas et al., 1988,
z Valette et al., 1990, f Mery et al., 1995,
v Lavan et al., 1989, h Hidaka and Endo, 1984,
^ Yamamoto et al., 1983b, i Weishaar et al., 1987,
xy Manganiello et al., 1992, l Schwabe et al., 1976,
m Torphy and Cieslinski, 1990, 2 Henkel-Tigges and Davis, 1989,
ô Wilson et al., 1994a, p Shakur et al., 1993,
vl Livi et al., 1990, r Michaeli et al., 1993,
vz Spence et al., 1995, t Mery et al., 1995,
u Podzuweit et al., 1995, v Pyne et al., 1986,
w Decourcelles et al., 1992, x Conti et al., 1995a,
ye McLoughlin et al., 1993, z Bolger et al., 1993.

NI=not inhibited;
ND=not determined.
<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>PDE1 IC₅₀ range (nM)</th>
<th>PDE2 IC₅₀ range (nM)</th>
<th>PDE3 IC₅₀ range (nM)</th>
<th>PDE4 IC₅₀ range (nM)</th>
<th>PDE5 IC₅₀ range (nM)</th>
<th>PDE7 IC₅₀ range (nM)</th>
<th>Competitive w</th>
<th>Competitive w</th>
<th>Competitive w</th>
<th>Competitive w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amrinone</td>
<td>&gt;950 µM</td>
<td>&gt;400 µM</td>
<td>&gt;150 µM</td>
<td>&gt;150 µM</td>
<td>&gt;150 µM</td>
<td>&gt;150 µM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Milrinone</td>
<td>&gt;&gt;30 µM</td>
<td>&gt;150 µM</td>
<td>&gt;150 µM</td>
<td>&gt;150 µM</td>
<td>&gt;150 µM</td>
<td>&gt;150 µM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chlormethiazide</td>
<td>22 µM</td>
<td>15 µM</td>
<td>NI µm</td>
<td>NI µm</td>
<td>NI µm</td>
<td>NI µm</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rolipram</td>
<td>&gt;1000 µM</td>
<td>&gt;1000 µM</td>
<td>&gt;500 µM</td>
<td>&gt;300 µM</td>
<td>&gt;300 µM</td>
<td>&gt;300 µM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ro20-1724</td>
<td>200 µM</td>
<td>200 µM</td>
<td>200 µM</td>
<td>200 µM</td>
<td>200 µM</td>
<td>200 µM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>EHNA</td>
<td>0.8 - 5 µM</td>
<td>0.8 - 5 µM</td>
<td>0.8 - 5 µM</td>
<td>0.8 - 5 µM</td>
<td>0.8 - 5 µM</td>
<td>0.8 - 5 µM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IBAK</td>
<td>2.5 - 25 mg/ml</td>
<td>2.5 - 25 mg/ml</td>
<td>2.5 - 25 mg/ml</td>
<td>2.5 - 25 mg/ml</td>
<td>2.5 - 25 mg/ml</td>
<td>2.5 - 25 mg/ml</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Table 3.2. Assessment of PDE1 activity in thymocytes.

PDE assays were performed in the presence of 1μM cyclic AMP as substrate, as described in ‘Materials and Methods’. Stimulatory concentrations of Ca^{2+}/CaM was added to the thymocyte extract, and fold stimulation of PDE activity was assessed. This was carried out in the presence and absence of EGTA (1mM). The data presented are from single experiments representative of three separate experiments. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
<table>
<thead>
<tr>
<th>Murine Thymocytes</th>
<th>Specific activity with 1μM cAMP (Tris/Mg(^{2+}) Buffer)</th>
<th>Specific activity with 1μM cAMP (Tris/Mg(^{2+})/EGTA Buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.1 +/- 0.4</td>
<td>19.1 +/- 0.5</td>
</tr>
<tr>
<td>Ca(^{2+})/CaM added</td>
<td>12.1 +/- 0.4</td>
<td>15.1 +/- 0.3</td>
</tr>
<tr>
<td>PDE1</td>
<td>Not stimulated</td>
<td>Not stimulated</td>
</tr>
</tbody>
</table>
Table 3.3. Basal PDE activities of thymocytes isolated from 4 week old Balb/c mice.

The cells were prepared, and the PDE assays were carried out as described in 'Materials and Methods'. PDE assays were performed with 1μM cyclic AMP as substrate. Table A shows the PDE activities in the absence of stimulatory concentrations (10μM) of cyclic GMP. PDE1 activity was expressed as the fold stimulation on addition of Ca^{2+}/CaM to thymocyte extract. PDE2 activity was assessed as a percentage of inhibited PDE activity on addition of 10μM EHNA. PDE3 activity was assessed as the percentage inhibition of PDE activity on addition of 5μM milrinone. PDE4 activity was assessed as a percentage inhibition of PDE activity on addition of 10μM rolipram. PDE7 activity was expressed as the percentage activity remaining after the addition of 100μM IBMX.

In the presence of 10μM cyclic GMP, table B shows the fold stimulation of PDE activity upon addition of cyclic GMP. The selective inhibitors were used in the same way as described above to calculate the relative PDE activities in murine thymocytes. The data is the average of at least four separate experiments. Assays were carried out in triplicate (errors are SEM). Specific activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein. Percentages are calculated as the average of percentage inhibition/stimulation from each separate experiment. ND = not determined.
<table>
<thead>
<tr>
<th></th>
<th>Specific activity with 1μM cAMP</th>
<th>PDE1</th>
<th>PDE2 %age total activity</th>
<th>PDE3 %age total activity</th>
<th>PDE4 %age total activity</th>
<th>PDE7 %age total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting cells</td>
<td>18 +/- 3</td>
<td>Not stimulated</td>
<td>17 +/- 2</td>
<td>7 +/- 2</td>
<td>75 +/- 5</td>
<td>7 +/- 1</td>
</tr>
<tr>
<td>Activated cells</td>
<td>18 +/- 2</td>
<td>Not stimulated</td>
<td>16 +/- 1</td>
<td>7 +/- 2</td>
<td>63 +/- 4</td>
<td>4 +/- 1</td>
</tr>
<tr>
<td>Mixed cells</td>
<td>19 +/- 1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>74 +/- 2</td>
<td>ND</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Specific activity with 1μM cAMP</th>
<th>PDE1</th>
<th>PDE2 %age total activity</th>
<th>PDE3 %age total activity</th>
<th>PDE4 %age total activity</th>
<th>PDE7 %age total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting cells</td>
<td>60 +/- 6</td>
<td>3.3</td>
<td>63 +/- 5</td>
<td>4 +/- 2</td>
<td>22 +/- 2</td>
<td>0.1</td>
</tr>
<tr>
<td>Activated cells</td>
<td>67 +/- 5</td>
<td>3.7</td>
<td>56 +/- 6</td>
<td>2.5 +/- 0.5</td>
<td>22 +/- 3</td>
<td>6 +/- 2</td>
</tr>
<tr>
<td>Mixed cells</td>
<td>77 +/- 3</td>
<td>3.8</td>
<td>58 +/- 7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Table 3.4. Basal PDE activities of lymphocytes and T cells isolated from 4 week old Balb/c mice.

The cells were prepared, and the PDE assays were carried out as described in 'Materials and Methods'. PDE assays were performed with 1μM cyclic AMP as substrate. Specific activities were assessed in the presence or absence of stimulatory concentrations (10μM) of cyclic GMP. The fold stimulation of PDE activity upon addition of cyclic GMP or Ca²⁺/CaM was assessed. The data is the average of at least three separate experiments. Assays were carried out in triplicate (errors are SEM). Specific activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein. Percentages are calculated as the average of percentage stimulation from each separate experiment.
<table>
<thead>
<tr>
<th>Lymphocytes from 4 week old mice</th>
<th>Specific activity with 1μM cyclic AMP (cpm/mg protein)</th>
<th>Fold stimulation by cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting lymphocytes</td>
<td>21 ± 2</td>
<td>Not stimulated</td>
</tr>
<tr>
<td>Activated lymphocytes</td>
<td>632 ± 33</td>
<td>6</td>
</tr>
<tr>
<td>Mixed T lymphocytes</td>
<td>232 ± 21</td>
<td>11</td>
</tr>
</tbody>
</table>

FPDE1

<table>
<thead>
<tr>
<th>Specific activity with 1μM cyclic AMP + 1μM cyclic GMP (cpm/mg protein)</th>
<th>Fold stimulation by cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.18 ± 32</td>
<td>8</td>
</tr>
</tbody>
</table>

cpm/mg protein: counts per minute per milligram of protein.

Fold stimulation: the ratio of the specific activity with cGMP to the specific activity without cGMP.
Table 3.5. Basal PDE activities of thymocytes and lymphocytes isolated from 12 week old Balb/c mice.

The cells were prepared, and the PDE assays were carried out as described in 'Materials and Methods'. PDE assays were performed with 1μM cyclic AMP as substrate. Specific activities were assessed in the presence or absence of stimulatory concentrations (10μM) of cyclic GMP. The fold stimulation of PDE activity upon addition of cyclic GMP or Ca^{2+}/CaM was assessed. The data is the average of at least three separate experiments. Assays were carried out in triplicate (errors are SEM). Specific activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein. Percentages are calculated as the average of percentage stimulation from each separate experiment.
<table>
<thead>
<tr>
<th>Cells from 12 week old mice</th>
<th>Specific activity with 1μM cyclic AMP</th>
<th>Fold stimulation with cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting thymocytes</td>
<td>12 ± 2</td>
<td>8</td>
</tr>
<tr>
<td>Activated thymocytes</td>
<td>11 ± 2</td>
<td>11</td>
</tr>
<tr>
<td>Resting lymphocytes</td>
<td>28 ± 4</td>
<td>360 ± 22</td>
</tr>
<tr>
<td>Activated lymphocytes</td>
<td>24 ± 3</td>
<td>290 ± 27</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specific activity with 1μM cyclic AMP + 10μM cyclic GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 ± 1</td>
</tr>
</tbody>
</table>
Table 3.6. Basal PDE activities of Jurkat T cell lymphoma and T3.2 thymoma cell lines.

The cell lines were maintained and the PDE assays were carried out as described in 'Materials and Methods'. PDE assays were performed with 1μM cyclic AMP as substrate. In Table A, specific activities were assessed in the presence or absence 10μM cyclic GMP. The percentage inhibition of PDE activity upon addition of cyclic GMP was assessed. PDE1 activity was expressed as the fold stimulation on addition of Ca^2+/CaM to thymocyte extract. In Table B, PDE2 was expressed as the difference between the activity 'inhibited' by cyclic GMP and the activity inhibited by 5μM milrinone. PDE3 activity was assessed as the activity which was inhibited by 5μM milrinone. PDE4 activity was assessed as the activity inhibited on addition of 10μM rolipram. PDE7 activity was the remaining after the addition of 100μM IBMX. The data is the average of four separate experiments. Assays were carried out in triplicate (errors are SEM). Specific activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein). Percentages are calculated as the average of percentage inhibition from each separate experiment.

ND= not determined.
### Table A

<table>
<thead>
<tr>
<th></th>
<th>Specific activity with 1μM cyclic AMP</th>
<th>Specific activity with 1μM cyclic AMP + 10μM cyclic GMP</th>
<th>%age inhibition with cGMP</th>
<th>PDE1 Fold stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat T cell</td>
<td>8 +/- 1</td>
<td>6 +/- 1</td>
<td>33 +/- 3</td>
<td>1.37</td>
</tr>
<tr>
<td>lymphoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3.2 thymoma cell</td>
<td>14 +/- 1</td>
<td>12 +/- 2</td>
<td>21 +/- 4</td>
<td>ND</td>
</tr>
<tr>
<td>line</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table B

<table>
<thead>
<tr>
<th></th>
<th>PDE2 Specific activity</th>
<th>PDE3 Specific activity</th>
<th>PDE4 Specific activity</th>
<th>PDE7 Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat T cell</td>
<td>2 +/- 0.4</td>
<td>5 +/- 1</td>
<td>0.5 +/- 0.2</td>
<td>1.8 +/- 0.2</td>
</tr>
<tr>
<td>lymphoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3.2 thymoma cell</td>
<td>5 +/- 0.5</td>
<td>4 +/- 1</td>
<td>8 +/- 1</td>
<td>ND</td>
</tr>
<tr>
<td>line</td>
<td></td>
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</tbody>
</table>
Table 3.7. Basal PDE activities of human tonsillar T lymphocytes.

The cells were isolated and the PDE assays were carried out as described in ‘Materials and Methods’. PDE assays were performed with 1μM cyclic AMP as substrate. Specific activities were assessed in the presence or absence of 10μM cyclic GMP. The percentage inhibition of PDE activity upon addition of cyclic GMP was assessed. PDE1 activity was expressed as the fold stimulation on addition of Ca^2+/CaM to the T lymphocyte extract. The data is the average of three separate experiments. Assays were carried out in triplicate (errors are SEM). Specific activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein. Percentages are calculated as the average of percentage inhibition from each separate experiment.
<table>
<thead>
<tr>
<th>Human tonsillar T lymphocytes</th>
<th>Specific activity with 1μM cyclic AMP</th>
<th>Specific activity with 1μM cyclic AMP plus cyclic GMP</th>
<th>%age inhibition with cGMP</th>
<th>PDE1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting T cells</td>
<td>9 +/- 2</td>
<td>7 +/- 1</td>
<td>35 +/- 10</td>
<td>Not stimulated</td>
</tr>
<tr>
<td>Activated T cells</td>
<td>10 +/- 2</td>
<td>5 +/- 1</td>
<td>40 +/- 2</td>
<td>Not stimulated</td>
</tr>
</tbody>
</table>
Table 3.8. Summary of cyclic AMP PDE isoforms present in the lymphoid cells studied.
<table>
<thead>
<tr>
<th>Summary</th>
<th>PDE1</th>
<th>PDE2</th>
<th>PDE3</th>
<th>PDE4</th>
<th>PDE7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine thymocytes</td>
<td>Abst</td>
<td>Prst</td>
<td>Abst</td>
<td>Abst</td>
<td>Abst</td>
</tr>
<tr>
<td>Murine lymphocytes</td>
<td>Abst</td>
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<td>ND</td>
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<tr>
<td>T3.2 thymoma cell line</td>
<td>Prst</td>
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<td>Present</td>
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<td>Jurkat leukemic cell line</td>
<td>Present</td>
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<td>ND</td>
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<tr>
<td>Human tonsillar T cells</td>
<td>Absen</td>
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Figure 3.1. Structure of cyclic AMP and non-selective PDE inhibitor, IBMX.
cyclic AMP

IBMX
Figure 3.2. Structure of PDE3 selective inhibitors.

Shown are the structures of amrinone [5-amino-(3',4'-bipyridin)-6(1H0-one], milrinone [1,6-Dihydro-2-methyl-6-oxo-(3,4'-bipyridine)-5-carbonitrile] and cilostamide [4,5-dihydro-6 [4-(1H-imadazol-1-yl)phenyl]-5-methyl-3(2H)-pyrazone].
Amrinone

Milrinone

Cilostamide
Figure 3.3. Structure of PDE4 selective inhibitors.

Shown are the structure of rolipram [4-(3-(cyclopentoxyl)-4-methoxyphenyl)-2-pyrrolidone] and Ro20-1724 [4-(Butoxy-4-methoxybenzyl)-2-imidazolidinone].
Figure 3.4. Identification of PDE4 activity in thymocytes: Inhibition by rolipram.

PDE assays were performed in the presence of 1μM cyclic AMP as substrate, as described in ‘Materials and Methods’. The dose dependent inhibition of cyclic AMP hydrolysis by rolipram is shown; a) in the absence of 10μM cyclic GMP and b) in the presence of 10μM cyclic GMP. The data presented are from single experiments representative of three separate experiments. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.)
Figure 3.5. Identification of PDE2 activity in thymocytes: Activation by low concentrations of cyclic GMP.

PDE assays were performed in the presence of 1μM cyclic AMP as substrate, as described in 'Materials and Methods'. The dose dependent action of cyclic GMP is shown. The data presented are from single experiments representative of three separate experiments. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.)
Figure 3.6. Identification of PDE2 activity in thymocytes: Inhibition by EHNA.

PDE assays were performed in the presence of 1μM cyclic AMP as substrate, as described in ‘Materials and Methods’. The dose dependent inhibition of PDE activity by EHNA is shown for assays done in the presence of stimulatory concentrations (10μM) of cyclic GMP. The data presented are from single experiments representative of three separate experiments. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.)
Figure 3.7. Dose response of thymocyte PDE activity by milrinone.

PDE assays were performed in the presence of 1μM cyclic AMP as substrate, as described in 'Materials and Methods'. The dose-dependent action of milrinone on cyclic AMP hydrolysis is shown, a) in the absence of 10μM cyclic GMP and b) in the presence of 10μM cyclic GMP. The data presented are from single experiments representative of three separate experiments. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 3.8. Dose-dependent inhibition of thymocyte PDE activity by IBMX.

PDE assays were performed in the presence of 1µM cyclic AMP as substrate, as described in ‘Materials and Methods’. The dose dependent inhibition of cyclic AMP hydrolysis by IBMX is shown, a) in the absence of 10µM cyclic GMP and b) in the presence of 10µM cyclic GMP. The data presented are from single experiments representative of three separate experiments. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.)
Figure 3.9. FPLC analysis of a soluble thymocyte extract using ion exchange chromatography (MonoQ).

This was carried out as described in 'Materials and Methods'. 1ml fractions were collected over a 120ml gradient, alternate fractions were assayed. PDE activity was assessed using 1μM cyclic AMP as substrate; a) in the absence (circles) and presence (diamonds) of stimulatory concentrations of cyclic GMP and b) in the absence (circles) and presence (triangles) of Ca^{2+}/CaM (50μM/20ng/ml). Peak 1 activity is stimulated in the presence of stimulating concentrations (10μM) of cyclic GMP. Peak 2 is activated in the presence or absence of cyclic GMP or Ca^{2+}/CaM. PDE activity is expressed as cpm. The data presented are from single experiments representative of results obtained three times.
Figure 3.10. FPLC analysis of a soluble thymocyte extract using ion exchange chromatography (MonoQ): The assessment of PDE4 or PDE7 activity.

This was carried out as described in 'Materials and Methods'. 1ml fractions were collected over a 120ml gradient, alternate fractions were assayed. PDE activity was assessed using 1μM cyclic AMP as substrate in the absence (circles) of presence (squares) of inhibitory concentrations of PDE4 selective inhibitor rolipram. PDE activity is expressed as cpm. The data presented are from single experiments representative of results obtained three times.
Figure 3.11. FPLC analysis of a soluble thymocyte extract using ion exchange chromatography (MonoQ): The assessment of PDE3 activity.

This was carried out as described in ‘Materials and Methods’. 1ml fractions were collected over a 120ml gradient, and fractions 31-80 were assayed. PDE activity was assessed using 1µM cyclic AMP as substrate a) in the absence (circles) and presence (diamonds) of stimulatory concentrations of cyclic GMP. Peak 1 activity is stimulated in the presence of stimulating concentrations (10µM) of cyclic GMP. Peak 2 is activated in the presence or absence of cyclic GMP. b) PDE activity was assessed in the absence (circles) or presence (triangles) of 5µM milrinone, the PDE3 selective inhibitor. PDE activity is expressed as cpm. The data presented are from single experiments representative of results obtained three times.
Figure 3.12. FPLC analysis of a soluble thymocyte extract using ion exchange chromatography (MonoQ): The assessment of PDE7 activity

This was carried out as described in ‘Materials and Methods’. 1ml fractions were collected over a 120ml gradient, and fractions 31-80 were assayed. PDE activity was assessed using 1μM cyclic AMP as substrate in the absence (circles) of presence (crosses) of inhibitory concentrations of non-selective inhibitor IBMX. PDE activity is expressed as cpm. The data presented are from single experiments representative of results obtained three times.
Figure 3.13. Dose-dependent inhibition of peak 1 PDE activity with EHNA.

Samples eluted in fractions 55-60 were pooled. PDE assays were performed in the presence of 1μM cyclic AMP as substrate, as described in 'Materials and Methods'. The dose dependent inhibition of PDE activity by EHNA is shown for assays carried out a) in the presence and b) the absence of stimulatory concentrations of cyclic GMP. The data presented are from single experiments representative of three separate experiments. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as percentage of control i.e. in the absence of cyclic GMP (100%).
Figure 3.14. Dose-dependent action of milrinone or rolipram on peak 1 PDE activity.

Samples eluted in fractions 55-60 were pooled. PDE assays were performed in the presence of 1 μM cyclic AMP as substrate, as described in 'Materials and Methods'. The dose dependent action of a) milrinone on PDE activity in peak 1 and of b) rolipram on PDE activity in peak 1 was assessed. The data presented are from single experiments representative of three separate experiments. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as percentage of control ie. without inhibitors (100%).
Figure 3.15. Dose-dependent inhibition of rolipram on peak 2 PDE activity.

Samples eluted in fractions 71-73 were pooled. PDE assays were performed in the presence of 1μM cyclic AMP as substrate, as described in 'Materials and Methods'. The dose dependent inhibition of PDE activity by rolipram on peak 2 is shown. The data presented are from single experiments representative of three separate experiments. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as percentage of the control ie. without rolipram (100%).
Figure 3.16. Dose-dependent action of milrinone or EHNA on peak 2 PDE activity.

Samples eluted in fractions 71-73 were pooled. PDE assays were performed in the presence of 1μM cyclic AMP as substrate, as described in ‘Materials and Methods’. The dose dependent action of a) milrinone on PDE activity in peak 2 and of b) EHNA on PDE activity in peak 2 was assessed. The data presented are from single experiments representative of three separate experiments. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as percentage of control ie. without inhibitors (100%).
CHAPTER 4.

REGULATION OF CYCLIC AMP PDEs FOLLOWING LIGATION OF THE TCR/CD3 COMPLEX.
4.1. Introduction.

It is well established that following ligation of the TCR/CD3 complex, the activation of PTKs such as p95Tcr, p56lck and p70 zap results in the rapid tyrosine phosphorylation, recruitment and activation of key signal transducers such as the inositol phospholipid-specific PLCγ-1 (June et al., 1990a; Secrist et al., 1991; Weiss and Litman, 1994). Hydrolysis of PIP2 generates the second messengers, IP3 and DAG leading to a release of calcium from intracellular stores and the activation of PKC. Other signalling events, downstream of PTK activation include the recruitment of the PI-3-K (Rudd et al., 1994), p21ras (Downward et al., 1992) and MAPK (Nel et al., 1991; Siegel et al., 1990) signalling cascades (see section 1.2.4).

As mentioned previously (see section 1.4), a central problem yet to be resolved in T cell signalling is how the TCR transduces differential signals in a maturation state-dependent manner (Hueber et al., 1994). Proliferation and differentiation into effector cells occurs upon ligation of the TCR in mature cells. However, within the thymus, TCR-derived signals in developing thymocytes can result in proliferation (selection) or programmed cell death (apoptosis) depending on the antigen specificity of the TCR (Hueber et al., 1994; Kaye and Ellenberger, 1992). The molecular mechanisms underlying these biological responses are, as yet, poorly understood. However, it has been proposed that the TCR may be partially uncoupled from PTK activation, tyrosine phosphorylation of PLCγ-1 and calcium mobilisation in immature thymocytes upon ligation (Finkel et al., 1991; Sancho et al., 1992). This ultimately leads to immunological unresponsiveness due to a lack of IL-2 receptor expression, IL-2 secretion and proliferation (Finkel et al., 1991; Ramarli et al., 1987). In addition, elevation of cyclic AMP levels has been implicated in both the differentiation of early thymocytes (Bach et al., 1975; Scheid et al., 1975) and the stimulation of thymic apoptosis (Kaye and Ellenberger, 1992; McConkey et al., 1990a). It is possible, therefore, that cyclic AMP may play a pivotal role in thymocyte maturation and/or cell death. As such, the regulation of
cyclic AMP hydrolysis by cyclic AMP PDEs may provide a route for achieving such a process. There is very little evidence concerning the early changes in cyclic AMP PDE activity occurring upon ligation of the TCR/CD3 complex. However, there is some evidence which suggests that these enzymes may play a central role in regulating cyclic AMP levels during lymphocyte activation and cell cycle progression (Epstein et al., 1980; Hurwitz et al., 1990; Marcoz et al., 1993; Meskini et al., 1992). There is a growing interest in the role that specific PDE isoforms may play in the maturation, proliferation and cell death of lymphocytes.

Anti-CD3 monoclonal antibodies and mitogenic lectins have been shown to be extremely useful in deciphering signalling pathways which lead to T cell proliferation. In vivo, T lymphocytes are activated by antigen presented in the context of processed peptide in self MHC molecules on the surface of antigen presenting cells. Such an interaction involves the recognition of the antigen by a TCR/CD3 complex. In vitro, T cells can be activated not only by antigen on appropriate antigen-presenting cells, but also by ligands that interact with the TCR/CD3 complex. These interactions can be provided by monoclonal antibodies that are raised against specific epitopes on TCR/CD3 complex or by mitogenic lectins.

Mitogens are a class of molecule which can activate T or B cells in a non-antigen-specific polyclonal manner. The majority of human and mouse T cells can be stimulated by phytohaemagglutinin (PHA), extracted from red kidney beans (Phaseolus vulgaris), and concanavalin A (Con A), extracted from castor beans. Since PHA was first discovered to induce blast transformation in normal leukocytes (Nowell, 1960), mitogenic stimulation has been studied extensively as a model for antigenic activation and growth control. It has been shown that lectins bind specific glycoproteins on the cell surface of the cells leading to polyclonal stimulation of lymphoid cells. Initial events in lectin-induced activation include; tyrosine phosphorylation (Hirata et al., 1984), calcium influx (Coffey et al., 1981; Parker et al., 1979) and inositol phosphate production (Taylor et al., 1984). Early studies
also indicated fluctuations in cyclic nucleotide levels (Hadden et al., 1972; Krishwaraj and Talwar, 1973; Wedner et al., 1975). Analysis of the mechanisms underlying PHA-stimulation of proliferation in both peripheral T cells and Jurkat cells has shown that two signals were required in order to obtain IL-2 production from these cells (Weiss et al., 1984b). The first involved ligation of the TCR/CD3 and the second was provided by accessory cells, CD28 ligation, or could be in the form of the phorbol ester, phorbol-12-myristate, 13-acetate (PMA) (Imboden et al., 1985; Sperling et al., 1993; Weiss et al., 1984b; Weiss et al., 1986b). Thus, Jurkat cells produce IL-2 upon addition of PMA in combination with the ligation of the TCR/CD3 complex. IL-2 production also occurred in the presence of PHA and PMA (Weiss et al., 1984b). In contrast, mutants of Jurkat cells that did not express the TCR/CD3 complex were unable to produce IL-2 in response to PHA and PMA (Weiss et al., 1984b; Weiss and Stobo, 1984). This indicates that the TCR/CD3 complex is necessary for T cell activation via PHA. Such a notion was confirmed when the TCR/CD3 complex was reconstituted back into the mutant Jurkat cell line, thus restoring its ability to respond to PHA and PMA. These data strongly support the contention that the mitogenic action of PHA requires its direct or indirect interaction with the T cell antigen receptor. Evidence now suggests that PHA binds with the αβTCR region of the receptor (Kanellopoulos et al., 1985). CD2 (sheep red blood cell receptor) has also been described to be a receptor for PHA (O'Flynn et al., 1985). This is of relevance to this study, as CD2 is expressed during the early stages of thymic development (Fox et al., 1985), present on 85-95% of all developing T cells (Lamier et al., 1986; Sewell et al., 1986) and has been suggested to play an essential role in the signalling pathways of TCR/CD3-immature thymocytes (Fox et al., 1985; O'Flynn et al., 1985).

Monoclonal antibodies raised against discrete epitopes of lymphocyte surface receptors have been shown to be useful tools that can be used to delineate signalling pathways during the activation of T cells (Oettgen and Terhorst, 1987;
Weiss and Winchurch, 1978). These have an advantage over mitogenic lectins, as they recognise a single cell surface receptor, as opposed to the lack of specific interaction seen with lectins. Antibodies directed against either the αβTCR heterodimer or CD3 complex cause a diverse range of responses. These include, increased tyrosine phosphorylation, increased PIP2 hydrolysis, PKC activation, the release of intracellular calcium release, calcium influx, a transient increase in cyclic AMP concentrations and IL-2 receptor induction and display. Studies by Ledbetter et al. (1986) demonstrated that the use of monoclonal antibodies raised against CD5 and CD28 can mimic the actions of accessory cells. This is because they induce IL-2 production and subsequent proliferation when used in concert with anti-CD3 antibodies. This suggests that the study of T cell signalling and activation with monoclonal antibodies may result in a response which can be compared to a physiological response.

4.1.1. Aims and objectives.

With the use of monoclonal antibodies (mAbs) directed against specific components of the TCR and the mitogenic lectin PHA, the regulation of cyclic AMP PDE activities were studied, focusing mainly on murine thymocytes. However, comparisons have been drawn between these cells and the proliferating Jurkat T cell line. Data are discussed in relation to cyclic AMP metabolism and the development of T cells.
4.2. Results.

4.2.1. Cyclic AMP PDE activities following stimulation of murine thymocytes with PHA.

Stimulation of murine thymocytes isolated from four week old Balb/c mice with mitogenic concentrations of PHA (50μg/ml) induced a rapid and dramatic decrease in PDE activity assayed at 1μM cyclic AMP (fig. 4.1a and 4.1b). A reduction of approximately 80% in total cellular cyclic AMP PDE activity was observed within 5 minutes of addition of PHA. Analysis of the rolipram inhibited component of this activity (fig. 4.1a and 4.1b) revealed that the decrease was due primarily to a reduction in the PDE4 activity. However, this decline in activity was only transient as, 7 minutes after PHA addition, PDE activity began to slowly rise. PDE activity had reached 60-70% of basal activity after 20 minutes of challenge with PHA.

Analysis of thymocyte PDE activity in the presence of stimulatory concentrations of cyclic GMP identified an immediate, but again transient, decrease in PDE activity (fig. 4.2). This transient decrease was also evident in analysis of PDE2 activity assessed as the cyclic GMP-stimulated component (fig. 4.2). Within 5 minutes of challenge with PHA, PDE2 activity fell to around 61% of its basal activity. However, unlike the PDE4 activity, the PDE2 component had regained its starting level after about 7 minutes. This level of activity was maintained for at least 20 minutes (fig. 4.2). Essentially similar results were obtained on analysis of activated (figs. 4.1a and 4.2a) and resting (figs. 4.1b and 4.2b) populations of cells, when thymocytes were stimulated with PHA.

PHA was shown therefore, to induce rapid inhibitory effects on both PDE2 and PDE4 activities. The PDE2 activity reduction was extremely transient on addition of PHA (fig. 4.2), whereas the reduction in PDE4 activity was slower to recover, not re-gaining basal levels until 20 minutes after PHA challenge (fig. 4.1). Over this 20 minute period of challenge with PHA, PDE1, PDE3 and PDE7 activities were not evident (table 4.1).
4.2.2. Cyclic AMP PDE activities following stimulation of murine thymocytes with anti-CD3 and anti-TCR antibodies.

Stimulation of thymocytes with anti-CD3 and anti-TCR mAbs appeared to cause a small transient decrease in PDE activity, assayed with 1μM cyclic AMP (figs. 4.3 and 4.5). This was rapidly followed by a substantial and sustained increase in PDE activity. Such changes could be attributed predominantly to the PDE4 activity because the activity was almost entirely abrogated (approx. 80%) by rolipram treatment. In striking contrast to the pattern of PDE responses observed with PHA, the initial small, transient inhibition of PDE activity caused by anti-CD3 and anti-TCR antibodies was followed by a profound increase in the PDE4 activity. This PDE activity was elevated 2-2.5 fold over the basal activity 20 minutes after challenge with anti-CD3 or anti-TCR mAbs (P<0.05; n=3) (Figs 4.3 and 4.5). Similar results were obtained using both soluble and crosslinked anti-CD3 antibodies (fig. 4.7). This implies that the differences observed between these responses and those seen with PHA cannot be attributed to differential levels of crosslinking of the TCR by these reagents.

In contrast to the response elicited by PHA, addition of anti-CD3 or anti-TCR mAbs had no significant effect on PDE2 activity. Whilst there were observable changes in the total cyclic AMP PDE activity in the presence of cyclic GMP (10μM), these effects could be accounted for by the increases in PDE4 activity (figs. 4.4 and 4.6). As found using PHA to stimulate thymocytes, no PDE1, PDE3 or PDE7 activities were identified following the ligation of the TCR/CD3 complex with anti-CD3 or anti-TCR antibodies (tables 4.2 and 4.3), respectively. Similar profiles of PDE activities were obtained with activated (figs. 4.3a, 4.4a, 4.5a and 4.6a) and resting cells (figs. 4.3b, 4.4b, 4.5b and 4.6b).

4.2.3. Cyclic AMP levels in PHA stimulated thymocytes.

In order to determine the effects of such PHA-mediated modulation of PDE activity on cyclic AMP homeostasis, cyclic AMP levels were measured in PHA-
stimulated thymocytes. A 2-fold elevation of cyclic AMP levels was obtained within 5 minutes of PHA treatment, which was consistent with the observed decrease in total cyclic AMP PDE activity (P<0.02; n=3) (fig. 4.8). In addition, the transient nature of this rise in cyclic AMP levels paralleled the kinetics of PDE4 modulation, reaching basal levels within 30 minutes of challenge with PHA. Assay of cyclic AMP levels in the presence of PDE inhibitors, such as IBMX and rolipram, caused an elevation in cyclic AMP above that seen in the absence these PDE inhibitors (table 4.4). This indicates that in addition to modulating PDE activities, PHA stimulated substantial adenylyl cyclase activity. The PDE3 selective inhibitor milrinone did not potentiate cyclic AMP levels in thymocytes, with no difference observed in cyclic AMP concentrations in the presence or absence of milrinone (table 4.4). Since only a small, transient increase in cyclic AMP was observed in the absence of PDE inhibitors, these results suggested that PHA modulated both cyclic AMP synthesis and degradation.

The use of the PDE4 selective inhibitor rolipram suggested that PDE4 activity was mainly responsible the regulation of cyclic AMP degradation (table 4.4). The finding that milrinone did not effect cyclic AMP levels within thymocytes supported the contention that PDE3 activities were not present in these cells. The potentiation of cyclic AMP levels, when assayed in the presence of rolipram or IBMX, indicated that adenylyl cyclase activity was increased upon challenge of thymocytes with PHA. Again, similar results were obtained with resting (fig. 4.8b and table 4.4) and activated populations of thymocytes (fig. 4.8a and table 4.4).

**4.2.4. Cyclic AMP levels in anti-CD3 antibody stimulated thymocytes.**

In contrast to the transient PHA-mediated increase in cyclic AMP levels, stimulation of thymocytes with anti-CD3 mAbs induced a slow, but sustained, rise in cyclic AMP levels following a lag of 3 minutes (fig. 4.9). This was despite (i) a rise in PDE4 activity and (ii) maintenance of PDE2 activity over the same period of
time. Such data suggest that ligation of the CD3 by anti-CD3 also induced a substantial activation of adenylyl cyclase activity in thymocytes. This was confirmed by the enhanced cyclic AMP signals observed in the presence of the PDE inhibitors IBMX and rolipram (table 4.5). However, once again milrinone did not potentiate any increase in cyclic AMP levels (table 4.5) above those seen in the presence of anti-CD3 mAb alone. Once again, similar results were obtained with activated (fig. 4.9a and table 4.5) and resting populations of thymocytes (fig. 4.9b and table 4.5).

4.2.5. Cyclic AMP PDE activities in Jurkat cells on stimulation with PHA.

Upon challenge of the human T cell line Jurkat with PHA, there was little change in cyclic AMP PDE activity throughout the 30 minute stimulation (fig. 4.10). However, addition of cyclic GMP (10μM) to PDE assays of the Jurkat cell extract (fig. 4.10a) identified a decrease in this form of PDE activity. This indicated the presence of cyclic GMP-inhibited PDE activity. A small decline in this cyclic GMP-inhibited proportion of PDE activity was observed when the cells were treated with PHA. Jurkat cell extracts treated with Ca\(^{2+}/CaM\) (50μM/20ng/ml) showed an increase in PDE activity (fig. 4.10b) indicating the presence of PDE1. This level of stimulation declined slightly when cells were challenged with PHA however it was not statistically significant, indicating that this ligand had little effect on PDE1 activity.

PDE2, PDE3, PDE4 and PDE7 activities all appeared to be present in the Jurkat cell extract (table 4.6) as indicated in section 3.2.5. Interestingly, an increase in rolipram sensitive, PDE4 activity was observed upon stimulation with PHA (~15% stimulation; \(P<0.02; n=4\)), and a decrease in cyclic GMP-inhibited PDE3 activity (~15% inhibition; \(P<0.05; n=4\)). Therefore, there appears to be differential regulation of specific PDE isoforms in the Jurkat cell line upon stimulation with PHA.
4.2.6. Proliferation studies on murine thymocytes and lymphocytes.

DNA synthesis experiments (thymidine incorporation) were carried out in order to ascertain the long-term effects of PHA and anti-CD3 stimulations on murine thymocytes. These studies were carried out with a view to investigating the effects of cyclic AMP level alterations on proliferation. However it was found that in vivo-activated and resting populations of thymocytes did not proliferate in response to PHA or anti-CD3 antibodies (table 4.7 and fig. 4.11) after stimulation for 48 hours. Instead, a low incorporation of $[^3]H$-thymidine was observed, suggesting that the cells were anergic i.e. non-responsive rather than dying. Although a dose-dependent increase in $[^3]H$-thymidine uptake were observed, it was in the range of hundreds of cpms, whereas one would have expected tens of thousands of cpms to constitute a proliferative response (Harding et al., 1992).

Research carried out in thymic cultures suggests that communication between stromal cells and maturing T cells is essential for the maintenance of both populations of cells (Ritter and Boyd, 1993). Indeed, thymocytes have been shown to proliferate in the presence of IL-1, but this is inhibited in purified thymocytes (Rock and Benacerraf, 1984) indicating the lack of proliferation seen in thymocytes is due to the lack of stromal cells.

Murine lymphocytes responded slightly better, increasing their $[^3]H$-thymidine uptake in response to PHA (table 4.7) and anti-CD3 antibodies (fig. 4.12). However there was not a substantial incorporation of $[^3]H$-thymidine, indicating the requirement of accessory cells.
4.3. Discussion.

In this study, the regulation of PDE activities within murine thymocytes has been investigated upon challenge of the cells with the mitogenic lectin PHA or anti-CD3/anti-TCR antibodies. It was shown that these two classes of TCR ligands appeared to regulate differentially the PDE2, PDE4 activities and cyclic AMP levels within these cells. The cyclic AMP PDE activities were also studied in Jurkat cells in response to PHA.

4.3.1. PDE activities upon ligation of the TCR/CD3 complex.

There were two key differences in the PDE activities stimulated in thymocytes following ligation of the TCR by anti-CD3/TCR mAbs or the mitogenic lectin PHA: (i) PHA mediated a rapid decrease in PDE4 activity followed by a slow recovery towards basal levels, whereas anti-CD3/TCR mAbs mediated an initial small and transient decrease in PDE4 activity followed by a slow, but sustained, increase in this activity, approximately 2 fold above basal. (ii) The kinetics of PDE2 modulation differed in PHA- and anti-CD3 stimulated thymocytes. PHA caused a dramatic, but transient loss in PDE2 activity. In contrast, there was no change in PDE2 activity upon ligation of TCR/CD3 complex. This might imply that signalling pathways emanating from the TCR/CD3 complex are not coupled to the regulation of PDE2 activity.

In contrast to murine thymocytes, the proliferating Jurkat T-cell line did not show altered total cyclic AMP PDE activity upon stimulation with PHA. However, upon analysis of specific PDE isoforms it was apparent that there was an increase in the cyclic AMP-specific PDE4 activity, together with a concomitant decrease in cyclic GMP-inhibited, PDE3 activity. This loss of cyclic GMP-inhibited PDE activity has been reported in peripheral blood lymphocytes within four hours of mitogenic stimulation (Takemoto et al., 1979). In Jurkat cells the stimulation of PDE4 activity and the loss of PDE3 activity occurred within 30 minutes. This may indicate some influence of the cell cycle-mediated regulation of PDE3 activity.
Engels et al. (1994) have identified PDE4A at the mRNA level in Jurkat cells. However, it has been shown here (see section 3.2.5) that little, if any PDE4 activity was observed in unstimulated Jurkat cells. It is therefore interesting to note an increase in this activity, as it suggests that this PDE activity is rapidly induced in response to PHA. Rapid induction of a Ca²⁺/CaM-stimulated, PDE1 activity has recently been shown in CHO cells in response to either challenge with the phorbol ester, PMA, or the selective overexpression of PKC isoforms (Spence et al., 1995). This PDE activity, which was found to be absent from untreated cells, could be detected within 25 minutes of their exposure to PMA. Thus it appears possible that rapid induction of PDE activities can occur in response to mitogenic agents.

Upon stimulation of murine thymocytes with anti-CD3/TCR antibodies, a PDE4 activity increases 2-2.5 fold within 20 minutes. An increase in PDE4 activity has been shown previously upon stimulation of rat thymocytes with Con A (Valette et al., 1990). This was found to due to an increase in the V_max of the PDE. In contrast, it has been found that PDE3 activity was selectively elevated within 10 minutes of stimulation of human peripheral blood lymphocytes (HPBLs) with Con A (Meskini et al., 1992). Interestingly, challenge of HPBLs with anti-CD3 antibodies also caused an elevation in PDE3 activity, although the rise in PDE activity was less strong (Meskini et al., 1992). Chapter 5 will address the mechanism which underlies the elevation in PDE4 activity in murine thymocytes.

Upon long-term stimulation of murine thymocytes, there was no evidence of proliferation in response to anti-CD3 antibodies or mitogenic lectins. Indeed anti-CD3 monoclonal antibodies have been shown to induce apoptosis in thymocytes in organ culture experiments (Smith et al., 1989) and in vivo (Shi et al., 1991). However, it has been reported that the addition of rolipram to purified rat thymic cells which were induced to proliferate with Con A, led to a reduction in proliferation, of 60% (Marcoz et al., 1993). Their report highlighted the importance of PDE4 activity in the control of proliferation in thymocytes, so it is
interesting that PDE4 activity has also been shown to be regulated and elevated in murine thymocytes and Jurkat cells upon ligation of the TCR/CD3 complex.

4.3.2. Cyclic AMP levels upon ligation of the TCR/CD3 complex.

Differences in cyclic AMP levels were also observed upon ligation of thymocytes with PHA or anti-CD3 antibodies. PHA stimulation of thymocytes led to a transient elevation of cyclic AMP levels, whereas anti-CD3 mAb treatment led to a slow, sustained increase in cyclic AMP. IBMX was used to inhibit PDE activity in the cells, allowing changes in cyclic AMP level to be regarded as an index of adenyl cyclase activity. Thus the increase in cyclic AMP levels observed upon anti-CD3 mAb or PHA treatment of thymocytes in the presence of IBMX, implied an activation of adenyl cyclase. Rolipram was used to inhibit PDE4 activity, therefore indicating the relevance of this PDE activity in the regulation of cyclic AMP degradation. An increase in cyclic AMP levels was seen in response to anti-CD3 mAbs and PHA in the presence of rolipram in thymocytes. This elevation in cyclic AMP was similar to that seen in the presence of IBMX, indicating that the PDE4 may be the main PDE isoform responsible for degrading cyclic AMP in thymocytes following stimulation with PHA or anti-CD3 mAbs. The addition of milrinone did not potentiate cyclic AMP concentrations, implying that the PDE3 isoform was not involved in the regulation of cyclic AMP levels in murine thymocytes. This supported the previous finding that PDE3 activity is not present in these cells. Consistent with PDE regulation studies, investigation of cyclic AMP concentrations revealed that PHA stimulated a transient 2-fold elevation of cyclic AMP levels, which correlated with the modulation of PDE activities within the cells. However, anti-CD3 ligation induced a slow sustained increase in cyclic AMP. This indicated that a complex balance of adenyl cyclase and PDE activities occurred following TCR activation. These results implied that fundamental differences exist in the PHA- and anti-CD3-stimulated cyclic AMP signalling pathways in thymocytes.
The differential cyclic AMP signalling responses resulting from stimulation of thymocytes with PHA and anti-CD3 may be due, at least in part, to the complement of cell surface receptors recruited by these agents. Whereas anti-CD3 acts solely through the TCR/CD3 complex, PHA is known to crosslink multiple cell surface receptors, including the TCR/CD3 complex and CD2 accessory molecule (O’Flynn et al., 1985). CD2 is expressed during the early stages of thymic development (Fox et al., 1985) and it has been suggested that CD2 may trigger differentiation and IL-2-dependent proliferation signals in immature thymocytes via interaction with its complementary molecule, LFA-3, on thymic epithelial cells (Denning et al., 1987; Fox et al., 1985; Reem et al., 1987). Therefore, a different array of signals is likely to be generated following stimulation of thymocytes with PHA or anti-CD3/TCR. McConkey et al. (1990a) have shown that a sustained elevation of cyclic AMP in thymocytes leads to apoptosis. Thus, the above finding that anti-CD3 induced sustained cyclic AMP generation, whilst PHA only elicited a transient cyclic AMP signal, might explain why thymocytes undergo apoptosis, rather than proliferation in response to anti-CD3 (Jondal et al., 1993; Shi et al., 1991; Smith et al., 1989). Anti-CD3 antibody stimulated thymocytes also apoptose in the presence of PMA or IL-2 (Sancho et al., 1992), whereas thymocytes can respond mitogenically to co-stimulation with lectins and PMA (Taylor et al., 1984). This suggests that PHA might rescue thymocytes from programmed cell death by co-ligating CD3 and CD2. CD3 and CD2 ligation has been shown to cause a significant, but not sustained, increase in cyclic AMP levels in mature T cells (Carrera et al., 1988; Hahn et al., 1991; Kammer et al., 1988; Ledbetter et al., 1986). Indeed, increased cyclic AMP concentrations subsequent to CD2 receptor ligation have been found to be dependent on the expression of TCR/CD3 complex (Kvanta et al., 1991). Furthermore the kinetics of anti-CD3-mediated cyclic AMP rises in thymocytes and in mature T cells may suggest that CD3 is coupled to differential cyclic AMP signalling pathways during T cell maturation. Thymocytes have been shown to have lower concentrations of cyclic AMP than lymphocytes.
However, they are more sensitive to stimulation via isoproterenol, leading to a substantial cyclic AMP response, indicating that the cyclic AMP generating pathways are more readily activated in the thymocyte (Bach, 1975; Niaudet et al., 1976).

The cyclic AMP generated in response to ligation of CD2 has been linked to the potential avidity that exists between cells (Hahn et al., 1993). This indicates that cyclic AMP may be important in modulating the interaction between mature cells, and possibly aiding the TCR/CD3 contact with APCs. Adhesion processes are essential in producing viable cell responses, as inhibition of these actions stops proliferation (Rabin et al., 1993). This would be of particular importance in thymocytes, where antigen is presented to the cells and apoptosis or proliferation of a clone of cells occurs in response to the interaction.

4.3.3. Signalling implications for T cell development.

A hypothesis has been proposed to explain how differential biological responses occur during T cell development (Finkel et al., 1991). It is thought that differential TCR signalling may be due to distinct signals being transmitted through different regions of the antigen receptor, leading to proliferation or deletion of particular thymocyte clones (Finkel et al., 1989). However, the studies presented here propose that PDE activities may be modulated in a similar fashion when murine thymocytes are stimulated with either anti-CD3 antibodies or anti-TCR antibodies. This suggests that thymocyte apoptosis may be the result of partial uncoupling of the TCR to the “activation” pathways of the mature T cells acting in concert with the coupling of the TCR to thymocyte of cell death-specific “negative” signalling pathways.

It is interesting to note that PHA did not lead to any comparable increase in PDE4 activity, despite the fact that this mitogen is known to interact with the TCR/CD3 complex (O’Flynn et al., 1985; Weiss et al., 1984a). This suggests that an additional signalling action of PHA may negate such regulation on thymocyte
PDE4 activity. It is possible that co-ligation of CD2 (and/or other surface receptors) by PHA can actively prevent an elevation in PDE4 activity by exerting a powerful inhibitory effect on the process triggered upon TCR/CD3 ligation. It is interesting to note however, that PDE4 activity appears to increase in Jurkat cells upon stimulation with PHA. This may support the idea, mentioned previously (see section 4.3.2) that CD2 can signal independently of the TCR in immature T cells but upon expression of the antigen receptor in mature T lymphocytes, CD2 signals are directed through the TCR/CD3 complex (Alcover et al., 1988; Blue et al., 1987; Fox et al., 1985). However, considering the complexity of T cell activation this may be a very simplistic view. The Jurkat cell line has been utilised in signalling studies relating to T cell activation pathways assessing the biochemical activation pathways coupled to the TCR/CD3 complex as these cells were reputed to mimic the responses of normal mature human T cells (Nel et al., 1991; Weiss et al., 1984a). However the original characterisation of the Jurkat T cell line suggests that these cells may have been derived from a thymic origin (Schneider, 1977).

Interestingly, PHA has been shown to cause apoptosis in the Jurkat T cell line (D. Williams and M. M. Harnett, personal communication). Therefore there may be a link between the elevation of PDE4 activity and apoptosis, as it is known that anti-CD3 stimulation of thymocytes also induces programmed cell death (Shi et al., 1991; Smith et al., 1989).

4.3.4. Cyclic GMP and implications for NO signalling.

Clearly, cyclic GMP could have an enormous effect on cyclic AMP hydrolysis in murine cells, with the possibility of a ‘molecular switch’ existing for the control of cyclic AMP hydrolysis in these cells as outlined previously (see section 3.3). Cyclic GMP has been documented to increase following stimulation of thymocytes and lymphocytes in the absence of adherent cells with PHA, Con A, PMA and calcium ionophore, A23187 (Coffey et al., 1977; Hadden and Coffey, 1982; Hadden et al., 1972). In the case of PHA, this may be due to its ability to

Cyclic GMP may be elevated through the stimulation of soluble guanylyl cyclase by NO (Furchgott and Zawadski, 1980). NO is a freely diffusible molecule synthesised from the amino acid L-arginine by NO synthase, and can function as an intercellular as well as an intracellular messenger (Lee et al., 1988; Moncada et al., 1991; Southam and Garthwaite, 1993). It appears to have a role in mediating cytotoxicity in the immune system, being responsible for stimulating the phagocytosis of pathogens by cytokine-activated macrophages (Nathan and Hibbs, 1991). However recently, NO has been shown to inhibit apoptosis in B lymphocytes (Genaro et al., 1995), indicating that nitric oxide may also have a role as a ‘survival’ signal in the immune system.

It has been established that sustained elevations of cyclic AMP by agents such as cyclic AMP analogues or E series prostaglandins, as well as glucocorticoid treatment, leads to apoptosis in thymocytes (Cohen and Duke, 1984; Dowd and Miesfeld, 1992; Kaye and Ellenberger, 1992; McConkey et al., 1989a; McConkey et al., 1990a; Wyllie, 1980). However, these cell death pathways can be linked, as cyclic AMP can potentiate apoptosis caused by glucocorticoids (McConkey et al., 1993). Interestingly, glucocorticoids have been shown to inhibit the induction, but not the activity, of calcium independent NO synthase in response to LPS alone or in combination with IFN-γ in vitro (Radomski et al., 1990; Rees et al., 1990; Di Rosa et al., 1990) and in vivo (Knowles et al., 1990). It is tempting to suggest that one of the actions of glucocorticoids may be to stimulate apoptosis by raising cyclic AMP levels by removing cyclic GMP regulation of cyclic AMP hydrolysis.

The connection between cyclic nucleotides and NO signalling pathways is exciting, especially in the context of the murine thymus, where it is apparent from these studies that cyclic AMP hydrolysis may be greatly influenced by the intracellular levels of cyclic GMP.
Table 4.1. PDE1, PDE3 and PDE7 activities in murine thymocytes upon challenge with PHA.

Thymocytes were stimulated with PHA (50μg/ml) for 20 minutes. PDE activity was observed in the presence of 1μM cyclic AMP as substrate. Shown is the percentage inhibition of activity in the presence of 5μM milrinone (PDE3 activity) or 100μM IBMX (PDE7 activity), and the stimulation of PDE1 activity in the presence of Ca²⁺/CaM (50μM/20ng/ml). This is shown for a) zero minutes (basal) and b) 20 minutes of stimulation with PHA. These data show an average of at least four experiments, with assays done in triplicate (errors are SEM). Percentages are calculated as the average of percentage stimulation from each separate experiment with basal PDE activity representing 100%, assayed with 1μM cyclic AMP.
<table>
<thead>
<tr>
<th>Table A</th>
<th>PDE1 Fold stimulation</th>
<th>PDE3 %age total activity</th>
<th>PDE7 %age total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>-PHA 0 min.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting thymocytes</td>
<td>Not stimulated</td>
<td>7 +/- 2</td>
<td>7 +/- 1</td>
</tr>
<tr>
<td>Activated thymocytes</td>
<td>Not stimulated</td>
<td>7 +/- 3</td>
<td>4 +/- 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table B</th>
<th>PDE1 Fold stimulation</th>
<th>PDE3 %age total activity</th>
<th>PDE7 %age total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ PHA 20min.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting thymocytes</td>
<td>Not stimulated</td>
<td>4 +/- 2</td>
<td>2 +/- 1</td>
</tr>
<tr>
<td>Activated thymocytes</td>
<td>Not stimulated</td>
<td>5 +/- 3</td>
<td>7 +/- 1</td>
</tr>
</tbody>
</table>
Table 4.2. PDE1, PDE3 and PDE7 activities in murine thymocytes upon challenge with anti-CD3 monoclonal antibodies.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) for 20 minutes. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). PDE activity was observed in the presence of 1μM cyclic AMP as substrate. Shown is the percentage inhibition of activity in the presence of 5μM milrinone (PDE3 activity) or 100μM IBMX (PDE7 activity), and the stimulation of PDE1 activity in the presence of Ca²⁺/CaM (50μM/20ng/ml). This is shown for a) zero minutes (basal) and b) 20 minutes of stimulation with anti-CD3 monoclonal antibodies. These data show an average of at least four experiments, with assays done in triplicate (errors are SEM). Percentages are calculated as the average of percentage stimulation from each separate experiment with basal PDE activity representing 100%, assayed with 1μM cyclic AMP.
<table>
<thead>
<tr>
<th>Table A</th>
</tr>
</thead>
<tbody>
<tr>
<td>- anti-CD3 0min.</td>
</tr>
<tr>
<td>PDE1 Fold stimulation</td>
</tr>
<tr>
<td>Resting thymocytes</td>
</tr>
<tr>
<td>Activated thymocytes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table B</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ anti-CD3 20min.</td>
</tr>
<tr>
<td>PDE1 Fold stimulation</td>
</tr>
<tr>
<td>Resting thymocytes</td>
</tr>
<tr>
<td>Activated thymocytes</td>
</tr>
</tbody>
</table>
Table 4.3. PDE1, PDE3 and PDE7 activities in murine thymocytes upon challenge with anti-TCR monoclonal antibodies.

Thymocytes were stimulated with anti-TCR monoclonal antibodies (1μg/ml) for 30 minutes. The antibody used was directed against the β chain of the TCR complex. PDE activity was observed in the presence of 1μM cyclic AMP as substrate. Shown is the percentage inhibition of activity in the presence of 5μM milrinone (PDE3 activity) or 100μM IBMX (PDE7 activity), and the stimulation of PDE1 activity in the presence of Ca²⁺/CaM (50μM/20ng/ml). This is shown for a) zero minutes (basal) and b) 30 minutes of stimulation with anti-TCR monoclonal antibodies. These data show an average of at least three experiments, with assays done in triplicate (errors are SEM). Percentages are calculated as the average of percentage stimulation from each separate experiment with basal PDE activity representing 100%, assayed with 1μM cyclic AMP.
<table>
<thead>
<tr>
<th>Table A</th>
<th>Table B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PDE</strong></td>
<td><strong>PDE</strong></td>
</tr>
<tr>
<td>Fold stimulation</td>
<td>Fold stimulation</td>
</tr>
<tr>
<td>%age total activity</td>
<td>%age total activity</td>
</tr>
<tr>
<td>Not stimulated</td>
<td>Not stimulated</td>
</tr>
<tr>
<td>7 ± 1</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Activated thymocytes</td>
<td>Activated thymocytes</td>
</tr>
<tr>
<td>7 ± 1</td>
<td>5 ± 2</td>
</tr>
</tbody>
</table>

*Note: The table data represents the fold change in %age total activity for different conditions.*
Table 4.4. Effect of PDE inhibitors on cyclic AMP concentrations upon stimulation with PHA.

Thymocytes were stimulated with PHA (50μg/ml) for 30 minutes. The table shows the cyclic AMP levels in 'activated thymocytes' and 'resting thymocytes' i.e. the in vivo-activated population of cells which were isolated on the basis of their buoyant density. The cells were stimulated in the presence of 1mM IBMX, 10μM rolipram or 5μM milrinone. Cyclic AMP concentrations were assayed in triplicate (errors are SEM). These data are representative values of three experiments. The results are expressed as pmols/cyclic AMP/10^6 cells against time with PHA (minutes).
<table>
<thead>
<tr>
<th></th>
<th>Control pmols cyclic AMP</th>
<th>PHA pmols cyclic AMP</th>
<th>+ IBMX pmols cyclic AMP</th>
<th>+ rolipram pmols cyclic AMP</th>
<th>+ milrinone pmols cyclic AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting thymocytes</td>
<td>0.54 +/- 0.10</td>
<td>0.75 +/- 0.11</td>
<td>2.28 +/- 0.34</td>
<td>2.69 +/- 0.13</td>
<td>0.75 +/- 0.07</td>
</tr>
<tr>
<td>Activated thymocytes</td>
<td>0.25 +/- 0.15</td>
<td>0.45 +/- 0.2</td>
<td>2.50 +/- 0.22</td>
<td>1.80 +/- 0.17</td>
<td>0.22 +/- 0.03</td>
</tr>
</tbody>
</table>
Table 4.5. Effect of PDE inhibitors on cyclic AMP concentrations upon stimulation with anti-CD3 antibodies.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) for 30 minutes. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). The table shows the cyclic AMP levels in ‘activated thymocytes’ and ‘resting thymocytes’ i.e. the *in vivo*-activated population of cells which were isolated on the basis of their buoyant density. The cells were stimulated in the presence of 1mM IBMX, 10μM rolipram or 5μM milrinone. Cyclic AMP concentrations were assayed in triplicate (errors are SEM). These data are representative values of three experiments. The results are expressed as pmols/cyclic AMP/10^6 cells against time with anti-CD3 antibodies (minutes).
<table>
<thead>
<tr>
<th>+ anti-CD3 30min.</th>
<th>Control pmols cyclic AMP</th>
<th>anti-CD3 pmols cyclic AMP</th>
<th>+ IBMX pmols cyclic AMP</th>
<th>+ rolipram pmols cyclic AMP</th>
<th>+ milrinone pmols cyclic AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting thymocytes</td>
<td>0.56 +/- 0.11</td>
<td>1.26 +/- 0.22</td>
<td>3.40 +/- 0.10</td>
<td>2.81 +/- 0.35</td>
<td>0.72 +/- 0.17</td>
</tr>
<tr>
<td>Activated thymocytes</td>
<td>0.21 +/- 0.032</td>
<td>0.82 +/- 0.23</td>
<td>3.10 +/- 0.17</td>
<td>1.57 +/- 0.30</td>
<td>0.93 +/- 0.09</td>
</tr>
</tbody>
</table>
Table 4.6. PDE2, PDE3, PDE4 and PDE7 activities in the Jurkat cell line upon stimulation with PHA.

Jurkat cells were stimulated with PHA (50μg/ml) for 30 minutes. PDE activity was observed in the presence of 1μM cyclic AMP as substrate. Shown is the percentage inhibition of activity in the presence of 10μM EHNA plus cyclic GMP (PDE2 activity), 5μM milrinone (PDE3 activity), 10μM rolipram (PDE4 activity) or 100μM IBMX (PDE7 activity). This is shown for a) zero minutes (basal) and b) 30 minutes of stimulation with PHA. These data show an average of at least four experiments, with assays done in triplicate (errors are SEM). Percentages are calculated as the average of percentage stimulation from each separate experiment with basal PDE activity representing 100%, assayed with 1μM cyclic AMP.
<table>
<thead>
<tr>
<th>+ PHA</th>
<th>PDE1 Fold stimulation</th>
<th>PDE2 %age total activity</th>
<th>PDE3 %age total activity</th>
<th>PDE4 %age total activity</th>
<th>PDE7 %age total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat Basal</td>
<td>1.37 fold</td>
<td>22 +/- 3</td>
<td>61 +/- 5</td>
<td>4 +/- 1</td>
<td>21 +/- 2</td>
</tr>
<tr>
<td>Jurkat 30min.</td>
<td>1.4 fold</td>
<td>23 +/- 3</td>
<td>46 +/- 5</td>
<td>28 +/- 4</td>
<td>23 +/- 1</td>
</tr>
</tbody>
</table>
Table 4.7. $[^3\text{H}]-\text{thymidine incorporation of murine thymocytes and lymphocytes upon stimulation with PHA.}$

Cells isolated from 4 week old Balb/c mice were stimulated for 48 hours in the presence of PHA (50μg/ml). $[^3\text{H}]-\text{thymidine}$ was added to the cells for the last 4 hours of the incubation, as outlined in 'Materials and Methods.' $2 \times 10^5$ cells were used per well (200μl). These data show results typical of experiments carried out three times, with assays done in triplicate (errors are SEM). The thymidine incorporation (a direct indication of proliferation state of the cells) is presented as cpm/well of cells.
<table>
<thead>
<tr>
<th>[³H]-Thymidine incorporation 48hours</th>
<th>- PHA cpm</th>
<th>+ PHA cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting thymocytes</td>
<td>134 +/- 16</td>
<td>235 +/- 17</td>
</tr>
<tr>
<td>Activated thymocytes</td>
<td>152 +/- 22</td>
<td>264 +/- 29</td>
</tr>
<tr>
<td>Resting lymphocytes</td>
<td>47 +/- 4</td>
<td>2500 +/- 144</td>
</tr>
<tr>
<td>Activated lymphocytes</td>
<td>242 +/- 50</td>
<td>4160 +/- 260</td>
</tr>
</tbody>
</table>
Figure 4.1. PDE4 activity in murine thymocytes upon challenge with PHA.

Thymocytes were stimulated with PHA (50μg/ml) for up to 20 minutes as indicated. The total PDE activity observed in the presence of 1μM cyclic AMP as substrate (circles) is shown. Also given is that fraction of activity (squares) which is inhibited by 10μM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity. The graphs show the PDE activities in:
a) 'activated thymocytes' i.e. the in vivo-activated population of cells which were isolated on the basis of their buoyant density, and b) 'resting thymocytes' i.e. the in vivo-resting population of cells which were isolated on the basis of their buoyant density. These data show results typical of experiments carried out at least five times. PDE assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
a) 

![Graph a)

PDE activity vs. Time (min.)

b) 

![Graph b)

PDE activity vs. Time (min.)
Figure 4.2. PDE2 activity in murine thymocytes upon challenge with PHA.

Thymocytes were stimulated with PHA (50μg/ml) for up to 20 minutes as indicated. Shown is the total PDE activity (diamonds) observed in the presence of stimulatory concentrations of cyclic GMP (10μM) and 1μM cyclic AMP as substrate. Also given is the magnitude of the increase in PDE activity elicited by cyclic GMP (triangles); this is taken to serve as an index of PDE2 activity. In this regard it must be taken as an underestimate as it does not take into account the basal activity of the enzyme. However, given the magnitude of the stimulation by cyclic GMP, then such a basal component is likely to be small. This approach has the advantage in that it solely represents PDE2 activity. The graphs show the PDE activities in: a) ‘activated thymocytes’ i.e. the in vivo-activated population of cells which were isolated on the basis of their buoyant density, and b) ‘resting thymocytes’ i.e. the in vivo-resting population of cells which were isolated on the basis of their buoyant density. These data show results typical of experiments carried out at least five times. PDE assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 4.3. PDE4 activity in murine thymocytes upon challenge with anti-CD3 monoclonal antibodies.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) for up to 20 minutes as indicated. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). The total PDE activity observed in the presence of 1μM cyclic AMP as substrate (circles) is shown. Also given is that fraction of activity (squares) which is inhibited by 10μM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity. The graphs show the PDE activities in: a) ‘activated thymocytes’ i.e. the in vivo-activated population of cells which were isolated on the basis of their buoyant density, and b) ‘resting thymocytes’ i.e. the in vivo-resting population of cells which were isolated on the basis of their buoyant density. These data show results typical of experiments carried out at least four times. PDE assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 4.4. PDE2 activity in murine thymocytes upon challenge with anti-CD3 monoclonal antibodies.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5µg/ml) for up to 20 minutes as indicated. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). Shown is the total PDE activity (diamonds) observed in the presence of stimulatory concentrations of cyclic GMP (10µM) and 1µM cyclic AMP as substrate. Also given is the magnitude of the increase in PDE activity elicited by cyclic GMP (triangles); this is taken to serve as an index of PDE2 activity. In this regard it must be taken as an underestimate as it does not take into account the basal activity of the enzyme. However, given the magnitude of the stimulation by cyclic GMP, then such a basal component is likely to be small. This approach has the advantage in that it solely represents PDE2 activity. The graphs show the PDE activities in; a) ’activated thymocytes’ i.e. the in vivo-activated population of cells which were isolated on the basis of their buoyant density, and b) ‘resting thymocytes’ i.e. the resting population of cells which were isolated on the basis of their buoyant density. These data show results typical of experiments carried out at least four times. PDE assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 4.5. PDE4 activity in murine thymocytes upon challenge with anti-TCR monoclonal antibodies.

Thymocytes were stimulated with anti-TCR monoclonal antibodies (1µg/ml) for up to 30 minutes as indicated. The antibody used was directed against the β chain of the TCR. The total PDE activity observed in the presence of 1µM cyclic AMP as substrate (circles) is shown. Also given is that fraction of activity (squares) which is inhibited by 10µM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity. The graphs show the PDE activities in: a) ‘activated thymocytes’ i.e. the \textit{in vivo}-activated population of cells which were isolated on the basis of their buoyant density, and b) ‘resting thymocytes’ i.e. the resting population of cells which were isolated on the basis of their buoyant density. These data show results typical of experiments carried out at least three times. PDE assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 4.6. PDE2 activity in murine thymocytes upon challenge with anti-TCR monoclonal antibodies.

Thymocytes were stimulated with anti-TCR monoclonal antibodies (1 μg/ml) for up to 30 minutes as indicated. The antibody used was directed against the β chain of the CD3 complex. Shown is the total PDE activity (diamonds) observed in the presence of stimulatory concentrations of cyclic GMP (100 μM) and 1 μM cyclic AMP as substrate. Also given is the magnitude of the increase in PDE activity elicited by cyclic GMP (triangles); this is taken to serve as an index of PDE2 activity. In this regard it must be taken as an underestimate as it does not take into account the basal activity of the enzyme. However, given the magnitude of the stimulation by cyclic GMP, then such a basal component is likely to be small. This approach has the advantage in that it solely represents PDE2 activity. The graphs show the PDE activities in: a) ‘activated thymocytes’ i.e. the in vivo-activated population of cells which were isolated on the basis of their buoyant density, and b) ‘resting thymocytes’ i.e. the in vivo-resting population of cells which were isolated on the basis of their buoyant density. These data show results typical of experiments carried out at least three times. PDE assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 4.7. PDE activities in murine thymocytes upon challenge with anti-CD3 monoclonal antibodies, and crosstlinking with anti-hamster IgG antibodies.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5µg/ml) for up to 30 minutes as indicated in the presence of antibodies directed against hamster IgG antibodies (10µg/ml). The first antibody (anti-CD3 antibody) used was directed against the ε chain of the CD3 complex (Philips et al. 1991). Shown is a) the total PDE activity observed in the presence of 1µM cyclic AMP as substrate, in the absence (circles) and that fraction of activity (squares) which is inhibited by 10µM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity. Graph b) shows the total PDE activity observed in the presence of stimulatory concentrations of cyclic GMP (10µM) (diamonds) and the magnitude of the increase in PDE activity elicited by cyclic GMP (triangles); this is taken to serve as an index of PDE2 activity. In this regard it must be taken as an underestimate as it does not take into account the basal activity of the enzyme. However, given the magnitude of the stimulation by cyclic GMP, then such a basal component is likely to be small. This approach has the advantage in that it solely represents PDE2 activity. These data show results typical of experiments carried out two times on 'mixed' populations of thymocytes PDE assays are carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 4.8. Cyclic AMP concentration in murine thymocytes upon stimulation with PHA.

Thymocytes were incubated with PHA (50μg/ml) for up to 30 minutes as indicated. The graphs show the cyclic AMP levels in; a) ‘activated thymocytes’ i.e. the in vivo-activated population of cells which were isolated on the basis of their buoyant density, and b) ‘resting thymocytes’ i.e. the in vivo-resting population of cells which were isolated on the basis of their buoyant density. These data show results typical of experiments carried out three times. Assays were carried out in triplicate (errors are SEM). The results are expressed as pmols/cyclic AMP/10^6 cells against time with PHA (minutes).
Figure 4.9. Cyclic AMP concentration in murine thymocytes upon stimulation with anti-CD3 monoclonal antibodies.

Thymocytes were incubated with anti-CD3 monoclonal antibodies (5µg/ml) for up to 30 minutes as indicated. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). The graphs show the cyclic AMP levels in: a) 'activated thymocytes' i.e. the in vivo-activated population of cells which were isolated on the basis of their buoyant density, and b) 'resting thymocytes' i.e. the in vivo-resting population of cells which were isolated on the basis of their buoyant density. These data show results typical of experiments carried out three times. Assays were carried out in triplicate (errors are SEM). The results are expressed as pmols/cyclic AMP/10^6 cells against time with anti-CD3 antibodies (minutes).
a) Time (min.)

b) Time (min.)
Figure 4.10. PDE activities in the human leukemic T cell line, Jurkat, upon stimulation with PHA.

Jurkat cells were stimulated with PHA (50μg/ml) for up to 30 minutes as indicated. The total PDE activity observed in the presence of 1μM cyclic AMP as substrate (circles) is shown. Also given is a) the fraction of activity (squares) remaining after addition of 10μM cyclic GMP and b) the total activity upon addition of Ca²⁺/CaM (50μM/20ng/ml) (triangles). These data show results typical of experiments carried out at least five times, with assays done in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 4.11. \[^{3}\text{H}\]-thymidine incorporation of murine thymocytes upon stimulation with anti-CD3 monoclonal antibodies.

Thymocytes isolated from 4 week old Balb/c mice were stimulated for 48 hours in the presence of increasing doses of anti-CD3 monoclonal antibodies, as indicated. The antibody used was directed against the \(\varepsilon\) chain of the CD3 complex (Philips et al. 1991). \[^{3}\text{H}\]-thymidine was added to the cells for the last 4 hours of the incubation, as outlined in ‘Materials and Methods.’ The graphs show \[^{3}\text{H}\]-thymidine incorporation in; a) ‘activated thymocytes’ i.e. the in vivo-activated population of cells which were isolated on the basis of their buoyant density, and b) ‘resting thymocytes’ i.e. the in vivo-resting population of cells which were isolated on the basis of their buoyant density. 2 x 10\(^5\) cells were used per well (200\(\mu\)l). These data show results typical of experiments carried out three times. Assays were carried out in triplicate (errors are SEM). The \[^{3}\text{H}\]-thymidine incorporation (a direct indication of proliferation state of the cells) is presented as cpm/well of cells.
Lymphocytes isolated from 4 week old Balb/c mice were stimulated for 72 hours in the presence of increasing doses of anti-CD3 monoclonal antibodies. The antibody used was directed against the \( \varepsilon \) chain of the CD3 complex (Philips et al. 1991). \(^3\)H-thymidine was added to the cells for the last 4 hours of the incubation, as outlined in 'Materials and Methods.' The graphs show \(^3\)H-thymidine incorporation in: a) 'activated thymocytes' i.e. the \textit{in vivo}-activated population of cells which were isolated on the basis of their buoyant density, and b) 'resting thymocytes' i.e. the \textit{in vivo}-resting population of cells which were isolated on the basis of their buoyant density. \( 2 \times 10^5 \) cells were used per well (200\( \mu \)l). These data show results typical of experiments carried out three times. Assays were carried out in triplicate (errors are SEM). The \(^3\)H-thymidine incorporation (a direct indication of proliferation state of the cells) is presented as cpm/well of cells.
CHAPTER 5.

MECHANISM OF PDE4 ACTIVITY ELEVATION UPON STIMULATION OF MURINE THYMOCYTES WITH ANTI-CD3 MONOCLONAL ANTIBODIES.
5.1. Introduction.

The regulation of a number of processes including, metabolism, DNA transcription and replication, protein synthesis, proliferation and differentiation in response to hormones and growth factors, is largely achieved by coordinated changes in protein phosphorylation of enzymes and structural components. It is well established that phosphorylation-dephosphorylation processes play an important role in signal transduction, altering enzyme activities upon receptor ligation. Processes controlled by reversible phosphorylation require not only a protein kinase, but also a protein phosphatase (PPT). It is now realised that these processes are regulated by a large group of enzymes, with mammals having as many as 2000 protein kinase genes (Hanks and Hunter, 1995) and, possibly, as many as 1000 PPT genes (Wilson et al., 1994b). In general these enzymes are divided into serine/threonine or tyrosine kinases or phosphatases, although some activities have the ability to phosphorylate or dephosphorylate all residues (Hanks et al., 1988; Hunter, 1987). Activities of protein kinases and PPTs may be regulated by protein phosphorylation, allosteric effectors and second messengers, therefore being sensitive to extracellular and intracellular signals. The first example illustrating the role of protein phosphorylation in signal transduction was that involving cyclic AMP and cyclic AMP-dependent protein kinase (see section 1.5.4) which was elucidated as a result of work on the mechanism of action of epinephrine on skeletal muscle (Krebs, 1973). This also constituted the first example of a protein kinase cascade, in which a kinase activated another by phosphorylation.

It is apparent that PPTs are not simply a reversal of a phosphorylation reaction, but also represent another level at which regulation can occur, leading to the control of cellular processes (Shenolikar, 1994). Transient production of second messengers upon receptor stimulation can result in proliferation and differentiation, which are long lasting effects and sometimes irreversible. Protein phosphorylation can regulate gene expression by modulating sequence specific transcription factors, whose activity is altered by phosphorylation. This form of
control has been established for CREB/ATF (see section 1.5.6), AP-1 (jun/fos) and NF-κB proteins. Signal-induced post-translational activation of these factors results in induction of downstream genes, some of which encode other transcription factors, which in turn effect the expression of target genes. Such signalling cascades result in long lasting nuclear signals.

5.1.1. Protein kinase C.

PKC is a serine/threonine kinase which was first discovered by Nishizuka et al. as a protein kinase activity which was only active after limited proteolysis (Inoue et al., 1977). Later, it was found to be activated by calcium and phospholipids (especially phosphatidyserine (PS)) (Takai et al., 1979) or phorbol esters and phospholipids (Castagna et al., 1982). Phorbol esters are a group of compounds which activate PKC in a similar manner to DAG, representing non-metabolisable analogs of DAG (Bell and Burns, 1991). With the use of phorbol esters as a pharmacological tool it has become recognised that PKC is involved in a variety of diverse processes within the cell. Initially, PKC was recognised to exist as three isoenzymes (α, β, γ), but now it is realised that there are at least twelve members of the PKC multigene family which can be divided into sub-species, possibly explaining why PKC has been implicated in so many cellular processes (Hug and Sarre, 1993; Nishizuka, 1992). These sub-species display subtly different enzymological properties, differential tissue expression and limited intracellular localisation, and structural properties (Nishizuka, 1988). Thus, three main groups are recognised, one containing α, βI, βII and γ which is the calcium dependent or 'conventional' PKC family (cPKCs), one group consisting of the novel or calcium-independent isoenzymes, δ, ε, θ, η (nPKCs) (Ohno et al., 1991), and the last group are the atypical PKCs, ζ, μ, τ, λ (aPKCs), which although structurally related to the PKC family, differ as their activities are not regulated by DAG or phorbol esters (Nishizuka, 1988; Ohno et al., 1994).
Marked differences are found with regard to tissue distribution of the various isoforms of PKC, with α, βI/II, δ, ε and ζ isoforms being widely expressed, found in the brain, thymus, spleen and lung (Niskizuka, 1988; Ohno et al., 1991; Wetsel et al., 1992; Yoshida et al., 1988). However, γ, τ, and θ have restricted distribution, with each isoform being found mainly in the brain (Niskizuka, 1988; Ohno et al., 1991; Wetsel et al., 1992), skin and lung (Bacher et al., 1991; Osada et al., 1990) and skeletal muscle (Osada et al., 1990), respectively. Indeed, human peripheral blood-derived T cells have been shown to be express PKC-α, βI, δ, ε, ζ, and τ subtypes (Lucas et al., 1990; Mischak et al., 1991a; Mischak et al., 1991b) which appear to be differentially translocated to intracellular membranes among T cell subsets (Gupta and Harris, 1994). These tissue specific variations in the pattern of expression may underline divergence in function occurring between isotopes.

Compartmentalisation of PKC isoforms appears to be dependent on the activation state of the cell. It has been generally thought in the past that stimulation of cells leads to the translocation of PKC from the cytosol to the Plasma membrane as this was seen upon stimulation with phorbol ester (Bell, 1986; Borner et al., 1992; Hoevar and Fields, 1991; Strulovici et al., 1991). However this picture is a little over simplified, as it has been noticed that isoforms tend to behave differently depending on the cell in which they are studied and the form of activation used. PKC isoforms have also been reported to be rapidly translocated from the cytosol to the nucleus (Cambier et al., 1987; Leach et al., 1989), cytoskeletal elements (Moehly-Rosen et al., 1990), golgi apparatus (Saito et al., 1989) as well as being down regulated (Kiley et al., 1991) or being non-responsive (Ha and Exton, 1993; Kiley et al., 1991; Olivier and Parker, 1994). Hence, it appears that there is no clear cut pattern of cellular distribution and behaviour of any one PKC isoform upon stimulation of cells with particular stimuli. In a similar fashion to that seen for PKAII (see section 1.5.5) intracellular PKC receptors have been described that act
as receptors for activated PKCs (RACKs) possibly contributing to the localisation of PKCs within the cell (Mochly-Rosen et al., 1991).

PKC isoforms show differing co-factor requirements and responsiveness to phospholipid metabolites in vitro (Bell and Burns, 1991; Nishizuka, 1992). The differences in calcium dependence is well established (see above), however there are some interesting differences in regulation between isoforms. PKC-ζ is not activated by phorbol ester or DAG, but has a low constitutive kinase activity (Liyanage et al., 1992), which can be enhanced markedly by PS or arachidonic acid (Nakanishi and Ezton, 1992). There is evidence for some PKCs being stimulated by PIP₃ (Nakanishi et al., 1993). This is of particular interest as it opens up the possibility of cross-talk between PKCs and PI-3-K, as well as breaking the restriction of PKC activation only in the presence of DAG generation. Activation of cPKCs is thought to require DAG as an activator and PS as a cofactor. The presence of both lowers the concentration of calcium required for activation. However in addition to this, PKC isotypes can be activated by fatty acids and lysophosphatidic acids in synergy with DAG, in vitro (Asaoka et al., 1992; Nakamura et al., 1993). It is evident that the array of PKC isotypes offer a multitude of prospects for interactions with signalling cascades upon stimulation.

5.1.2. Control of gene expression.

Protein phosphorylation is known to regulate gene expression within cells upon stimulation. A growing list of nuclear effector proteins includes proto-oncogene products which function as transcription factors as well as tumour suppressor proteins, which may be involved in regulating the onset of DNA replication. Control of activities can occur through phosphorylation and dephosphorylation of transcription factors, which can respond to extracellular signals transduced via signalling pathways to the nucleus. Among the proteins controlled are CREB/ATF family that mediates the stimulation of gene expression in
response to factors which elevate intracellular levels of cyclic AMP (see section 1.5.6), and activator protein 1 (AP-1).

AP-1 is a collection of homodimeric and heterodimeric complexes composed of jun and fos gene products. These products interact with a common DNA binding site (TGAC/GTCA) (Angel and Karin, 1991), termed the PMA responsive element (TRE), since this motif was first identified in the promoters of genes induced by phorbol esters such as PMA (Lee et al., 1987). Thus, activation of gene transcription occurs in response to activators of PKC, growth factors and transforming oncogenes (Angel et al., 1987). The jun and fos genes are immediate-early genes whose transcription is rapidly induced by the same signals which induce AP-1 activity (Angel and Karin, 1991). In several cell types the basal level of fos and jun is very low, but expression can be induced rapidly and transiently (Morgan and Curran, 1991). Indeed c-Fos accumulation has been reported within an hour of the initial stimulus (Greenberg and Ziff, 1984; Kruijer et al., 1984). The induction of c-jun is longer lasting and can elevated for days depending on the manner of the cell type and stimulus provided (Karin, 1990).

Functional AP-1 consists of dimers of proteins of the Fos and Jun families (Bohmann et al., 1987; Chiu et al., 1988; Franz et al., 1988). The Jun family proteins c-Jun, JunB and JunD have the ability to homo- or hetero-dimerise with other AP-1 proteins (Nakabeppu et al., 1988). In contrast, Fos family proteins c-Fos, Fra-1, Fra-2 and FosB need one of the Jun family proteins for dimerisation. The hetero-dimers formed are more stable than Jun homodimers, and are consequently more efficient in binding to DNA (Chiu et al., 1988; Smeal et al., 1989).

Increases in AP-1 activity can occur in the absence of de novo protein synthesis by a process involving modulation of pre-existing AP-1. PMA directly activates most PKC isotypes, leading to phosphorylation of target proteins which results in the up-regulation of AP-1 activity. Resting cells contain a latent form of phosphorylated c-Jun, which is unable to effectively bind to TRE (Boyle et al., 1987).
Upon stimulation with PMA, there is a rapid site specific dephosphorylation of c-Jun close to the DNA binding domain, and an activation of DNA binding (Boyle et al., 1991). Two N-terminal serines are phosphorylated upon phorbol ester treatment of cells, and this phosphorylation event appears to be important for the activation of c-Jun activity (Pulverer et al., 1991). As c-Jun is positively regulated, these phosphorylations result in the production of c-Jun protein. PKC activation also stimulates the phosphorylation of c-Fos (Barber and Verma, 1987). In addition to its role in transcriptional activation, it also has a repressor activity (Sassone-Corsi et al., 1988). The phosphorylation sites are present in the C-terminal end of the protein, in the same region as its activation and repression domains. Substitution of these phosphorylatable residues for alanines abolishes the repressor activity, without affecting the activation of transcription (Offir et al., 1990). The biological consequence is that c-Fos is likely to contribute to its own transient synthesis, whereas in the case of c-Jun it prolongs its production and therefore generates a longer lasting signal.

Clearly, the regulation of transcription factor AP-1 by different mitogenic signals can occur through the phosphorylation of c-Jun and c-Fos protein families. Although other signalling pathways are involved in the regulation of these proteins, PKC appears to play an important role in the control of gene transcription through TRE-regulated genes.

5.1.3. Protein phosphatase and protein kinase inhibitors.

A number of selective protein kinase inhibitors are available, but it has become apparent with the generation of more sensitive techniques, that some inhibitors are not as selective for a particular type of kinase as was first thought. With this in mind, it is essential when administering kinase inhibitors to treat results obtained with extreme caution.

Intense efforts have been made to find protein kinase inhibitors which target PKC or PTKs, as the action of many oncogenes involves the aberrant activation of
these protein kinases. The specificity of each a selection of the protein kinase inhibitors is summarised in table 5.1. It can be seen that chelerythrine, which was used in this study displays a high selectivity for PKC, with an IC₅₀ approximately 100 fold lower than that of any other protein kinase tested. This inhibitor targets the substrate binding site of the kinase (Herbert et al., 1990), being a competitive inhibitor with respect to the phosphate acceptor, and a non-competitive inhibitor with respect to ATP. Staurosporine, which binds to the ATP binding site was also used in this study, although, this kinase cannot be considered to be a selective PKC inhibitor, as it is obvious from table 5.1 that this inhibitor shows little selectivity for PKC over any other serine/threonine or tyrosine kinase (Kiss and Deli, 1992; Wilkinson and Hallum, 1994). H-89 was utilised as a selective PKA inhibitor, as it shows an IC₅₀ of 40nM against PKA activity (Chijiwa et al., 1990; Hidaka et al., 1991). The structures of chelerythrine, staurosporine and H89 are outlined in figure 5.1.

Tyrophostins are a large group of inhibitors that have different potencies towards different PTKs (Lyall et al., 1989; Margolis et al., 1989). They are synthetic derivatives of the naturally occurring aromatic compound eribastin, produced by strains of Streptomyces. Two such inhibitors were used in this study, tyrophostin A1 and tyrophostin A25. The former does not have an inhibitory action against PTKs and was therefore utilised as a negative control. Genistein has been shown to inhibit PTKs that regulate T cell proliferation (Akiyama and Ogawara, 1991). However, genistein has also been shown to inhibit PKC at higher concentrations (Akiyama and Ogawara, 1991). The structures of these PTK inhibitors are represented in figure 5.2.

The tumour promoter okadaic acid is a polyether fatty acid produced by marine dinoflagellates, which potently and specifically inhibits protein phosphatases 1 and 2A (Bialojan and Takai, 1988) (IC₅₀ of 0.01 and 1µM respectively), two of the four major protein serine/threonine phosphatases in mammalian cells (Cohen and Cohen, 1989). Ca²⁺/CaM dependent PP2B (calcineurin) is far less sensitive to
this inhibitor (IC$_{50}$ of 10mM), whilst Mg$^{2+}$ dependent PP2C is unaffected (Bialojan and Takai, 1988). The structure of this PPT inhibitor is outlined in figure 5.3. Okadaic acid was used in this study to ascertain whether or not dephosphorylation via PP1/PP2A played any role in the regulation of PDE activities in murine thymocytes.

5.1.4. Aims and objectives.

It is evident from chapter 4 that, upon stimulation of activated and resting population of murine thymocytes with anti-CD3 monoclonal antibodies, there was a significant increase in PDE4 activity. It was therefore of interest to study the mechanism behind this elevation. The studies were carried out on 'mixed' populations of thymocytes, as it was apparent that both in vivo-activated and resting cells behave similarly with respect to the modulation of their PDE activities upon stimulation with anti-CD3/TCR antibodies or with PHA. As changes in PDE activity differed upon PHA stimulation or anti-CD3 stimulation, it was decided to concentrate on the regulation of the anti-CD3-antibody induced stimulation of PDE4 activity. This is because the mechanism could be directly attributed to the CD3 component of the TCR/CD3 complex. PHA is known to cross-link a number of cell surface receptors, including CD2 and the TCR/CD3 complex (O'Flynn et al., 1985), therefore making interpretation of the results, and assigning an effect to a single cell surface receptor more difficult.
5.2. Results.

With the use of selective inhibitors towards particular serine/threonine or tyrosine kinases, the underlying mechanism of TCR-mediated elevation of PDE4 activity was studied in murine thymocytes. As mentioned previously, 'mixed' populations of thymocytes were used in this part of the study as opposed to 'in vivo-activated' and 'resting' populations of cells (see chapters 3 and 4). Figure 5.4 shows murine thymocytes stimulated with anti-CD3 antibodies (5μg/ml) for up to 30 minutes. In a similar fashion as described before (see section 4.2 and fig. 4.3), there was an elevation in total PDE activity, which upon analysis with rolipram, the PDE4 selective inhibitor, was shown to be due to the PDE4 component of the thymocyte extract (fig 5.4). Upon addition of stimulatory concentrations of cyclic GMP (10μM), total PDE activity was elevated about 4 fold, in keeping with the presence of cyclic GMP-stimulated, PDE2 activity (fig. 5.5). This activity remained unchanged upon stimulation with anti-CD3 antibodies, although a slight increase in total PDE activity is observed, due to the elevation of PDE4 activity (fig. 5.5). This result was similar to that shown previously (fig. 4.4).

5.2.1. Modulation of PKC activities.

Selective inhibitors and activators of PKC were employed to ascertain whether or not these serine/threonine kinases had a role in the activation of this PDE4 activity elevation. Stimulating murine thymocytes with anti-CD3 antibodies in the presence of chelerythrine (10μM), which shows selectivity for inhibition of PKC at low concentrations (Herbert et al., 1990), abrogated the elevation in PDE4 activity (fig. 5.6b). With the use of rolipram, it was seen that chelerythrine was responsible for blocking the rise in PDE4 activity specifically (fig. 5.6a). Intriguingly, abrogation of PKC activity caused a decrease in PDE activity (fig. 5.6b). Staurosporine also abrogated the elevation in PDE4 activity (table 5.2).

Upon addition of PMA (100nM) alone, there was an elevation in PDE4 activity (fig. 5.7a), which was similar to that seen upon stimulation of anti-CD3
antibodies alone (fig. 5.7b). This implies that the rise in PDE4 activity was a PKC-mediated response. Interestingly, addition of PMA alone appeared to block the initial decrease in PDE activity observed upon stimulation of the cells with anti-CD3 mAbs. This may indicate that a pathway which is inhibitory to PDE4 is initially activated prior to PKC activation following ligation of the TCR and can be bypassed by the direct activation of PKC. An elevation of PDE4 activity was observed upon addition of PMA and anti-CD3 antibodies together (fig 5.8). The increase in PDE4 activity was of the same magnitude as that seen in the presence of PMA or anti-CD3 mAbs alone suggesting they are using the same pathway (fig. 5.7b). PDE2 activities remained unchanged following PMA treatment of thymocytes (table 5.3).

Basal PDE4 activities were generally unaffected by incubation with the inhibitors alone (table 5.4) and PDE2 activities were unchanged by protein kinase inhibitors in the absence (table 5.5) and presence (table 5.3) of anti-CD3 mAbs.

5.2.2. Modulation of PKA activities.

In order to determine whether PKA played a role in controlling the activity of PDE activities, the selective PKA inhibitor H89 was employed in the assays (Chijiwa et al., 1990). It was found to have no effect on the elevation of PDE4 activity when incubated in the presence of anti-CD3 antibodies (fig 5.9). This was also true of PDE2 activity which remained unchanged in the presence of H89 (table 5.3).

5.2.3. Effect of protein phosphatase inhibitor, okadaic acid.

Okadaic acid is a PPT inhibitor which is selective for the serine/threonine phosphatases-1 and -2A (Bialojan and Takai, 1988). Murine thymocytes stimulated with anti-CD3 mAbs showed an increase in PDE4 activity (fig. 5.10a) in the presence of okadaic acid (100nM). This was similar to that seen in the absence of the PPT inhibitor (fig. 5.10b). Thus implying that a serine/threonine
dephosphorylation event, involving PP1 and PP2A, is not necessary for the activation of PDE4 activity upon ligation of the TCR/CD3 complex. Once again, PDE2 activity was unaffected by the addition of okadaic acid to anti-CD3 antibody stimulated thymocytes (table 5.3). Okadaic acid alone was found to be without effect on PDE activity throughout the incubation time (table 5.4 and 5.5).

5.2.4. Effect of tyrosine kinase inhibitors on PDE activities.

The elevation in PDE4 activity resulting from anti-CD3 ligation was inhibited by addition of genistein (10μM) (table 5.6). This result indicated that tyrosine phosphorylation was also probably involved in the process of PDE4 activation. Similar results were observed on addition of 100μM genistein (table 5.6). Indeed, at the higher concentration the inhibition by genistein was greater, which may imply that this tyrosine kinase inhibitor is non-specifically inhibiting serine/threonine kinases (Akiyama and Ogawara, 1991). However, this may also indicate that a higher concentration of genistein is required to inhibit PTK activities in murine thymocytes. Tyrophostins A1 and A25 were also used to determine whether tyrosine phosphorylation played a role in the elevation of PDE activity. Tyrophostin A1 was chosen as it is a non-inhibitory tyrophostin and should therefore be without effect when incubated with anti-CD3 stimulated thymocytes. However, tyrophostin A1 and A25 revealed similar results to those results obtained with 10μM genistein (table 5.6). The inhibition seen with tyrophostin A1 indicated that these tyrophostins may be acting in a non-specific manner in murine thymocytes.

However, the inhibition of PDE4 activity was evident on addition of genistein. This indicated that tyrosine phosphorylation was involved with the elevation of PDE4 activity in murine thymocytes. Neither type of PTK inhibitor affected basal PDE activities of murine thymocytes (table 5.4 and 5.6).
5.2.4. Effect of RNA and protein synthesis inhibition.

Both actinomycin D and cyclohexamide abolished the elevation of PDE4 activity, seen in response to either anti-CD3 antibodies (fig 5.11 and 5.14a), PMA (fig. 5.12 and 5.14b) or anti-CD3 antibodies plus PMA (fig. 5.13 and 5.14c). This is exciting, as it implies that the increase in PDE4 activity is derived by rapid induction of a PDE4 gene generating de novo enzyme upon ligation of the TCR/CD3 complex.
5.3. Discussion

It has previously been established that upon ligation of the TCR in murine thymocytes there is an increase in PDE4 activity (see chapter 4). The results presented above suggest that the PDE4 activity is elevated in a PKC-dependent manner. This is indicated by three lines of evidence: (i) Upon addition of PKC selective inhibitor, chelerythrine, the rise in PDE activity was abolished. (ii) Upon addition of the phorbol ester, PMA, which activates most isoforms of PKC (Ohno et al., 1994), there was an immediate increase in PDE4 activity in the absence of stimulation with anti-CD3 antibodies. This elevation of activity was of a similar magnitude to that seen with anti-CD3 antibodies alone. (iii) The addition of TPA and anti-CD3 antibodies was not additive regarding PDE4 activation.

Staurosporine and the PTK inhibitor, genistein also led to an abrogation of PDE4 increase in activity, in the presence of anti-CD3 antibodies. This result implied that a tyrosyl phosphorylation event may be involved in the activation of PDE4 activity. Tyrophostin A25 also inhibited the elevation of PDE4 activity, however, this activity was also affected by the negative control tyrophostin A1 indicating that tyrophostins may have non-specific effects in these cells. The selective PKA inhibitor, H-89 did not effect PDE4 elevation, suggesting that PKA is not involved in the elevation of PDE4 activity. Serine/threonine residue dephosphorylation is also not thought to be implicated in the increase of PDE4 activity, as the PP1 and PP2A inhibitor, okadaic acid had no effect.

In order to test whether enzyme induction might have been responsible for the increase in PDE activity, stimulations were carried out in the presence of either actinomycin D or cyclohexamide. It was exciting to note that the incubation of murine thymocytes with either of these agents inhibited the PMA/anti-CD3-mediated elevation in PDE4 activity. This result was also somewhat surprising when one considers that the elevation in PDE activity, above basal, occurred within ten minutes of anti-CD3 stimulation. However this is not to say that the activity is being induced this quickly. There is clearly PDE activity present in the extracts of
unstimulated thymocytes, which may be further activated by post-translational modification such as phosphorylation, as has been described previously for PDE4D activity (Sette et al., 1994a). However in this case, the rise in PDE activity did appear to be inhibited by incubation with actinomycin D or cyclohexamide. This suggests a rapid induction of a PDE4 gene. This rapid induction bears a similarity to the ability of phorbol esters to activate immediate early genes (Greenberg and Ziff, 1984; Hata et al., 1993). It may be that a PDE4 gene is under the same transcriptional control as c-jun (Angel et al., 1988) and c-fos, as well as other proteins (Angel et al., 1987; Imbra and Karin, 1988). Therefore, PDE4 gene expression may be regulated by a cis-acting PMA responsive element which is bound by AP-1 transcription factors (see section 5.1.2). In several cell types the basal level of Fos and Jun is very low, but expression can be induced rapidly and transiently, with transcriptional activation (Greenberg et al., 1985; Greenberg and Ziff, 1984). PMA has been shown to induce Fos in CHO cells in a transient fashion within 30 minutes of exposure (Stumpo et al., 1988). Indeed, a recent paper has established the induction of PDE1 activity within 25 minutes of stimulation with PMA in CHO cells (Spence et al., 1995). This was transient in nature, similar to that of c-fos induction (Greenberg et al., 1985). This cell line was found to be devoid of type-I PDE activity in the resting state, unlike the situation presented here. This study was of particular interest, as it identified PKC- α and -ε to be essential in mediating the induction of the PDE activity, whilst PKC- βI and -γ were ineffectual (Spence et al., 1995). Interestingly, PKC-ε has been shown to be expressed at high concentrations in murine thymocytes (Strulovici et al., 1991). Therefore, it may be the case that an early event in stimulation of murine thymocytes is the activation of a PDE4 activity mediated by a specific PKC isoform, possibly, PKC-ε, which is known to be activated early in T cell stimulation.
5.3.1. The role of PKC in T cell activation

It is well established that PKC plays an important role in downstream signalling pathways of T cell activation (Farrar and Ruscetti, 1986; Gupta et al., 1991; Weiss et al., 1986a). It has been reported that early in T cell activation, PKC-α was activated, whilst PKC-β activation was prolonged after a lag phase. PKC-α activation was sufficient in induce the expression of IL-2Ra, whereas the sustained activation of PKC-β was required for the expression of IL-2 and subsequent proliferation (Berry et al., 1990; Szamel and Resch, 1992). Signalling through the TCR/CD3 complex initiates a complex series of events resulting in the activation of immediate early genes that are involved in the proliferation of T cells, the development of immune function and a variety of cellular interactions that contribute to immune responses (Crabtree, 1989). In addition, signalling through the antigen receptor is essential for several transitions in thymic development. Upon TCR/CD3 ligation, c-fos, but not c-jun gene product is induced. Both genes exhibit binding sites for transcription factor NF-κB which is bound to I-κB in the inactive state. This inhibitory factor can be removed by a phosphorylation event, therefore PKC may act in a dual way to aid in the activation of T cells. PMA stimulation of T cells has been shown to up-regulate c-jun and c-fos (Kontny et al., 1992). However, PMA incubation with an elevation in cyclic AMP only up-regulated c-fos, and decreased c-jun, thus elevating AP-1 activity (Kontny et al., 1992). This is interesting when one considers that upon ligation of the TCR/CD3 complex in murine thymocytes an elevation in cyclic AMP levels was observed (see section 4.3).

It is believed that there are two signalling pathways which are activated from the TCR: one mediated by PLC-γ1 and the other mediated by p21ws ((Izquierdo et al., 1992) and see section 1.2.4). PKC activation via phorbol ester or DAG has also been demonstrated to activate raf-1 (Sozeri et al., 1992). The expression of AP-1 driven promoters and genes is thought to require raf-1 kinase activity (Bruder
et al., 1992) and may indicate the existence of a protein kinase cascade consisting of PKC isoenzymes and the Raf-1 kinase during T cell activation.

It is interesting to note that the PTK inhibitor genistein abrogated PDE4 activation, as it is thought that a PTK-mediated event is responsible for activating PLC-γ1, resulting in the activation of PKC via DAG (Secrist et al., 1991; Weiss et al., 1991). However, recent studies indicate that this is not the only pathway responsible for activating PKC upon stimulation of thymocytes with anti-CD3 antibodies. Indeed, there appears to be a stimulation of phospholipase D activity which may cause the activation of PKC via phosphatidic acid or DAG derived from phosphatidic acid (P. Reid and M. M. Harnett, personal communication). Anti-CD3 antibodies have also been shown to activate PLA_2 activity which is responsible for the generation of arachidonic acid (J. J. Gilbert and M. M. Harnett, personal communication). Arachidonic acid is known to stimulate PKC-ζ (Nakanishi and Ezton, 1992). In light of this, it was interesting to note that the PKC inhibitor chelerythrine was more effective at inhibiting the elevation of PDE4 activity than genistein, indicating that PDE4 may be activated by a mechanism which involves PKC which has been stimulated via different pathways.

5.3.2. Regulation of cyclic AMP PDEs.

PDE4D activities have been shown to be activated on stimulation with thyroid-stimulating hormone (Sette et al., 1994a) via an increase in cyclic AMP levels in thyroid cells. This investigation was interesting, as it demonstrated that splice variants from the same locus could be differentially regulated on stimulation of the cell by a particular hormone (Sette et al., 1994b). Differential regulation led to short-term activation of PDE4D3.3, via changes in phosphorylation status, and long-term regulation by stimulating de novo protein synthesis of two other splice variants PDE4D3.1 and PDE4D3.2. An elevation of cyclic AMP has also been shown to cause an increase in ratPDE3 (PDE4D) expression in immature Sertoli cells on stimulation with follicle stimulating hormone (FSH) (Monaco et al., 1994;
Sette et al., 1994b; Swinnen et al., 1991b) and in cardiac myoblasts (Kovala et al., 1994). In these cases, cyclic AMP has also been shown to play a role in the regulation of its own degradative enzyme, via PKA. PDE4D has also been induced in Jurkat cells upon incubation of these cells with a cyclic AMP analog (Engels et al., 1994). An interesting point to note about that study was the strong mRNA signal that was observed in unstimulated Jurkat cells for PDE4A. In chapter 4 it was demonstrated that the activity of PDE4 was extremely low in unstimulated cells, but that the proportion of activity increased within 20 minutes of stimulation with cyclic AMP. It may be the case that murine thymocytes have a high concentration of PDE4 mRNA. This could at least partially explain the rapid onset of PDE4 induction in these cells. However actinomycin D blocked the induction of PDE4 activity, therefore indicating that RNA synthesis is also necessary for the rise in PDE activity. It is not known which PDE4 is induced in murine thymocytes, although studies are currently underway in the laboratory to investigate this further with the use of PCR amplification and Western blotting techniques. These investigations will, hopefully, identify which PDE4(s) are present in murine thymocytes, as well as shedding light on the PDE4 species being rapidly induced upon stimulation with anti-CD3 antibodies and PMA.

The situation presented above represents a connection between lipid and cyclic AMP signalling within murine thymocytes. The complex family of PDEs lends itself to the ability to partake in the interactions between signalling pathways, due to its ability to be regulated by a number of different effectors (see section 1.5.7) and (Beavo, 1990; Bolger, 1994; Conti et al., 1995b; Houslay and Kilgour, 1990; Manganiello et al., 1990a)). This, coupled with the enormous capacity for differential regulation of adenylyl cyclase (Chen and Iyengar, 1993; Krupinski et al., 1992), demonstrates the immense opportunity for dynamic control of a vast network of intracellular signals. The action of PKC has been shown to regulate a number of elements of the cyclic AMP signalling pathway (Houslay, 1991), leading to an inhibition (Beckner and Farrar, 1986; Kelleher et al., 1984) or an
enhancement (Cronin et al., 1986; Yoshimasa et al., 1987) of agonist induced increases in cyclic AMP, depending on the cell type. The regulation of cyclic AMP signalling by PKC can occur at various different stages of the signalling pathway, such as the modification of stimulatory and inhibitory receptors coupled to adenylyl cyclase such as the β2-adrenoceptor (Bouvier et al., 1987), the α2-adrenoceptor (Convents et al., 1989), and the inhibitory G protein (Bell and Brunton, 1986; Morris et al., 1994; O'Brien et al., 1987). Adenylyl cyclase has been shown to be modulated by PKC phosphorylation recently in intact cells (Jacobowitz and Iyengar, 1994; Kawabe et al., 1994; Williams et al., 1987), as well as in purified cell membranes (Nagshineh et al., 1986; Pyne et al., 1994). Moreover, differing sensitivities of the cells may occur due to the different complement of PKC and adenylyl cyclase/PDE isoforms which are expressed within the cell types. It has recently been established that PKC-ζ and PKC-α selectively activated type-V adenylyl cyclase (Kawabe et al., 1994). Cyclic AMP PDE activities have been shown to be influenced dramatically by treatment with phorbol esters in hepatocytes. For example, glucagon-treated cells become sensitised to cyclic AMP elevation upon addition of PMA, resulting from an inhibition of PDE activity (Irvine et al., 1986).

Therefore, it is apparent that upon ligation of the TCR/CD3 complex in murine thymocytes, there is a rapid induction of a PDE4 activity. The activation of this PDE activity appears to be mediated by a PKC-dependent process, as PMA activates the activity in a fashion similar to that of anti-CD3 stimulation. Furthermore, PTK activity(s) also appears to play a role in the activation of PDE4. An early event in T cell activation is the stimulation of PKC-α, and it may be this isoform that is responsible for the induction of PDE activity within these cells. Overexpression of this PKC isoform, and PKC-ε, have been shown to induce PDE1 activity previously (Spence et al., 1995). In this regard, it is interesting to note that PKC-ε has been found to play an active role during thymocyte activation by PMA (Strulovici et al., 1991).
Table 5.1. Summary of IC₅₀ values of protein kinase inhibitors.

The potency and selectivity of protein kinase inhibitors is compared. The values are expressed as IC₅₀, i.e. the concentration of the inhibitor at which there is half maximal inhibition.

a Herbert et al., 1990, b Kiss and Deli, 1992,
c Schachtele et al., 1988, d Tischler et al., 1990,
e Tamaoki et al., 1986, f Yanagihara et al., 1991,
s Girault, 1994, h Chijiwa et al., 1990,
i Akiyama et al., 1987, j Gazit et al., 1989,
k Lyall et al., 1989, l Margolis et al., 1989,
m Bilder, 1991.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Protein kinase C</th>
<th>Protein kinase A</th>
<th>Protein kinase G</th>
<th>Myosin light chain kinase</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;/CaM dependent kinase</th>
<th>EGF receptor kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chelerythrine</td>
<td>0.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>170&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>&gt; 100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>0.0007&lt;sup&gt;b,c,d,e,g&lt;/sup&gt;</td>
<td>0.007&lt;sup&gt;c,d,e,g&lt;/sup&gt;</td>
<td>0.0085&lt;sup&gt;c,d,e,g&lt;/sup&gt;</td>
<td>0.0013&lt;sup&gt;c,d,e,g&lt;/sup&gt;</td>
<td>0.02&lt;sup&gt;c,d,e,g&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>H89</td>
<td>31.7&lt;sup&gt;a,h&lt;/sup&gt;</td>
<td>0.048&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.48&lt;sup&gt;h&lt;/sup&gt;</td>
<td>28.3&lt;sup&gt;h&lt;/sup&gt;</td>
<td>29.7&lt;sup&gt;h&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Genistein</td>
<td>15&lt;sup&gt;i&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.6&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tyrophostin A1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&gt; 400&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tyrophostin A25</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>15&lt;sup&gt;i,k,l,m&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 5.2. PDE4 activity upon stimulation of murine thymocytes with anti-CD3 monoclonal antibodies, in the presence of protein kinase inhibitor, staurosporine.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) for 30 minutes, in the presence of 100nM staurosporine. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). PDE activities were assessed with 1μM cyclic AMP substrate. The tables show a) total PDE activity as a percentage of the basal, and b) the fraction of activity inhibited by 10μM rolipram, expressed as a percentage of the PDE4 basal activity. These experiments were carried out on a ‘mixed’ population of cells as described in ‘Materials and Methods.’ The data shown is an average of three separate experiments. Individual assays were carried out in triplicate (errors are SEM). Percentages are calculated as the average of percentage stimulation from each separate experiment with basal PDE activity representing 100%, assayed with 1μM cyclic AMP.
<table>
<thead>
<tr>
<th>Total PDE activity</th>
<th>anti-CD3 %age zero activity</th>
<th>+ Staurosporine %age zero activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 +/- 5</td>
<td>100 +/- 6</td>
</tr>
<tr>
<td>30</td>
<td>150 +/- 6</td>
<td>104 +/- 9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PDE4 activity</th>
<th>anti-CD3 %age zero activity</th>
<th>+ Staurosporine %age zero activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 +/- 5</td>
<td>100 +/- 7</td>
</tr>
<tr>
<td>30</td>
<td>184 +/- 7</td>
<td>94 +/- 10</td>
</tr>
</tbody>
</table>
Table 5.3. PDE2 activity upon stimulation with anti-CD3 antibodies and/or PMA in the presence of various inhibitors.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) for 30 minutes, in the presence of various inhibitors. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). PDE activities were assessed with 1μM cyclic AMP substrate in the presence of stimulatory concentrations of cyclic GMP (10μM), and the magnitude of the increase in PDE activity elicited by cyclic GMP is shown. This is taken to serve as an index of PDE2 activity. In this regard it must be taken as an underestimate as it does not take into account the basal activity of the enzyme. However, given the magnitude of the stimulation by cyclic GMP, then such a basal component is likely to be small. This approach has the advantage in that it solely represents PDE2 activity. Inhibitors used are chelerythrine (10μM), staurosporine (100nM), PMA (100nM), okadaic acid (100nM), H89 (200nM), Genistein (10μM and 100μM), tyrophostin 1 (100μM) and tyrophostin 25 (100μM), actinomycin D (100μg/ml) and cyclohexamide (1μM). These experiments were carried out on a ‘mixed’ population of cells as described in ‘Materials and Methods.’ The data shown is an average of three separate experiments. Individual assays were carried out in triplicate (errors are SEM). Percentages are calculated as the average of percentage stimulation from each separate experiment with basal PDE activity representing 100%, assayed with 1μM cyclic AMP.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>0min. (100%)</th>
<th>30min. %age of 0min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-CD3 antibodies</td>
<td>100 +/- 5</td>
<td>90 +/- 4</td>
</tr>
<tr>
<td>Chelerythrine + anti-CD3</td>
<td>100 +/- 10</td>
<td>94 +/- 3</td>
</tr>
<tr>
<td>Stauroporine + anti-CD3</td>
<td>100 +/- 9</td>
<td>103 +/- 1</td>
</tr>
<tr>
<td>H89 + anti-CD3 antibodies</td>
<td>100 +/- 5</td>
<td>94 +/- 8</td>
</tr>
<tr>
<td>PMA</td>
<td>100 +/- 8</td>
<td>104 +/- 1</td>
</tr>
<tr>
<td>PMA + anti-CD3 antibodies</td>
<td>100 +/- 1</td>
<td>96 +/- 7</td>
</tr>
<tr>
<td>Okadaic acid + anti-CD3</td>
<td>100 +/- 8</td>
<td>89 +/- 3</td>
</tr>
<tr>
<td>Genistein (10μM) + anti-CD3</td>
<td>100 +/- 14</td>
<td>81 +/- 7</td>
</tr>
<tr>
<td>Genistein (100μM) + anti-CD3</td>
<td>100 +/- 13</td>
<td>78 +/- 7</td>
</tr>
<tr>
<td>Tyrophostin A1 + anti-CD3</td>
<td>100 +/- 9</td>
<td>109 +/- 2</td>
</tr>
<tr>
<td>Tyrophostin A25 + anti-CD3</td>
<td>100 +/- 13</td>
<td>106 +/- 10</td>
</tr>
<tr>
<td>Actinomycin D + anti-CD3</td>
<td>100 +/- 11</td>
<td>81 +/- 3</td>
</tr>
<tr>
<td>Actinomycin D + PMA</td>
<td>100 +/- 15</td>
<td>96 +/- 2</td>
</tr>
<tr>
<td>Actinomycin D + anti-CD3</td>
<td>100 +/- 13</td>
<td>102 +/- 22</td>
</tr>
<tr>
<td>Cyclohexamide + anti-CD3</td>
<td>100 +/- 8</td>
<td>110 +/- 12</td>
</tr>
<tr>
<td>Cyclohexamide + PMA</td>
<td>100 +/- 11</td>
<td>80 +/- 5</td>
</tr>
<tr>
<td>Cyclohexamide + anti-CD3</td>
<td>100 +/- 12</td>
<td>127 +/- 22</td>
</tr>
</tbody>
</table>
Table 5.4. PDE4 activities upon incubation with inhibitors alone.

PDE4 activities were assessed upon incubation of murine thymocytes with various inhibitors for 30 minutes, at 1μM cyclic AMP substrate. Inhibitors used are chelerythrine (10μM), staurosporine (100nM), PMA (100nM), okadaic acid (100nM), H89 (200nM), Genistein (10μM and 100μM), tyrophostin A1 (100μM) and tyrophostin A25 (100μM), actinomycin D (100μg/ml) and cyclohexamide (1μM).

These experiments were carried out on a 'mixed' population of cells as described in 'Materials and Methods.' The data shown is an average of three separate experiments. Individual assays were carried out in triplicate (errors are SEM). Percentages are calculated as the average of percentage stimulation from each separate experiment with basal PDE activity representing 100%, assayed with 1μM cyclic AMP.
<table>
<thead>
<tr>
<th>PDE4 activity -anti-CD3 antibodies</th>
<th>0min. 100%</th>
<th>30min. % of 0min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation</td>
<td>100 +/- 10</td>
<td>117 +/- 5</td>
</tr>
<tr>
<td>Chelerythrine</td>
<td>100 +/- 9</td>
<td>91 +/- 4</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>100 +/- 5</td>
<td>116 +/- 11</td>
</tr>
<tr>
<td>H89</td>
<td>100 +/- 10</td>
<td>100 +/- 6</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>100 +/- 12</td>
<td>119 +/- 14</td>
</tr>
<tr>
<td>Genistein (10μM)</td>
<td>100 +/- 8</td>
<td>94 +/- 3</td>
</tr>
<tr>
<td>Genistein (100μM)</td>
<td>100 +/- 10</td>
<td>95 +/- 7</td>
</tr>
<tr>
<td>Tyrophostin A1</td>
<td>100 +/- 9</td>
<td>86 +/- 7</td>
</tr>
<tr>
<td>Tyrophostin A25</td>
<td>100 +/- 7</td>
<td>77 +/- 5</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>100 +/- 15</td>
<td>93 +/- 2</td>
</tr>
<tr>
<td>Cyclohexamide</td>
<td>100 +/- 4</td>
<td>89 +/- 9</td>
</tr>
</tbody>
</table>
Table 5.5. PDE2 activities upon incubation with inhibitors alone.

PDE2 activities were assessed upon incubation of murine thymocytes with various inhibitors for 30 minutes, at 1μM cyclic AMP substrate in the presence of stimulatory concentrations of cyclic GMP (10μM). Inhibitors used are chelerythrine (10μM), staurosporine (100nM), PMA (100nM), okadaic acid (100nM), H89 (200nM), Genistein (10μM and 100μM), tyrophostin A1 (100μM) and tyrophostin A25 (100μM), actinomycin D (100μg/ml) and cyclohexamide (1μM). These experiments were carried out on a ‘mixed’ population of cells as described in ‘Materials and Methods.’ The data shown is an average of three separate experiments. Individual assays were carried out in triplicate (errors are SEM). Percentages are calculated as the average of percentage stimulation from each separate experiment with basal PDE activity representing 100%, assayed with 1μM cyclic AMP.
<table>
<thead>
<tr>
<th>PDE2 activity - anti-CD3 antibodies</th>
<th>0min. (100%)</th>
<th>30min. %age of 0min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation</td>
<td>100 +/- 5</td>
<td>105 +/- 9</td>
</tr>
<tr>
<td>Chelerythrine</td>
<td>100 +/- 10</td>
<td>92 +/- 10</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>100 +/- 9</td>
<td>94 +/- 1</td>
</tr>
<tr>
<td>H89</td>
<td>100 +/- 5</td>
<td>100 +/- 10</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>100 +/- 8</td>
<td>107 +/- 2</td>
</tr>
<tr>
<td>Genistein (10μM)</td>
<td>100 +/- 14</td>
<td>91 +/- 9</td>
</tr>
<tr>
<td>Genistein (100μM)</td>
<td>100 +/- 13</td>
<td>96 +/- 9</td>
</tr>
<tr>
<td>Tyrophostin A1</td>
<td>100 +/- 9</td>
<td>84 +/- 7</td>
</tr>
<tr>
<td>Tyrophostin A25</td>
<td>100 +/- 13</td>
<td>93 +/- 9</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>100 +/- 11</td>
<td>97 +/- 2</td>
</tr>
<tr>
<td>Cyclohexamide</td>
<td>100 +/- 8</td>
<td>107 +/- 8</td>
</tr>
</tbody>
</table>
Table 5.6. PDE4 activity upon stimulation of murine thymocytes with anti-CD3 monoclonal antibodies, in the presence of PTK inhibitors.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) for 30 minutes, after a pretreatment of 1 hour with 100μM tyrophostin 1 and tyrophostin 25, and genistein (10μM and 100μM). The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). PDE activities were assessed with 1μM cyclic AMP substrate. The tables show a) total PDE activity as a percentage of the basal, and b) the fraction of activity inhibited by 10μM rolipram, expressed as a percentage of the PDE4 basal activity. These experiments were carried out on a ‘mixed’ population of cells as described in ‘Materials and Methods.’ The data shown is an average of three separate experiments. Individual assays were carried out in triplicate (errors are SEM). Percentages are calculated as the average of percentage stimulation from each separate experiment with basal PDE activity representing 100%, assayed with 1μM cyclic AMP.
<table>
<thead>
<tr>
<th>Table A</th>
<th>Total PDE activity</th>
<th>anti-CD3 antibodies</th>
<th>+10µM genistein</th>
<th>+100µM genistein</th>
<th>+tyrophostin A1</th>
<th>+tyrophostin A25</th>
</tr>
</thead>
<tbody>
<tr>
<td>0min.</td>
<td></td>
<td>100 +/- 5</td>
<td>100 +/- 14</td>
<td>100 +/- 11</td>
<td>100 +/- 1</td>
<td>100 +/- 3</td>
</tr>
<tr>
<td>30min.</td>
<td></td>
<td>150 +/- 6</td>
<td>113 +/- 6</td>
<td>85 +/- 1</td>
<td>127 +/- 14</td>
<td>119 +/- 4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table B</th>
<th>PDE4 activity</th>
<th>anti-CD3</th>
<th>+10µM genistein</th>
<th>+100µM genistein</th>
<th>+tyrophostin A1</th>
<th>+tyrophostin A25</th>
</tr>
</thead>
<tbody>
<tr>
<td>0min.</td>
<td></td>
<td>100 +/- 5</td>
<td>100 +/- 8</td>
<td>100 +/- 10</td>
<td>100 +/- 9</td>
<td>100 +/- 7</td>
</tr>
<tr>
<td>30min.</td>
<td></td>
<td>184 +/- 7</td>
<td>151 +/- 10</td>
<td>104 +/- 3</td>
<td>154 +/- 8</td>
<td>150 +/- 4</td>
</tr>
</tbody>
</table>
Figure 5.1. Structures of the serine/threonine protein kinase inhibitors chelerythrine, staurosporine and H89.
Chelerythrine Chloride

Staurosporine

H89 Dihydrochloride
Figure 5.2. Structures of the protein tyrosine kinase inhibitors genistein, tyrophostin A1 and tyrophostin A25.
Figure 5.3. Structure of the protein phosphatase inhibitor okadaic acid.
Figure 5.4. PDE4 activity in murine thymocytes upon stimulation with anti-CD3 monoclonal antibodies.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) for up to 30 minutes as indicated. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). The total PDE activity observed in the presence of 1μM cyclic AMP as substrate (circles) is shown. Also given is that fraction of activity (squares) which is inhibited by 10μM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity. These experiments were carried out on a 'mixed' population of cells as described in 'Materials and Methods.' These data show results typical of experiments carried out five times. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 5.5. PDE2 activity in murine thymocytes upon challenge with anti-CD3 monoclonal antibodies.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) for up to 30 minutes as indicated. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). Shown is the total PDE activity (diamonds) observed in the presence of stimulatory concentrations of cyclic GMP (10μM) and 1μM cyclic AMP as substrate. Also given is the magnitude of the increase in PDE activity elicited by cyclic GMP (triangles); this is taken to serve as an index of PDE2 activity. In this regard it must be taken as an underestimate as it does not take into account the basal activity of the enzyme. However, given the magnitude of the stimulation by cyclic GMP, then such a basal component is likely to be small. This approach has the advantage in that it solely represents PDE2 activity. These experiments were carried out on a ‘mixed’ population of cells as described in ‘Materials and Methods.’ These data show results typical of experiments carried out at least four times. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 5.6. PDE4 activity in murine thymocytes upon stimulation with anti-CD3 monoclonal antibodies in the presence of PKC inhibitor, chelerythrine.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) for up to 30 minutes as indicated, in the presence of 10μM chelerythrine. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). The graphs show: a) the total PDE activity observed in the presence of 1μM cyclic AMP as substrate (inverted triangles), compared with that fraction of activity (squares) which is inhibited by 10μM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity.

b) This graph compares the PDE activity upon stimulation of the cells with anti-CD3 antibodies in the absence (circles) and presence (inverted triangles) of chelerythrine. These experiments were carried out on a 'mixed' population of cells as described in 'Materials and Methods.' These data show results typical of experiments carried out five times. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 5.7. PDE4 activity in murine thymocytes upon stimulation with PKC activator, PMA.

Thymocytes were stimulated with 100nM PMA. The graphs show: a) the total PDE activity observed in the presence of 1μM cyclic AMP as substrate (inverted triangles), compared with that fraction of activity (squares) which is inhibited by 10μM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity. b) This graph compares the PDE activity upon stimulation of the cells with anti-CD3 antibodies (circles) or PMA (inverted triangles). These experiments were carried out on a 'mixed' population of cells as described in 'Materials and Methods.' These data show results typical of experiments carried out four times. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 5.8. PDE4 activity in murine thymocytes upon stimulation with anti-CD3 monoclonal antibodies in the presence of PKC activator, PMA.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) for up to 30 minutes as indicated, in the presence of 100nM PMA. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). The graphs show; a) the total PDE activity observed in the presence of 1μM cyclic AMP as substrate (inverted triangles), compared with that fraction of activity (squares) which is inhibited by 10μM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity. b) This graph compares the PDE activity upon stimulation of the cells with anti-CD3 antibodies in the absence (circles) and presence (inverted triangles) of PMA. These experiments were carried out on a 'mixed' population of cells as described in 'Materials and Methods.' These data show results typical of experiments carried out four times. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 5.9. PDE4 activity in murine thymocytes upon stimulation with anti-CD3 monoclonal antibodies in the presence of PKA inhibitor, H89.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) for up to 30 minutes as indicated, in the presence of 200nM H89. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). The graphs show: a) the total PDE activity observed in the presence of 1μM cyclic AMP as substrate (inverted triangles), compared with that fraction of activity (squares) which is inhibited by 10μM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity. b) This graph compares the PDE activity upon stimulation of the cells with anti-CD3 antibodies in the absence (circles) and presence (inverted triangles) of H89. These experiments were carried out on a ‘mixed’ population of cells as described in ‘Materials and Methods.’ These data show results typical of experiments carried out three times. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 5.10. PDE4 activity in murine thymocytes upon stimulation with anti-CD3 monoclonal antibodies in the presence of PPT inhibitor, okadaic acid.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) for up to 30 minutes as indicated, in the presence of 100nM okadaic acid. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). The graphs show; a) the total PDE activity observed in the presence of 1μM cyclic AMP as substrate (inverted triangles), compared with that fraction of activity (squares) which is inhibited by 10μM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity. b) This graph compares the PDE activity upon stimulation of the cells with anti-CD3 antibodies in the absence (circles) and presence (inverted triangles) of okadaic acid. These experiments were carried out on a 'mixed' population of cells as described in 'Materials and Methods.' These data show results typical of experiments carried out three times. Assays were carried out in triplicate (errors are SEM). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 5.11. PDE4 activity in murine thymocytes upon stimulation with anti-CD3 monoclonal antibodies in the presence of RNA synthesis inhibitor, actinomycin D.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5µg/ml) for up to 30 minutes as indicated, after a preincubation of 45 minutes in the presence of 100µg/ml actinomycin D. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). The graphs show: a) the total PDE activity observed in the presence of 1µM cyclic AMP as substrate (filled), compared with that fraction of activity (hatched) which is inhibited by 10µM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity. b) This graph compares the PDE activity upon stimulation of the cells with anti-CD3 antibodies in the absence (spots) and presence (filled) of actinomycin D. These experiments were carried out on a 'mixed' population of cells as described in 'Materials and Methods.' These data show results typical of experiments carried out three times. Assays were carried out in duplicate (range >12%). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 5.12. PDE4 activity in murine thymocytes upon stimulation with PKC activator, PMA, in the presence of actinomycin D.

Thymocytes were stimulated with 100nM PMA in the presence of 100μg/ml actinomycin D. The graphs show; a) the total PDE activity observed in the presence of 1μM cyclic AMP as substrate (filled), compared with that fraction of activity (hatched) which is inhibited by 10μM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity. b) This graph compares the PDE activity upon stimulation of the cells PMA in the absence (spots) or presence (filled) of actinomycin D. These experiments were carried out on a 'mixed' population of cells as described in 'Materials and Methods.' These data show results typical of experiments carried out three times. Assays were carried out in duplicate (range >12%). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 5.13. PDE4 activity in murine thymocytes upon stimulation with anti-CD3 monoclonal antibodies in the presence of PMA and actinomycin D.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5μg/ml) and PMA (100nM) for up to 30 minutes as indicated, after a preincubation of 45 minutes in the presence of 100μg/ml actinomycin D. The antibody used was directed against the ε chain of the CD3 complex (Phillips et al. 1991). The graphs show: a) the total PDE activity observed in the presence of 1μM cyclic AMP as substrate (filled), compared with that fraction of activity (hatched) which is inhibited by 10μM rolipram. This is not the residual rolipram-insensitive fraction, but the fraction of PDE activity which was inhibited by rolipram and, as such, can be taken as an index of PDE4 activity.
b) This graph compares the PDE activity upon stimulation of the cells with anti-CD3 antibodies and PMA in the absence (spots) and presence (filled) of actinomycin D. These experiments were carried out on a ‘mixed’ population of cells as described in ‘Materials and Methods.’ These data show results typical of experiments carried out three times. Assays were carried out in duplicate (range >11%). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
Figure 5.14. Cyclic AMP PDE activity in murine thymocytes upon stimulation with anti-CD3 monoclonal antibodies and/or PMA in the presence of protein synthesis inhibitor, cyclohexamide.

Thymocytes were stimulated with anti-CD3 monoclonal antibodies (5µg/ml) and/or PMA (100nM) for up to 30 minutes as indicated, after a preincubation of 45 minutes in the presence of 1µM cyclohexamide. The antibody used was directed against the ε chain of the CD3 complex (Philips et al. 1991). The graphs show the total PDE activity observed with 1µM cyclic AMP as substrate in the presence (filled) and absence (spots) of 1µM cyclohexamide. Graphs:

a) thymocytes are stimulated with anti-CD3 antibodies; b) thymocytes are stimulated with PMA; and c) thymocytes are stimulated with both anti-CD3 antibodies and PMA. These experiments were carried out on a ‘mixed’ population of cells as described in ‘Materials and Methods.’ These data show results typical of experiments carried out three times. Assays were carried out in duplicate (range >15%). PDE activity is expressed as pmols cyclic AMP hydrolysed/min/mg protein.
CHAPTER 6.

DISCUSSION.
6. Discussion

This study has illuminated some exciting points regarding the expression and regulation of cyclic AMP PDEs in T cells from different sources. It has been determined that: (i) the profile of cyclic AMP PDE isoforms varies between different species, (ii) differential regulation of cyclic AMP PDE activities and cyclic AMP levels occur upon stimulation of murine thymocytes with the TCR ligating mitogenic lectin, PHA or anti-CD3/anti-TCR antibodies, (iii) anti-CD3 stimulated changes in PDE4 activity appear to be mediated by a PKC-dependent pathway, involving rapid induction of a PDE4 activity.

6.1. Implications of different PDE activities in T cells.

Ca\(^{2+}\)/CaM-stimulated, PDE1 activity has not been found to be present in either primary murine lymphocytes, thymocytes or human tonsillar T cells. This finding is supported by other investigations which indicate the absence of type-I PDE activity in human and bovine peripheral blood lymphocytes (Hurwitz et al., 1990; Robicsek et al., 1989; Thompson et al., 1976), rat thymocytes (Marcoz et al., 1993) or rat splenic lymphocytes (Hait and Weiss, 1976). However this activity does appear to be present in Jurkat cells and other proliferating lymphoid cell lines (Epstein et al., 1987). Furthermore, expression can be induced by mitogenic stimulation of primary T cells (Hurwitz et al., 1990). This indicated that Ca\(^{2+}\)/CaM-stimulated, PDE1 activity may have a role in proliferating cells. These studies demonstrate a possible regulatory interface for the metabolism of calcium and cyclic nucleotides during lymphocyte mitogenesis.

It has been demonstrated that cyclic GMP-stimulated, PDE2 activities exist in primary murine thymocytes, T3.2 thymoma cells and the human T cell line, Jurkat. From the studies presented above and others (Franks and MacManus, 1971; Meskini et al., 1992; Robicsek et al., 1989; Valette et al., 1990) it is clear that cyclic GMP may play a very influential role in the regulation of hydrolysis of cyclic AMP. In murine thymocytes it is evident that upon increasing cyclic GMP levels
there is an enormous increase in cyclic AMP breakdown. In future studies, it would intriguing to study the effects of increasing cyclic GMP concentrations with the use of sodium nitroprusside, an activator of guanylyl cyclase activity, or cyclic GMP analogues on cyclic AMP levels and PDE activities within the murine thymocytes. It has been previously found in studies on rat thymocytes, that inhibiting PDE3 in conjunction with PDE4 had anti-proliferative effects on the proliferation of rat thymocytes (Marcoz et al., 1993). This effect was mirrored by inhibiting the PDE4 activity and elevating cyclic GMP with sodium nitroprusside, indicating that cyclic GMP has a role as a negative effector in lymphocyte mitogenic response. However the effect was not seen when 8-bromo-cyclic GMP, a potent activator of PKG, was used to elevate cyclic GMP levels. Due to the predominance of PDE2 activity in murine thymocytes, and the apparent lack of PDE3 activity, it would be interesting to investigate PDE2 function in relation to proliferation within murine thymic organ cultures. These studies may illuminate key differences in the control of cyclic AMP homeostasis between murine and rat models.

As eluded to above, rat thymocytes have been shown to contain both cyclic GMP-inhibited and cyclic GMP-stimulated PDE activities (Franks and MacManus, 1971; Valette et al., 1990), implying that there is a complex control of cyclic GMP regulation of cyclic AMP PDE activities. The investigations here suggest an absence of cyclic GMP-inhibited PDE3 activity in murine thymocytes, demonstrating that there are species specific differences in PDE expression. In systems where both cyclic GMP-stimulated, and cyclic GMP-inhibited PDE isoforms exist, differential regulation of these PDEs may occur with differing levels of cyclic GMP. At low concentrations of cyclic AMP, the cyclic GMP-inhibited PDE activity would predominate whereas, if levels were to increase, cyclic GMP stimulated PDE activity would be activated in the presence of cyclic GMP (Franks and MacManus, 1971).

T3.2 thymoma cells give an interesting comparison to murine thymocytes, as the predominant PDE activity in this murine cell line is not the cyclic GMP
stimulated PDE. Indeed these cells appear to express a cyclic GMP-inhibited PDE3 activity and therefore appear to be more similar to rat thymocytes (Valette et al., 1990), Jurkat cells and primary human lymphocytes (Meskini et al., 1992; Robicsek et al., 1991), with regard to the complement of cyclic AMP PDEs expressed. This difference may relate to the prominence of a particular developmental stage within the cell line, in contrast to thymocyte preparations which are made up of cells from several different maturation stages. Unlike Jurkat cells where PDE3 activity is dominant at basal levels, PDE4 is the predominant activity in T3.2 cells. This implies that in the absence (or at sub-micromolar concentrations) of cyclic GMP, T3.2 cells will behave in a similar manner to that of primary murine thymocytes in a similar situation with regard to PDE activities. The situation changes dramatically however, upon introduction of cyclic GMP. It is likely that cyclic AMP catabolism would increase dramatically within primary cells, but would not be affected considerably in the proliferating cell line. It would be of interest, therefore, to study the effects of cyclic GMP and cyclic AMP elevating agents on this cell line in parallel with studies carried out on thymic cultures. This would be particularly exciting, as it is known that upon stimulation of these cells with Con A, PHA and ionomycin there is an induction of apoptosis. However, this does not occur upon stimulation with PMA or anti-CD3 antibodies, unless the cells are sufficiently cross-linked (R. Zamoyska personal communication). This points to a difference in signalling networks between this cell line and primary thymocytes (Shi et al., 1991; Smith et al., 1989; Taylor et al., 1984).

Nitric oxide may have an important influence on cyclic AMP concentrations within cells, as it has been shown to elevate cyclic GMP levels within and between cells upon stimulation of soluble guanylyl cyclase in the brain. The generation of NO could be important in controlling apoptosis (Genaro et al., 1995), which is particularly relevant in the thymic environment, where 95% of developing T cells apoptose, with the remaining 5% entering the periphery (Hueber et al., 1994; Kaye and Ellenberger, 1992). It would therefore be of interest to study the effect of nitric
oxide on cyclic AMP metabolism, particularly in T3.2 cells, in order to identify a possible link between nitric oxide and T cell apoptosis.

PDE4 activities appeared to be apparent in all of the cells looked at in this study, dominating the PDE activities of murine thymocytes and T3.2 thymomas in the absence of cyclic GMP. Thus indicating the importance of this isozyme in the homeostasis of cyclic AMP in lymphoid cells. However, in Jurkat cells there appeared to be an IBMX insensitive activity present also. Without further analysis of these cells it cannot be defined as a PDE7 activity, although it is interesting to note that Ichimura et al. (1993) have reported a high affinity PDE activity in Jurkat cells, and other T cell lines that is insensitive to cyclic GMP and rolipram. This activity is absent from B cell lines, illustrating a lineage-specific difference in PDE expression in cells of lymphoid origin.

It was rather surprising to find that ‘in vivo-activated’ and ‘resting’ populations of thymocytes have a similar basal PDE activity in the absence and presence of stimulatory concentrations of cyclic GMP. This was not found to be the case with murine lymphocytes, where the activated populations of cells were seen to have higher PDE activities. This is consistent with studies carried out on leukemic cells, where PDE activities are seen to be enormously elevated in comparison to normal peripheral blood lymphocytes (Epstein and Hachisu, 1984; Epstein et al., 1977; Hait and Weiss, 1976). A possible reason for the difference between thymocytes and lymphocytes could be that ‘in vivo-activated’ thymocytes isolated on the basis of their buoyant density may reflect a population of ‘activated thymocytes’ that are undergoing activation-induced cell death, rather than proliferation, thus having unaltered PDE activities.

The pattern of PDE expression and activities have been observed to undergo profound changes after stimulation and during cell differentiation in cells such as Sertoli cells (Conti et al., 1982; Swinnen et al., 1989b), 3T3-L1 cells (Manganiello et al., 1983), fibroblasts (Manganiello and Vaughan, 1972) and lymphoma cells (Bourne et al., 1974). This indicates that changes in the pattern of cyclic AMP
degradation may be an integral part of the cells long term response to stimulation and possibly of the differentiating process. Indeed, PDE activity has also been seen to be elevated in Jurkat cells after stimulation with cyclic AMP elevating agents are applied for three hours (S. Erdogan, personal communication).

6.2. Cyclic AMP PDE activities on stimulation with TCR ligating agents.

Cyclic AMP PDE activities are differentially regulated upon stimulation of thymocytes with PHA or with monoclonal antibodies directed against the TCR/CD3 complex. One of the main reasons for this could be the complement of cell surface receptors which are being ligated by these agents. PHA is known to coligate CD2 and the TCR/CD3 complex (O'Flynn et al., 1985), therefore it would be interesting to try ligating CD2 alone with antibodies raised against specific epitopes of this molecule, to ascertain the effects on PDE activities. There is a range of anti-CD2 monoclonal antibodies available, whose effects are dependent on the pairing of antibodies used, from unresponsiveness to proliferation (Yang et al., 1986).

Another reason for the differing effects of these agents may be that CD2 is present at the early stages of thymocyte differentiation, being expressed on 85-95% of all developing T cells (Fox et al., 1985; Lanier et al., 1986; Sewell et al., 1986). This suggests that a greater proportion of the cells will be ligated by PHA than with monoclonal antibodies directed against the TCR. Anti-CD3/anti-TCR antibodies will target the more mature populations of cells, thus opening the possibility of different signalling. Cyclic AMP has been implicated in early thymocyte differentiation (Bach et al., 1975; Scheid et al., 1975) and stimulation of thymic apoptosis (Kaye and Ellenberger, 1992; McConkey et al., 1990a); two very divergent physiological events. This indicates that at different developmental stages, cyclic AMP signalling mediates a different end result. In order to study this, it may be possible to study sub-populations of cells within the thymus, in order to ascertain how the PDE activities are regulated following stimulation with...
PHA. This would have the advantage of studying a more homogeneous population of cells. It was hoped that these studies could be carried out on the T3.2 thymoma cell line, but the PDE activities expressed within the cells meant that comparisons between this cell system and primary thymocytes may be difficult.

In these studies it was interesting that upon ligation of TCR/CD3 complex there was a 2-fold increase in PDE4 activity within 20 minutes of incubation with anti-CD3/anti-TCR antibodies. This was accompanied by a rise in cyclic AMP concentration. It is known that ligating CD3 in thymocytes leads to apoptosis \textit{in vitro} (Smith et al., 1989) and \textit{in vivo} (Shi \textit{et al.}, 1991), whereas thymocytes respond mitogenically to co-stimulation with lectins and PMA (Taylor \textit{et al.}, 1984). Indeed, Con A has been found to cause proliferation of rat thymocytes, with PDE4 being essential for this mitogenic response (Marcoz \textit{et al.}, 1993). This indicates that sustained elevations of cyclic AMP are inhibitory to proliferation, and that PDE4 activities are central to controlling cyclic AMP levels within thymocytes (Marcoz \textit{et al.}, 1993). In mature lymphocytes it is tempting to suggest that cyclic AMP levels are important in the control of proliferation, as basal PDE activities are elevated in \textit{in vivo-activated} populations of cells.

Cyclic AMP homeostasis may play a role in controlling adherence of T lymphocytes to accessory cells. This would occur in a form of \textquoteleft inside-out\textquoteright signalling, where cyclic AMP produced intracellularly would signal an increase in adhesiveness extracellularly (Hahn \textit{et al.}, 1993). Indeed this has been suggested to occur upon ligation of the CD2 molecule (Hahn \textit{et al.}, 1993), increasing its avidity for its natural ligand LFA-3 and may be of importance in the thymic selection.

Upon stimulation of murine thymocytes with PHA, a rapid transient increase in cyclic AMP was observed, possibly mediated through CD2 (Carrera \textit{et al.}, 1988; Hahn \textit{et al.}, 1991). It may be the case that the elevation of cyclic AMP is compartmentalised, thus giving a very high local concentration of cyclic AMP sufficient to trigger localised membrane signalling events. Indeed, cyclic AMP has been shown to play a central role in controlling the mobility of cell surface
receptors, polarising cell surface molecules to the point of interaction of the cell (Kammer et al., 1988).

6.3. Mechanism of regulation.

The mechanisms involved in mediating the rapid changes in PDE2 and PDE4 activity upon stimulation of the thymocytes with PHA may involve an alteration of phosphorylation status. In this regard, increased activity correlating with PDE phosphorylation, has been noted for particular splice variants of PDE4D (Sette et al. 1994a) and for a PDE4 activity found in hepatocytes (Marchmont et al., 1981; Pyne et al., 1989). No studies, however, have thus far characterised any phosphorylation of PDE2, although it has been suggested that insulin may lead to transient activation of this activity in hepatocytes (Pyne and Houslay, 1988). It is possible that the transient decrease in PDE activity might ensue by virtue of a dephosphorylation event rather than any phosphorylation. It would therefore be interesting to study the mechanism behind the stimulation of PHA. Although, this research may be difficult due to the differing complement of cell surface receptors being ligated in addition to the TCR/CD3 complex.

The mechanism by which anti-CD3 antibody stimulation leads to PDE4 induction appears to be mediated by PKC, as selective inhibitors of PKC inhibit this increase, and the phorbol ester, PMA replicates the elevation of PDE4 activity observed upon stimulation with anti-CD3 antibodies. In contrast to this, there is evidence which shows that phorbol ester treatment can cause a rapid and profound inhibitory effect on PDE activity in hepatocytes, modulation which presumably occurs through the action of protein kinase C (Irvine et al., 1986). However, PMA appears to cause an immediate increase in PDE4 activity, whereas anti-CD3-mediated PDE4 elevation is preceded by an slight decrease in PDE activity. This is exciting, as it may imply that PDE4 activity is regulated by two pathways upon ligation of the TCR/CD3 complex, one of which is inhibitory to PDE4 activity and the other which is stimulatory. This is supported by the observation that upon
addition of chelerythrine, there was not only a block of the rise in PDE4 activity, but a decrease in PDE activity. Furthermore, addition of PTK inhibitors also abrogated the elevation of PDE4 activity. This implied that a tyrosine phosphorylation-mediated event might also be involved in the stimulation of PDE4 activity. Indeed, tyrosine phosphorylation has previously been shown to activate the peripheral plasma membrane PDE (PDE4) in intact hepatocytes, upon stimulation of the cells with insulin (Pyne et al., 1989). At this stage, it is impossible to assess whether the activation of PDE4 activity in murine thymocytes is due to a tyrosine or a serine/threonine kinase-mediated pathway. However, it is tempting to suggest that early activation of PDE4 may be due at least partially to the PTK-mediated activation of PLC-γ1 and the subsequent activation of PKC (Secrist et al., 1991). Further studies in this area should illuminate the pathway from the TCR/CD3 complex to PDE4 activation.

The situation described here differs from that seen by Sette et al. (1994b) where PKA has been shown to mediate the expression of certain splice variants of PDE4D (PDE4D3.1 and PDE4D3.2), whilst increasing activity of PDE4D3.3 by phosphorylation in response to thyroid-stimulating hormone. This investigation demonstrated that splice variants from the same locus could be differentially regulated on stimulation of the cell by a particular hormone. An elevation of cyclic AMP has also been shown to cause an increase in ratPDE3 (PDE4D) expression in immature Sertoli cells on stimulation with FSH (Monaco et al., 1994; Sette et al., 1994b; Swinnen et al., 1991b), in cardiac myoblasts (Kovala et al., 1994) and in Jurkats (Engels et al., 1994). It would be of interest to see whether different PDE4 activities are regulated by different signalling networks. At this stage, it is not known which PDE4 is present, or induced in murine thymocytes. Studies are currently underway in the laboratory to investigate this further.

The induction of the PDE4 is extremely fast, indicating that its gene expression, like that of c-jun (Angel et al., 1988) may be controlled by a cis-acting TRE to which AP-1 transcription factors bind. This rapid induction is supported by
work carried out on the induction of PDE1 in CHO cells (Spence et al., 1995). The PDE1 induction process occurred within 25 minutes of phorbol ester treatment of the cells, and could also be achieved by over-expression of selected isoforms of PKC (α and ε) within the cells. Both of these PKC isoforms are found to be present in T cells, and are implicated in the early events of T cell activation (Szamel and Resch, 1992). Indeed, PKCε is found to be expressed at high concentrations in murine thymocytes (Strulovici et al., 1991).

6.4. Prospects.

It is becoming increasingly evident that cyclic AMP PDEs play an active role in regulating the metabolism of cyclic AMP (Conti et al., 1995b; Houslay and Kilgour, 1990). Recently, it has become clear that at least PDE4 sub-families have the ability to be specifically targeted to the plasma membrane by virtue of an alternatively spliced N-terminal domain (Shakur et al., 1995). Research is currently underway which suggests that the PDE4A splice variant (RPDE6) binds via a proline/arginine rich region in its N-terminal tail to SH3 domains of the non-receptor tyrosine kinase v-src, p56^ck and p50^ck (McCallum et al., 1995). The importance of these kinases is well appreciated in T cell activation, implying that cyclic AMP PDEs may be centrally located to participate in T cell signalling. Indeed the results presented here suggest that PDE4 activity may be regulated by a PTK-mediated pathway.

Recent research has rekindled interest in cyclic AMP signalling pathways with the observation that cyclic AMP can inhibit raf-1 kinase activity in fibroblasts (Cook and McCormick, 1993; Hafner et al., 1994; Wu et al., 1993). This protein kinase is a central player in the activation of the MAP kinase signalling cascade (Gupta et al., 1994). Raf-1 kinase has recently been found associated with the TCR/CD3 complex (Loh et al., 1994). In addition, PKA type I isoform as been found to be associated with the TCR/CD3 complex (Skalhegg et al., 1994). PKA is known to mediate stimulatory as well as inhibitory effects on lymphocyte...
proliferation (see section 1.6). It has been suggested that cyclic AMP-mediated stimulation of PKA may release this kinase activity from the TCR/CD3 complex, and through phosphorylation uncouple the TCR from intracellular signalling pathways (Skalhegg et al., 1994). The finding that PDE4 isoforms may be localised to this area of the cell by their association with src-family non-receptor PTKs, indicates that cyclic AMP PDEs may play a positive role in T cell proliferation pathways. These findings could be the basis of a molecular mechanism for the modulatory effects of cyclic AMP in T cell biology.
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