CHARACTERIZATION OF VOLTAGE-DEPENDENT CALCIUM CHANNELS IN T LYMPHOCYTES

by

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ABSTRACT

In T lymphocytes, sustained calcium (Ca\(^{2+}\)) influx through Ca\(^{2+}\) channels localized in the plasma membrane is critical for T cell activation and proliferation. Previous studies intimated that L-type voltage-dependent Ca\(^{2+}\) channels (VDCCs) play a role in Ca\(^{2+}\) mobilization during T lymphocyte activation. However, the function of VDCCs in these non-excitable cells is still poorly understood. In order to explore the role of L-type VDCCs in T lymphocytes, molecular and pharmacological analyses were employed to identify L-type VDCCs, and to define their contribution to Ca\(^{2+}\) influx pathways in T lymphocytes.

In human T lymphocytes, two novel splice isoforms of the channel-forming \(\alpha_{1F}\)-subunit of retinal L-type VDCC were identified. It was found that both of the \(\alpha_{1F}\)-subunit splice isoforms contain unique structural features, distinct from the \(\alpha_{1F}\)-subunit originally isolated from human retina that may render these channel variants insensitive to changes in membrane depolarization. Through cDNA cloning with a human spleen library, the complete cDNA sequence of one of the \(\alpha_{1F}\)-subunit splice variants was isolated for future functional studies. The mRNA expression of the \(\alpha_{1F}\)-subunit splice isoforms appeared to be regulated by T cell receptor (TCR)-induced activation in the human Jurkat T cell leukemia line, and to a lesser extent in human peripheral blood T lymphocytes (PBTs). In addition, the \(\alpha_{1F}\)-subunit protein was detected in Jurkat T cells and human PBTs.

To further investigate the contribution of Ca\(^{2+}\) influx through L-type VDCCs, the effects of the 1,4-dihydropyridine (DHP) L-type Ca\(^{2+}\) channel agonist, (+/-) Bay K 8644, and antagonist, nifedipine, on Jurkat T cells, human PBTs and mouse splenocytes were
assessed. It was found that treatment of T lymphocytes with (+/-) Bay K 8644 increased intracellular Ca\(^{2+}\) and induced the activation of phospho-extracellular regulated kinase 1/2 (Erk1/2), whereas nifedipine blocked Ca\(^{2+}\) influx, the activity of Erk1/2 and nuclear factor of activated T cells, interleukin-2 (IL-2) production and IL-2 receptor expression. Nifedipine also significantly suppressed splenocyte proliferation in an \textit{in vitro} mixed lymphocyte reaction.

Since patients receive nifedipine for the treatment of cardiovascular diseases and other clinical disorders, it was important to further examine the potential immunosuppressive effects associated with nifedipine administration. It was found that nifedipine inhibited the proliferation of male antigen (H-Y)-specific TCR-transgenic CD\(^{8+}\) T cells in transplanted male mice \textit{in vivo}. Finally, a study exploring the effects of nifedipine administration on circulating T lymphocytes in renal disease patients showed T cells from renal patients secreted less IL-2 compared to T cells isolated from a healthy individual. This suggested that nifedipine therapy may act as an immunosuppressant.

The results demonstrate that alternative splicing of the human retina \(\alpha_{1F}\)-subunit has led to the expression of structurally unique \(\alpha_{1F}\)-subunits in T lymphocytes. Furthermore, the pharmacological studies indicate that L-type Ca\(^{2+}\) channels play a significant role in the Ca\(^{2+}\) influx pathways mediating T lymphocyte activation and proliferation \textit{in vitro} and \textit{in vivo}. 
TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................... ii
TABLE OF CONTENTS ........................................................................................................................ iv
LIST OF FIGURES ............................................................................................................................... vii
LIST OF TABLES .................................................................................................................................... x
LIST OF ABBREVIATIONS ................................................................................................................... xi
ACKNOWLEDGEMENTS ....................................................................................................................... xiv
DEDICATION ........................................................................................................................................ xv

CHAPTER 1: GENERAL INTRODUCTION ......................................................................................... 1
  1.1 Role of Calcium in Regulating Distinct Cellular Processes ......................................................... 1
  1.2 T Cell Activation ............................................................................................................................ 4
    1.2.1 Early Signaling Events Proximal to the T Cell Receptor ...................................................... 4
    1.2.2 Immunological Synapse Formation and Sustained T Cell Signaling ................................. 9
    1.2.3 Calcium’s Role as an Intracellular Secondary Messenger ............................................... 12
    1.2.4 Calcium-Dependent Transcriptional Regulation ............................................................... 14
  1.3 Intracellular Calcium Release and Regulation in T Cells ........................................................... 19
    1.3.1 IP₃ Receptor Calcium Channels ......................................................................................... 19
    1.3.2 Ryanodine Receptor Calcium Channels .......................................................................... 23
  1.4 Communication between Calcium Store Release and Calcium Influx Pathways ...................... 26
    1.4.1 Diffusible Messenger Model ............................................................................................... 27
    1.4.2 Conformational Coupling Model ....................................................................................... 29
    1.4.3 Secretion Model .................................................................................................................. 31
  1.5 Calcium Influx Pathways in T Cells ............................................................................................ 32
    1.5.1 IP₃ Receptor Calcium Channels ......................................................................................... 33
    1.5.2 Transient Receptor Potential Calcium Channels ............................................................. 34
    1.5.3 Voltage-Dependent Calcium Channels ............................................................................ 39

CHAPTER 2: MATERIALS AND METHODS ....................................................................................... 45
  2.1 Cell Lines and Culture Conditions ............................................................................................... 45
  2.2 Isolation and Culture of Human Peripheral Blood T Lymphocytes .......................................... 45
  2.3 Cell Separations ........................................................................................................................... 46
  2.4 Nested RT-PCR of α₁F-Subunit and DNA Sequencing ............................................................... 48
  2.5 Cloning of α₁F-Subunit cDNA from Human Retina and Spleen Libraries ............................. 50
  2.6 Production of Carboxyl-Terminal FLAG-tagged α₁F-Subunit ................................................ 52
  2.7 Immunofluorescence Staining ....................................................................................................... 54
  2.8 Immunoprecipitation Analysis of α₁F-Subunit .......................................................................... 54
  2.9 Flow Cytometry Analysis of α₁F-Subunit .................................................................................... 56
  2.10 Real-Time PCR ........................................................................................................................... 56
  2.11 Construction of Plasmids for Stable siRNA Expression ............................................................ 58
  2.12 Generation of Stable Cell Lines Expressing siRNA ................................................................. 61
  2.13 Immunoprecipitation Analysis of p53 ...................................................................................... 62
  2.14 Measurement of Intracellular Calcium Levels .......................................................................... 63
  2.15 Immunoblot Analysis of Phospho-p44/p42 MAP Kinase ........................................................ 64
2.16 NFAT-Luciferase Assay ................................................................. 65
2.17 IL-2 Assay ...................................................................................... 65
2.18 Flow Cytometry Analysis of IL-2R and CD69 ............................ 68
2.19 Mice ............................................................................................... 68
2.20 Mixed Lymphocyte Reaction ....................................................... 68
2.21 In Vivo Proliferation Assay ........................................................ 69
2.22 Statistical Analysis ......................................................................... 71

CHAPTER 3: MOLECULAR IDENTIFICATION OF L-TYPE VOLTAGE-
DEPENDENT CALCIUM CHANNELS IN T LYMPHOCYTES ............ 72
3.1 Introduction ....................................................................................... 72
3.2 Results .............................................................................................. 76
  3.2.1 L-Type α1F-Subunit mRNA Transcript is Expressed in T Cells .... 76
  3.2.2 Identification of Novel Alternative Splice Variants of the α1F-Subunit in T
       Cells ................................................................................................... 78
  3.2.3 Carboxyl-Terminus of the Retina α1F-Subunit Resides in the Cytoplasm 84
  3.2.4 Alternative Splice Variants of α1F-Subunit are Differentially Expressed in
       Human Leukocytes ............................................................................ 87
  3.2.5 α1F-Subunit Protein is Expressed in Human T Cells ................. 88
  3.2.6 Comparison of α1F-Subunit Alternative Splice Variant mRNA Expression
       in Resting and Activated T Cells ...................................................... 93
  3.2.7 Comparison of α1F-Subunit Protein Expression in Resting and Activated
       Jurkat T Cells .................................................................................. 100
3.3 Discussion .......................................................................................... 102

CHAPTER 4: DEFINING THE CONTRIBUTION OF L-TYPE CALCIUM
CHANNELS TO CALCIUM INFLUX DURING T LYMPHOCYTE ACTIVATION
IN VITRO .............................................................................................. 113
4.1 Introduction ....................................................................................... 113
4.2 Results .............................................................................................. 119
  4.2.1 Examination of the Effects of siRNA on α1F-Subunit Expression in Jurkat
       T Cells ................................................................................................ 119
  4.2.2 Induction of Calcium Influx in Jurkat T Cells and Human PBTs by (+/-)
       Bay K 8644 ....................................................................................... 121
  4.2.3 Nifedipine Inhibits Anti-CD3 Induced Calcium Influx in Jurkat T Cells
       and Human PBTs ............................................................................ 124
  4.2.4 (+/-) Bay K 8644 and Nifedipine Modulate Phospho-p44/p42 MAP Kinase
       Activation in Jurkat T Cells and Human PBTs ............................... 127
  4.2.5 Nifedipine Blocks NFAT-Transcriptional Activity in Jurkat T Cells .... 129
  4.2.6 IL-2 Production and IL-2R Expression is Inhibited by Nifedipine in
       Jurkat T Cells and Human PBTs ..................................................... 131
  4.2.7 Nifedipine Suppresses Splenocyte Proliferation .......................... 138
4.3 Discussion .......................................................................................... 138

CHAPTER 5: DETERMINING THE ROLE OF L-TYPE CALCIUM CHANNELS
IN T LYMPHOCYTES IN VIVO .............................................................. 146
5.1 Introduction ....................................................................................... 146
5.2 Results .............................................................................................. 151
  5.2.1 Nifedipine Inhibits T Cell Proliferation in Mice ........................ 151
LIST OF FIGURES

Figure 1-1: T cell signaling events proximal to the TCR .............................................. 6
Figure 1-2: Ca$^{2+}$-mediated signaling events during T cell activation ......................... 15
Figure 1-3: Three proposed models linking ER Ca$^{2+}$ store-depletion to CRAC channel activation ................................................................. 28
Figure 1-4: Phylogenic tree of the three mammalian TRP subfamilies ......................... 35
Figure 1-5: Membrane topology and structure of the three mammalian TRP subfamilies. ................................................................. 37
Figure 1-6: Nomenclature, chromosome location and tissue expression of the different channel-forming $\alpha_i$-subunits .................................................. 41
Figure 1-7: Model of the subunit structure and composition for VDCCs ..................... 42
Figure 2-1: PCR cloning strategy used to isolate $\alpha_{IF}$-subunit cDNA sequences from human retina and spleen cDNA libraries ........................................ 51
Figure 2-2: The pIRES-hrGFP-1a mammalian expression vector ............................ 53
Figure 2-3: The mammalian expression pSUPER directs stable synthesis of siRNA transcripts ................................................................. 59
Figure 2-4: The pNFAT-TA-Luc vector monitors NFAT-mediated signaling transduction pathways in mammalian cells .............................. 66
Figure 2-5: Experimental design for the in vivo proliferation assay ............................. 70
Figure 3-1: The channel-forming $\alpha_{IF}$-subunit of L-type VDCCs is expressed in T lymphocytes ................................................................. 77
Figure 3-2: Schematic representation of mRNA splice sites and putative protein topology of the voltage negative splice variant of $\alpha_{IF}$-subunit isolated from human spleen .............. 80
Figure 3-3: Schematic representation of mRNA splice sites and putative protein topology of the voltage positive splice variant of $\alpha_{IF}$-subunit isolated from human spleen .......... 83
Figure 3-4: FLAG-tagged retina $\alpha_{IF}$-subunit is expressed in HeLa cells .................. 86
Figure 3-5: Expression of alternatively spliced isoforms of α₁F-subunit mRNA in human leukocytes.............................................................. 89

Figure 3-6: Detection of α₁F-subunit protein in Jurkat T cells and human PBTs.......... 91

Figure 3-7: Anti-CD3 stimulation alters mRNA expression levels of α₁F-subunit splice variants and LTRPC2 channel in Jurkat T cells and human PBTs........................................ 94

Figure 3-8: Activation-induced splicing may control the mRNA expression of the voltage negative splice variant of the α₁F-subunit in Jurkat T cells....................................... 96

Figure 3-9: mRNA expression of different TRP subfamily members in Jurkat T cells, human PBTs and human spleen.......................................................... 99

Figure 4-1: Chemical Structures of L-Type Ca\(^{2+}\) Channel Modulators.................. 115

Figure 4-2: Stable expression of siRNAs targeted to the α₁F-subunit in Jurkat T cells did not knock-down α₁F-subunit protein expression........................................... 120

Figure 4-3: (+/-) Bay K 8644 induces Ca\(^{2+}\) influx in a dose-dependent manner in the human Jurkat T cell leukemia line and human PBTs.................................................. 123

Figure 4-4: Nifedipine blocks Ca\(^{2+}\) influx in the human Jurkat T cell leukemia line and human PBTs. .............................................................. 125

Figure 4-5: (+/-) Bay K 8644 modulates phospho-p44/42 MAP kinase activation in Jurkat T cells and human PBTs............................................................... 128

Figure 4-6: Nifedipine modulates phospho-p44/42 MAP kinase activation in Jurkat T cells...................................................................................... 130

Figure 4-7: Inhibition of NFAT transcription by nifedipine in the human Jurkat T cell leukemia line................................................................. 132

Figure 4-8: Nifedipine prevents IL-2 secretion from Jurkat T cells and human PBTs... 134

Figure 4-9: Decreased IL-2R expression in Jurkat T cells and human PBTs after treatment with nifedipine................................................................. 137

Figure 4-10: Nifedipine suppresses splenocyte proliferation..................................... 139

Figure 5-1: T cell proliferative response to H-Y male antigen is decreased in mice following repeated nifedipine treatment............................... 153

Figure 5-2: Anti-proliferative effect of nifedipine is dependent on the number of doses administered to mice.................................................. 155
Figure 5-3: One later dose of nifedipine suppresses T cell proliferation in response to the H-Y male antigen in mice. ........................................................................................................ 158

Figure 5-4: Anti-proliferative effect of nifedipine is not due to non-specific drug toxicity. .................................................................................................................. 160

Figure 5-5: Nifedipine treatment does not suppress antigen-independent T cell recovery in female mice. ........................................................................................................ 161

Figure 5-6: DHP administration may decrease IL-2 secretion from PBTs of renal disease patients .................................................................................................................................. 165

Figure 5-7: Uremic serum from renal disease patients reduces IL-2 secretion from human PBTs. ...................................................................................................................... 167

Figure 6-1: Proposed model for Ca\(^{2+}\) channels involved in distinct phases of Ca\(^{2+}\) signaling in T lymphocytes ........................................................................................................ 178
LIST OF TABLES

Table 2-1: pSUPER constructs used for stable expression of siRNA in Jurkat T cells... 60

Table 5-1: Summary of pharmacokinetic parameters of nifedipine and amlodipine in humans... 148

Table 5-2: Summary of the DHP dosage, creatinine levels and T cell characteristics of the human subjects used in the study... 163
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<td>actin related proteins 2 and 3</td>
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<td>Analysis of Variance</td>
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<td>Ca²⁺</td>
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<td>CFSE</td>
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<td>Ca²⁺-influx factor</td>
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<td>DHP</td>
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<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<td>Erk1/2</td>
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<td>ExPASy</td>
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<td>EYFP</td>
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<td>FACS</td>
<td>fluorescent-activated cell sorter</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
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<td>hrGFP</td>
<td>humanized recombinant green fluorescent protein</td>
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<tr>
<td>H-Y antigen</td>
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<td>GAPDH</td>
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<td>intracellular adhesion molecule</td>
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<td>Src-homology</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>SLP-76</td>
<td>SH2-containing leukocyte protein 76</td>
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<tr>
<td>SMAC</td>
<td>supramolecular activation cluster</td>
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<tr>
<td>SNAP-25</td>
<td>synaptosome-associated protein of MW25</td>
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<tr>
<td>SOCs</td>
<td>store-operated Ca²⁺ channels</td>
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<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>Tg</td>
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<td>Th</td>
<td>T helper</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol 13-acetate</td>
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<tr>
<td>TRP</td>
<td>transient receptor potential</td>
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<td>TRPC</td>
<td>TRP canonical</td>
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<td>TRPM</td>
<td>TRP melastatin</td>
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<td>TRPV</td>
<td>TRP vanilloid</td>
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<tr>
<td>VDCC</td>
<td>voltage-dependent Ca²⁺ channel</td>
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<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
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<tr>
<td>ZAP-70</td>
<td>zeta-associated protein-70</td>
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DEDICATION

I would like to dedicate this work to my loving parents, Susan Mary Kotturi and Murthi Sreerama Kotturi, and my dear brother, Gopaul Kotturi. Without their unending love and support I would not have been able to accomplish my goal of becoming a successful scientist.
CHAPTER 1: GENERAL INTRODUCTION

1.1 Role of Calcium in Regulating Distinct Cellular Processes

The Ca\(^{2+}\) ion is a highly versatile signaling agent that regulates many complex cellular processes in the human body involved in life and death (1). For instance, Ca\(^{2+}\) signaling initiates fertilization, controls muscle contraction and secretion of neurotransmitters, and mediates cellular proliferation, necrosis and apoptosis (1). The “on” and “off” switches of these Ca\(^{2+}\)-dependent processes are coordinated at one level by alterations in the intracellular Ca\(^{2+}\) concentration \([\text{Ca}^{2+}]_i\) of cells. In resting cells, the \([\text{Ca}^{2+}]_i\) is approximately 10-100 nM, which is 20,000 fold-less than the \([\text{Ca}^{2+}]\) found extracellularly (2). Upon cellular stimulation, intracellular Ca\(^{2+}\) levels rapidly rise to 0.5-2 \(\mu\)M from the release of Ca\(^{2+}\) through channels located in intracellular membrane Ca\(^{2+}\) stores and the influx of Ca\(^{2+}\) from the extracellular compartment through plasma membrane Ca\(^{2+}\) channels (2). Since \([\text{Ca}^{2+}]_i\) is critical for cell function, the events surrounding intracellular Ca\(^{2+}\) release and removal are tightly regulated through numerous ion channels, buffers, exchangers and pumps.

Further control of Ca\(^{2+}\)-dependent processes is achieved by modifying spatial and temporal patterning of the Ca\(^{2+}\) signal (2). The spatial aspects of Ca\(^{2+}\) signaling have been categorized as either elementary Ca\(^{2+}\) events, or intracellular and intercellular global Ca\(^{2+}\) waves (1). Elementary Ca\(^{2+}\) events and Ca\(^{2+}\) waves are visualized by imaging cells loaded with Ca\(^{2+}\) indicator dyes through confocal microscopy (3). An elementary Ca\(^{2+}\) event is defined as a localized release of Ca\(^{2+}\) from second-messenger operated Ca\(^{2+}\) channels.
found in intracellular Ca$^{2+}$ stores, such as the endoplasmic reticulum (ER) and the sarcoplasmic reticulum (SR) in muscle (4). In response to low second-messenger concentrations, elementary Ca$^{2+}$ events include single-channel opening of either inositol 1,4,5-trisphosphate receptor (IP$_3$R) or ryanodine receptor (RyR) Ca$^{2+}$ channels in intracellular stores, which are visualized as blips or quarks, respectively (2). Higher concentrations of second-messengers induce the coordinated opening of a group of IP$_3$Rs or RyRs that are visualized as puffs and sparks, respectively (2). Since elementary Ca$^{2+}$ signals are localized intracellular events, the result is a specific physiological outcome near the vicinity of Ca$^{2+}$ release. An example of a cellular process that is regulated by elementary Ca$^{2+}$ events is neuronal excitability. In the neuron cell body, transient, highly localized puffs of Ca$^{2+}$ released from IP$_3$R Ca$^{2+}$ channels located in the ER activate Ca$^{2+}$-activated potassium (K$^+$) channels in the plasma membrane (5). The activation of the K$^+$ channels induces K$^+$ ion efflux across the plasma membrane, which in turn directly alters the membrane potential, preventing further electrical activity (1, 5). Elementary Ca$^{2+}$ release from IP$_3$Rs may also augment other neuronal processes, such as secretion of synaptic vesicles containing neurotransmitters. There is accumulating evidence that elementary Ca$^{2+}$ release from IP$_3$Rs enhances localized [Ca$^{2+}$]$_i$ that is necessary to trigger neurotransmitter exocytosis (5).

Long-lasting elementary Ca$^{2+}$ events that produce puffs and sparks from either the ER or SR lead to an intracellular global Ca$^{2+}$ wave, that moves swiftly through the cytoplasm of an entire cell (4). During fertilization in mammals, sperm fusing with the egg plasma membrane generates repetitive increases in cytosolic Ca$^{2+}$ that last for approximately two hours (2). Each increase in intracellular Ca$^{2+}$ from IP$_3$Rs is a global
Ca^{2+} wave that begins at the sperm attachment site and spreads throughout the egg (6). As a result of the Ca^{2+} wave, exocytosis of enzyme-containing cortical granules that are responsible for "hardening" the structure of the fertilized egg is initiated, preventing additional sperm fusion (6). Another example of a cellular process regulated by intracellular global Ca^{2+} waves is the excitation-contraction coupling of cardiac muscle. When a motor nerve triggers an action potential in a muscle cell, voltage-operated plasma membrane Ca^{2+} channels open in response to membrane depolarization, initiating a small Ca^{2+} influx, that stimulates the release of a Ca^{2+} spark from four to six RyRs located in the SR (7). This response is amplified following the release of more Ca^{2+} sparks from RyRs. A global Ca^{2+} wave is then generated, inducing the contraction of each myofibril (7). When cells are connected to one another through gap junctions, intracellular Ca^{2+} waves can spread to many neighboring cells, leading to the generation of intercellular global Ca^{2+} waves (2). The first recorded intercellular Ca^{2+} waves were in hepatocytes from intact liver tissue after stimulation with the agonist, vasopressin (8). Intercellular Ca^{2+} waves through coordinated release of Ca^{2+} from IP_{3}Rs in the liver are thought to directly effect hepatic functions, including the movement and secretion of bile (8).

The temporal characteristics of Ca^{2+} signaling, such as speed, amplitude and frequency modulation, generate additional diversity of the Ca^{2+} signal. Furthermore, both elementary and global Ca^{2+} events can have distinct oscillation frequencies and durations depending on the cellular process. For instance, altering the Ca^{2+} oscillation frequency in lymphocytes can directly result in differential activation of proinflammatory transcription factors (9). High frequency oscillations are necessary to activate the transcription factor, nuclear factor of activated T cells (NFAT), whereas infrequent oscillations only stimulate
the transcription factor nuclear factor κB (NFκB) in T lymphocytes (9). The prolonged activation and nuclear retention of NFAT for up to two hours is required to promote lymphocyte proliferation (10). However, very high concentrations of Ca\(^{2+}\) or disruptions in Ca\(^{2+}\) homeostasis can be deleterious to cells, inducing cell death by either necrosis or apoptosis (11).

Together, both spatial and temporal patterning of the Ca\(^{2+}\) signal produce complex Ca\(^{2+}\) signaling networks with durations of seconds to many hours depending on the cellular process. In order to generate distinct physiological outputs, the intricate Ca\(^{2+}\) signals are "decoded" by various intracellular signaling molecules (1). The intracellular "decoders" provide a final level of control over the physiological outcome of Ca\(^{2+}\)-dependent processes. As an example of the complexity of Ca\(^{2+}\) signals in a single cell type, the precise nature and regulation of Ca\(^{2+}\) signals during T lymphocyte activation will be discussed.

1.2 T Cell Activation

1.2.1 Early Signaling Events Proximal to the T Cell Receptor

T lymphocyte activation is initiated through the TCR. The TCR complex is multi-subunit transmembrane receptor, consisting of the disulfide-linked, clonotypic TCR-αβ or TCR-γδ heterodimer, non-covalently associated with the TCR invariant chains; the TCR-ζ homodimer, and the CD3-δ, -ε, and -γ chains (12). Assembly of the TCR-αβ/CD3 complex occurs in the ER through a series of tightly coordinated protein interactions (13,
14). Once assembled on the cell surface, it is believed that the minimal TCR/CD3 complex contains one TCR-αβ or TCR-γδ heterodimer associated with a CD3-εδ and a CD3-εγ dimer, since the stoichiometry is one δ-, one γ- and two ε-chains per CD3 complex (15) (Figure 1-1). However, when quantitative flow cytometry was used to study subunit stoichiometry, it was demonstrated that there may be three CD3-ε chains for every two TCR heterodimers (16). Since the crystal structure of the entire TCR/CD3 complex has not been completed, the exact stoichiometry of the subunits is still under debate. It is well established that the TCR/CD3 complex is coupled to intracellular signaling machinery by the large cytoplasmic domains of the CD3-δ, -ε, and -γ chains and the TCR-ζ homodimer. T lymphocytes expressing TCR-αβ become activated when the TCR/CD3 complex recognizes antigen peptide bound to the major histocompatibility complexes (MHC) on antigen presenting cells (APCs), such as B lymphocytes, dendritic cells and macrophages. The extracellular domains of CD8 expressed on cytotoxic T cells, and CD4 found on T helper (Th) cells bind to membrane-proximal regions of MHC Class I and II, respectively (17). CD28 is a T cell surface receptor that binds to the costimulatory molecules, CD80 (B7.1) and CD86 (B7.2) expressed on APCs (17). Engagement of the coreceptors, CD4 and CD8, and the costimulatory receptor, CD28 present on αβ T lymphocytes by their appropriate ligands on APCs is also necessary to achieve full activation responses (17). In the absence of the CD28 costimulation signal, TCR engagement induces a state of T cell anergy or unresponsiveness (18).

Following ligation of the TCR/CD3 by a peptide-MHC complex, nonreceptor protein tyrosine kinases (PTKs) associated with the TCR/CD3 complex are activated
Following peptide-MHC recognition by the TCR, ITAM sequences in the CD3-ζδ and CD3-εγ dimers, and the TCR-ζ invariant chains are phosphorylated (designated as P) by Lck. ZAP-70 is activated by Lck phosphorylation and subsequently phosphorylates the adapter protein SLP-76. SLP-76 in conjunction with LAT recruits PI3K to the plasma membrane, which converts the membrane phospholipid PIP₂ to PIP₃. Newly synthesized PIP₃ interacts with the Tec family kinases, Itk and Rtk, which in turn phosphorylate PLC-γ₁. The intracellular messengers, IP₃ and DAG, are released into the cytoplasm when activated PLC-γ₁ hydrolyzes PIP₂. This T cell signal transduction diagram was adapted from Winslow et al. (19).
(Figure 1-1). Since the TCR/CD3 complex does not contain enzymatic activity in the cytoplasmic domains, the association and recruitment of PTKs is necessary for the activation of signaling cascades downstream of the antigen receptor. Three classes of PTKs implicated in T cell activation are the Src, zeta-associated protein (ZAP)-70 and Tec family kinases (12). The Src family members, lymphocyte-specific PTK p56<sup>Lck</sup> (Lck) and Fyn, noncovalently associated with the cytoplasmic tails of both CD4 and CD8 coreceptors, are activated through dephosphorylation of their negative regulatory tyrosine residue by the transmembrane receptor, protein tyrosine phosphatase, CD45 (20). In addition, CD4 and CD28 induce autophosphorylation of Lck by recruiting Lck to the TCR and sustaining Lck activation (21). Once activated, Lck and Fyn specifically phosphorylate tyrosine residues on CD3-δ, -ε, and -γ, and TCR-ζ chains at a motif known as the immunoreceptor tyrosine-based activation motif (ITAM) (22). The specific consensus sequence for an ITAM motif is YxxI/L(x)<sub>6</sub>-8YxxI/L, where Y denotes a tyrosine, I is isoleucine, L is leucine, and x is any amino acid (22). There is one ITAM motif present on each CD3 chain and three on a TCR-ζ chain, therefore a minimal TCR/CD3 complex contains ten ITAMs. ITAM sequences on TCR-ζ can either be phosphorylated once (TCR-ζ p21) or dual phosphorylated (TCR-ζ p23), generating two distinct ITAM isoforms (12). The ITAMs are predominantly phosphorylated by Lck and to a lesser extent by Fyn (12).

Dual-phosphorylated ITAMs recruit ZAP-70 from the cytoplasm, causing ZAP-70 to bind to ITAMs in the TCR-ζ chain through its tandem, phosphotyrosine recognition domains, the Src-homology (SH2) domains (22). ZAP-70 becomes tyrosine phosphorylated and activated as a result of autophosphorylation and phosphorylation by
Lck (23). Phosphorylation of ZAP-70 generates docking sites for the recruitment of the SH2-containing adapter or linker proteins that lack enzymatic activity, but contain numerous binding domains, which allow the formation of multiprotein signaling complexes (22). Proteins that bind to phosphotyrosine residues of ZAP-70 include; the transmembrane adapter protein, linker for activation of T cells (LAT); the scaffolding adapter protein, SH2-containing leukocyte protein 76 (SLP-76); and the Grb2 family of adapter proteins (12). The Grb2 family consists of Grb2, Grap, and Gads that are small linker proteins, containing both SH2 and SH3 domains (22). SLP-76 interacts with LAT through binding to Gads (22).

When tyrosine phosphorylated predominantly by ZAP-70, LAT and SLP-76 recruit additional proteins to the plasma membrane, including; phosphoinositol 3-kinase (PI3K); the guanine nucleotide exchange factor for the Rho-family of small GTPases, Vav; and the adapter protein, Nck (12). PI3K converts phosphoinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) (24). The amount of PIP3 synthesized at the plasma membrane is tightly regulated by PI3K and the lipid phosphatase, PTEN (phosphatase and tensin homologue deleted on chromosome ten), which converts PIP3 to PIP2 (19). The Tec family kinase, inducible T cell kinase (Itk), associates with PIP3 in the membrane via an amino-terminal pleckstrin homology (PH) domain (12). An additional Tec family kinase that lacks a PH domain, Rlk, is also recruited to the plasma membrane through palmitoylation of a cysteine-string motif (12). Once Itk and Rlk are phosphorylated and activated by Src family kinases, the Tec kinases in turn directly phosphorylate phospholipase C (PLC)-γ1. Activated PLC-γ1 catalyzes the hydrolysis of the plasma membrane PIP2, generating two cytoplasmic second messengers,
inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (19). While IP3 stimulates the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores in the ER, DAG activates protein kinase C (PKC), particularly PKC\(\theta\), and the downstream Ras signal transduction pathway (19).

TCR engagement also induces polarized actin cytoskeleton rearrangement in T lymphocytes. The trimolecular complex of SLP-76, Vav and Nck is critical for this process. Through the SH3 domains of Nck, the serine/threonine kinase Pak (p21-activated kinase) and the actin-binding protein, WASP (Wiskott-Aldrich syndrome protein) bind to the trimolecular complex (12). Vav and Nck also recruit GTP-bound Rac and Cdc42, which subsequently activate Pak and WASP (25). Once in its active state, WASP recruits and activates the actin related proteins 2 and 3 (Arp2/3) complex, which binds actin-ATP and initiates actin polymerization (25).

1.2.2 Immunological Synapse Formation and Sustained T Cell Signaling

The tyrosine phosphorylation cascade initiated through the TCR/CD3 complex occurs within seconds following TCR engagement by peptide-MHC complexes. However, sustained T cell signaling through the TCR/CD3 complex for many hours is necessary for the transcriptional activation of cytokine genes required for T cell proliferation and clonal expansion. In fact, naïve T cells require approximately 20 h of sustained signaling through the TCR to be committed to proliferation (26). In order to establish and maintain long-lasting T cell signaling, the contact zone between T cells and APCs must overcome many physical obstacles. First of all, large glycoproteins on the T cell surface, such as CD43 and CD45, impose a steric hindrance to the TCR and peptide-MHC interaction due to the smaller size of both the TCR and MHC molecules (27).
Second, there is a low-affinity interaction with a dissociation constant of 0.2 to 200 μM between the TCR and peptide-MHC (28). The continual movement of T cells is also preventative in maintaining a sustained signal (27). Furthermore, T cells must search for a minimum of 10 antigenic peptides amongst many thousands of peptides presented by MHC molecules (29). Remarkably, only 10 peptide-MHC complexes are necessary to sustain T cell signaling (28). It is thought that the mechanism used to overcome all of these barriers is the formation of the immunological synapse.

The immunological synapse is the specialized contact area between T cells and the interacting APCs, which contains highly organized molecular structures called supramolecular activation clusters (SMACs) (30). SMACs are micrometer scale domains, consisting of lymphocyte antigen receptors, costimulatory receptors, adapter proteins, cytoskeletal proteins, intracellular signaling molecules, and unique membrane domains (29). Using peptide-MHC and the adhesion ligand, intracellular adhesion molecule (ICAM)-1, integrated into a glass-supported planar bilayer, it was established that the formation of SMACs and the immunological synapse is a multiple step process (27). During the first 30 seconds, the junction between T cells and APCs is formed with peptide-MHC and TCR engagement in an outer ring, and the interacting T cell β2-integrin, lymphocyte function-associated antigen (LFA)-1 with ICAM-1 on APCs in the center of the ring. CD4 is also involved in the first stage of SMAC formation since CD4 deficient cells are less capable of stopping and forming immunological synapses (27). In the next 5 minutes, TCRs and peptide-MHC complexes translocate to the central (c)-SMAC, while adhesion molecules move to the outer ring or peripheral (p)-SMAC through an actin-mediated process. Translocation of cell surface receptors relies on actin
cytoskeletal reorganization through the previously described trimolecular SLP-76, Vav and Nck complex (25). Finally, the third stage involves the stabilization of the immunological synapse for several hours by maintaining c-SMAC and p-SMAC structures. Since immune synapse stabilization occurs 5 minutes after the TCR engagement, synapse formation is not necessary for inducing tyrosine kinase activation, but is important for sustaining signaling events. Sustained signaling through the TCR is maintained by a process termed the serial triggering mechanism. Once TCR engagement has initiated tyrosine phosphorylation, the peptide-MHC can disengage and then become available to a new TCR/CD3 complex (26). This explains how only 10 peptide-MHC complexes can serially trigger a T cell through hundreds of TCRs (27).

Further investigations on the T cell synapse have revealed that the c-SMAC contains not only the TCR/CD3 complex, but also CD28, protein kinases Lck, Fyn and PKCθ, and the adapter protein CD2 associated protein (30). The p-SMAC consists of LFA-1, the cytoskeletal protein talin, and CD45 (30). Interestingly, although there are 10 different PKC isoforms in T lymphocytes, only PKCθ translocates to the c-SMAC upon TCR engagement (30). Additional support for a role for PKCθ in the c-SMAC came when mature T cells from PKCθ knock-out mice failed to respond to TCR induced activation (31). CD45 is also recruited to the early c-SMAC prior to synapse stabilization in order to dephosphorylate and activate Lck (32). Smaller, alternatively spliced isoforms of CD45 may also be present in the c-SMAC of the mature immune synapse, potentially sustaining Lck activity (25).

Immunological synapse formation also involves the reorganization of plasma membrane structures into specialized “membrane domains” termed lipid rafts at the T cell
and APC interface. Lipid rafts are detergent-insoluble, liquid-ordered membrane structures that are highly enriched in cholesterol, GM1 gangliosides and glycosylphosphatidylinositol anchored proteins (25). In resting T cells, lipid rafts contain very few proteins and are randomly distributed in the plasma membrane with a diameter of less than 70 nm (33). Upon TCR engagement, proteins such as the TCR/CD3 complex, acylated Lck and LAT selectively associate with aggregated lipid rafts, whereas CD45 is generally considered to be excluded from rafts at the immunological synapse (33). Lipid raft coalescence is thought to bring signaling molecules such as Lck and LAT into close proximity with one another to enable more efficient signaling (33). Therefore, the aggregation of lipid rafts is likely an integral component of the T cell signaling process.

1.2.3 Calcium's Role as an Intracellular Secondary Messenger

In T lymphocytes, a critical event during immunological synapse formation is the sustained increase in $[\text{Ca}^{2+}]_i$. Intracellular Ca$^{2+}$ mobilization is a binary response involving an immediate, transient release of Ca$^{2+}$ through second messenger-gated Ca$^{2+}$ channels located in the ER, followed by prolonged Ca$^{2+}$ influx from the extracellular milieu for up to two hours, through Ca$^{2+}$ channels in the plasma membrane (34). Sustained Ca$^{2+}$ mobilization has many physiological consequences within T lymphocytes, including enzymatic activation, granule exocytosis and gene transcription (35). For instance, the Ca$^{2+}$-dependent protease, calpain, is activated following binding of this enzyme to Ca$^{2+}$. Once in its active form, calpain releases LFA-1 from its cytoskeletal anchor, allowing LFA-1 to move into the contact area between T cells and APCs (35). Increases in $[\text{Ca}^{2+}]_i$ are also thought to induce cytolytic granule exocytosis from cytotoxic
T cells since sustained Ca\(^{2+}\) gradients are present in cytotoxic T cells during lysis of target cells (35). In addition, a rise in intracellular Ca\(^{2+}\) is necessary for the long-term activation of cytokine genes in Th cells, which will be discussed in more detail below (36).

Elevated cytoplasmic Ca\(^{2+}\) levels also promote the binding of Ca\(^{2+}\) to a ubiquitous, 17 kilo Dalton (kDa) Ca\(^{2+}\)-dependent regulatory protein called calmodulin (37). In turn, Ca\(^{2+}\)/calmodulin complexes regulate the activity of several enzymes required for transcription factor activation, including the serine/threonine Ca\(^{2+}\)/calmodulin-dependent kinase (CaMK) family members, CaMKII and CaMKIV, and the serine/threonine protein phosphatase, calcineurin, also known as protein phosphatase 2B (19). Although the role of CaMKII in T cells is not well defined, CaMKIV may indirectly mediate the transcriptional upregulation of fos, which associates with jun forming the heterodimeric activating protein (AP)-1 transcription factor (37). CaMKIV is suggested to activate fos transcription through phosphorylation and subsequent activation of cAMP response element binding protein, CREB that binds to a cAMP responsive element upstream of fos (37).

The calcineurin isoforms functioning in T cells are calcineurin A\(\alpha\), and A\(\beta\) (38). A predominant role for calcineurin A\(\beta\) has been suggested since calcineurin A\(\beta\)^+ mice show a more severe defect in T cell maturation and function compared to calcineurin A\(\alpha\)^+ mice (39, 40). The activity of calcineurin is tightly regulated through Ca\(^{2+}\)/calmodulin and endogenous calcineurin inhibitors. Calcineurin is activated by interacting with Ca\(^{2+}\)/calmodulin complex through its catalytic domain, and direct binding of Ca\(^{2+}\) to its regulatory subunit (38). In contrast, the activity of calcineurin is attenuated by
upregulation of the calcineurin inhibitor, calcipressin 1, following calcineurin signaling (38). Calcipressin 1 exhibits its inhibitory action by directly binding between the catalytic and regulatory domains of calcineurin, inhibiting calcineurin phosphatase activity (38). The calcineurin binding protein, Cabin1/Cain-1, also binds to calcineurin and inhibits its phosphatase activity potentially through a negative-regulatory feedback mechanism involving Ca$^{2+}$ (35).

Pharmacological inhibitors, such as the fungal metabolites cyclosporin A (CsA) and tacrolimus (FK506) are widely used to inhibit the phosphatase action of calcineurin in human patients (41). Both CsA and FK506 serve as potent immunosuppressive agents that are used largely to prevent immune responses in transplant therapy. CsA and FK506 do not bind to calcineurin directly. Instead, CsA and FK506 bind to the intracellular immunophilin receptors, cyclophilin and FK506-binding protein, respectively (41). The drug/immunophilin complexes then interact with the regulatory domain of calcineurin, abolishing its catalytic activity and any downstream dephosphorylation events mediated by this phosphatase (38).

1.2.4 Calcium-Dependent Transcriptional Regulation

Through the regulation of calcineurin activity, Ca$^{2+}$ in turn, mediates the activation of many transcription factors, including NFAT, NFκB and myocyte enhancer factor (MEF)-2 in T lymphocytes (Figure 1-2). When calcineurin is activated through elevated [Ca$^{2+}$]i, the cytoplasmic component (NFATc) of NFAT transcription complexes is dephosphorylated by calcineurin at multiple phosphoserine residues in the amineterminus (38, 42). Calcineurin binds to the regulatory domain of NFATc through a
Figure 1-2: Ca^{2+}-mediated signaling events during T cell activation.

Upon TCR engagement by peptide-MHC, protein tyrosine kinases (designated as TK) proximal to the TCR activate PLC-γ1, which cleaves PIP_{2} from plasma membrane phospholipids to generate DAG and IP_{3}. Elevated levels of IP_{3} in the cytosol lead to the release of Ca^{2+} from IP_{3}R Ca^{2+} channels located in the ER. TCR stimulation also leads to the generation of cADPR that binds and opens RyR Ca^{2+} channels also located in the ER, leading to further Ca^{2+} release. Through an undetermined mechanism, Ca^{2+} release from the ER causes sustained Ca^{2+} influx from the extracellular space through plasma membrane CRAC channels. Elevated intracellular Ca^{2+} activates calcineurin and several transcription factors, including NFAT, NFκB and MEF2. The level of cytoplasmic Ca^{2+} is regulated through the removal of Ca^{2+} by sarco-ER Ca^{2+}-ATPase (SERCA) and plasma membrane Ca^{2+}-ATPase (PMCA) pumps. Intracellular Ca^{2+} is redistributed by the mitochondria to prevent Ca^{2+}-dependent inactivation of the CRAC channels. Plasma membrane potential is maintained by the efflux of K^{+} from Kv and Ca^{2+-activated K^{+} channels. This Ca^{2+} signaling diagram was adapted from Lewis et al. (10).
conserved calcineurin-binding sequence motif, PxIxIT, where P denotes a proline, I is isoleucine, T is threonine and x is any amino acid (38). Dephosphorylation of NFATc leads to the uncovering of a nuclear localization signal in the regulatory domain, which permits the activated form of NFATc to translocate from the cytoplasm to the nucleus (43). Since there is no delay in NFATc dephosphorylation and nuclear translocation it has been suggested that NFATc and calcineurin associate in resting T cells (35). Prolonged levels of intracellular Ca\(^{2+}\) are required to maintain NFATc activation and nuclear retention; however it is unclear how NFATc remains dephosphorylated in the nucleus. One possibility is that a small amount of calcineurin translocates with NFATc into the nucleus (38). Alternatively, it has also been suggested that a portion of calcineurin may reside in the nucleus of resting cells prior to TCR stimulation (38).

Once in the nucleus, NFATc associates with the newly synthesized nuclear subunit of NFAT (NFATn) (44). The NFATc and NFATn complex regulates the expression of several genes, through binding to response elements in gene promoter/enhancer regions, usually in association with AP-1 (41). The established cytokine gene targets of NFAT in T cells are IL-2, IL-3, IL-4, IL-5, IL-8, IL-13, granulocyte-macrophage colony-stimulating factor, and interferon-\(\gamma\). Other gene targets of NFAT are the cell surface receptors; CD40 ligand, Fas ligand and IL-2 receptor (IL-2R)\(\alpha\) (41). It should be noted that CsA and FK506 prevent NFATc nuclear translocation, and consequently block NFAT-mediated transcription of cytokine and cell surface receptor genes (41). CsA and FK506 do not block the synthesis of the NFATn component (44).
In T cells, the existence of three calcineurin regulated NFATc proteins that are capable of recognizing the same DNA element was thought to reflect redundancy of function, but recent evidence supports the idea that the family members may have gene-specific activities. The argument for gene-specific activities of NFAT has been strengthened by the phenotypic analysis of NFATc1\(^{-/-}\), NFATc2\(^{-/-}\), and NFATc3\(^{-/-}\) mice. NFATc1\(^{-/-}\) mice have an impaired ability to produce IL-4 in T cells, whereas IL-2 production was only slightly affected (41). The T cells from NFATc2\(^{-/-}\) mice have increased IL-4 production and hyperproliferative responses to TCR engagement, which is likely due to a lack of Fas ligand expression (42). Lymphocytes from mice lacking both NFATc2 and NFATc3 have a greatly enhanced Th2-type cytokine phenotype and acute allergic responses (45). Therefore the phenotypic analysis suggests that NFATc1 is a positive regulator of IL-4 transcription, but NFATc2 and NFATc3 may eventually repress IL-4 gene expression through a negative-regulatory feedback mechanism (41).

Upon TCR disengagement and the subsequent absence of sustained intracellular Ca\(^{2+}\) signaling, NFATc proteins are rapidly exported from the nucleus and NFATc-dependent gene transcription is terminated. Prior to nuclear export, NFATc is phosphorylated in three conserved serine/proline rich motifs in the amino-terminus predominantly by the constitutively active glycogen synthase kinase-3\(\beta\) (GSK-3\(\beta\)) (34, 46). Phosphorylation of NFATc is speculated to expose a nuclear export signal, permitting NFATc to rapidly leave the nucleus through the nuclear export receptor, Crm1 (41). Although nuclear inhibitors of NFATc activity have been identified, the regulation of these protein kinases is not clear. However, it has been established that the activity of
GSK-3β is rapidly downregulated to about 50% of basal levels upon TCR stimulation (47).

Additional transcription factors regulated by Ca\(^{2+}\)/calcineurin in T lymphocytes are NFκB and MEF2 (38). Previous studies have shown that the calcineurin inhibitors CsA and FK506 are capable of blocking upstream IκB kinase activation, as well as TCR-induced NFκB activity (48, 49). As expected, Ca\(^{2+}\)-independent activation of NFκB is not inhibited by CsA and FK506 following tumor necrosis factor α (TNFα) stimulation (50). Although the mechanism of Ca\(^{2+}\)/calcineurin-induced NFκB activation has not been clearly identified in T lymphocytes, it has been suggested that calcineurin-mediated IκB dephosphorylation induces the specific degradation of IκB (38). As a result of IκB degradation, active NFκB is released, allowing NFκB to translocate into the nucleus and bind to promoter/enhancer regions. Unlike NFAT and NFκB, MEF2 is a transcription factor in lymphocytes that is constitutively bound to DNA-response elements in the nucleus (51). MEF2, which is expressed in several tissues including brain and muscle, was recently identified as a Ca\(^{2+}\)/calcineurin-dependent transcription factor in T cells (52). Interestingly, generation of MEF2-dependent transcription requires direct association with NFATc2 (52). In coordination with NFAT, MEF2 is capable of inducing transcriptional activation of IL-2 (51).

In summary, T cell activation is a multi-step process involving numerous intricately woven signal transduction pathways, many of which are dependent upon the elevation of [Ca\(^{2+}\)]. The mechanisms of how increased intracellular Ca\(^{2+}\) levels are generated add to the complexity of the activation process.
1.3 Intracellular Calcium Release and Regulation in T Cells

During T cell activation, there are several mechanisms that introduce bursts of Ca\(^{2+}\) into the intracellular space. Immediately following TCR engagement, Ca\(^{2+}\) is transiently released from ER Ca\(^{2+}\) stores. As a direct consequence of intracellular Ca\(^{2+}\) store-depletion, a sustained influx of Ca\(^{2+}\) enters into the cytoplasm from the extracellular space. This mechanism of Ca\(^{2+}\) entry across the plasma membrane and subsequent store refilling is termed "capacitative Ca\(^{2+}\) entry" or "store-operated Ca\(^{2+}\) entry" (35). Ca\(^{2+}\) ions enter the cytoplasm from either the ER Ca\(^{2+}\) stores or through the plasma membrane by specific Ca\(^{2+}\) channels. In T lymphocytes, there are two important second-messenger operated Ca\(^{2+}\) channels that mediate Ca\(^{2+}\) mobilization from intracellular ER stores; IP\(_3\)R and RyR Ca\(^{2+}\) channels.

1.3.1 IP\(_3\) Receptor Calcium Channels

Immediately following TCR/CD3 complex stimulation, cytoplasmic IP\(_3\) levels rapidly rise, and remain elevated within the first 10 to 20 minutes after T cell activation (10). IP\(_3\) binds to the IP\(_3\)R Ca\(^{2+}\) channel located in the ER membrane, releasing Ca\(^{2+}\) from its intracellular store (Figure 1-2). IP\(_3\)Rs are large tetrameric intracellular Ca\(^{2+}\) channels, composed of four subunits of approximately 2700 amino acids (~300 kDa) (53). The amino-terminus of each subunit contains a single IP\(_3\) binding site that resides in the cytoplasm. It has been proposed that the negatively charged phosphate groups of IP\(_3\) interact with the IP\(_3\)R through the basic residues that are scattered throughout the IP\(_3\) binding site (53). Several IP\(_3\) molecules are required to bind to the IP\(_3\)R to induce a
relatively large conformational change, causing channel opening and Ca\textsuperscript{2+} release (54).
Near the cytoplasmic-residing carboxyl-terminus there are six transmembrane domains
that anchor the channel in the reticular membrane. The last two transmembrane domains
and the membrane-associated loop between these domains form the pore of the channel
(54). In between the IP\textsubscript{3} binding site and the channel pore is the modulatory domain. This
domain is composed of 1700 amino acids, and contains phosphorylation and protein
binding sites that directly mediate channel opening (54).

Three isoforms of human IP\textsubscript{3}Rs have been identified in lymphocytes (55). The
human Jurkat T cell leukemia line, the chicken DT40 B cell line, and mouse thymocytes
express type 1 IP\textsubscript{3}R (IP\textsubscript{3}R1), IP\textsubscript{3}R2, and IP\textsubscript{3}R3, whereas rat thymocytes and splenocytes
only express IP\textsubscript{3}R2 and IP\textsubscript{3}R3 (56-58). IP\textsubscript{3}R2 and IP\textsubscript{3}R3 share approximately 69% and
64% identity, respectively, with the amino acid sequence of IP\textsubscript{3}R1 (59). Interestingly, the
IP\textsubscript{3}R isoforms show differences in their sensitivities to IP\textsubscript{3} and their regulation by [Ca\textsuperscript{2+}].
IP\textsubscript{3}R1 is rapidly activated by cytosolic IP\textsubscript{3} and shows a biphasic response to [Ca\textsuperscript{2+}]\textsubscript{i} (10).
IP\textsubscript{3}R1 is activated by \(~300\) nM [Ca\textsuperscript{2+}]\textsubscript{i}, but low and high [Ca\textsuperscript{2+}]\textsubscript{i} cause channel
inactivation, which leads to the generation of regular Ca\textsuperscript{2+} oscillations (54, 60). IP\textsubscript{3}R2
responds more effectively to IP\textsubscript{3} and Ca\textsuperscript{2+} than IP\textsubscript{3}R1 (10). Surprisingly, both IP\textsubscript{3}R2 and
IP\textsubscript{3}R3 are not inhibited by Ca\textsuperscript{2+} (10). Since the IP\textsubscript{3}R isoforms have distinct sensitivities to
IP\textsubscript{3} and [Ca\textsuperscript{2+}], their differential expression may confer unique patterns of oscillatory
Ca\textsuperscript{2+} signaling through IP\textsubscript{3}-sensitive and -insensitive Ca\textsuperscript{2+} pools in the ER (35, 60).

The Src family of non-receptor PTKs also seems to regulate IP\textsubscript{3}R-controlled Ca\textsuperscript{2+}
release from intracellular stores in T lymphocytes. Src and Fyn were shown to increase
channel pore opening through phosphorylation of tyrosine residues on the IP\textsubscript{3}R1 Ca\textsuperscript{2+}
channel in Jurkat T cells (61). The IP₃R1 contains two potential tyrosine phosphorylation sites, one located adjacent to the putative channel pore and the other near the IP₃ binding region (61). *In vivo* analysis with thymocytes from fyn⁻/⁻ mice showed decreased phosphorylation of IP₃R1 correlated with reduced intracellular Ca²⁺ release and defective TCR signaling (61).

Although it is well established that three IP₃R isoforms are expressed in T lymphocytes, the exact contribution of each IP₃R isoform to the initial Ca²⁺ release is still relatively unclear. In addition, both homotetrameric and heterotetrameric IP₃Rs have been detected (62). Several studies have been conducted examining the role of IP₃R1 during T cell activation. Inhibiting expression of IP₃R1 by stable transfection of antisense IP₃R1 complementary deoxyribonucleic acid (cDNA) in Jurkat T cells prevented an increase in intracellular Ca²⁺ and IL-2 secretion after TCR stimulation (63). The predominant role of IP₃R1 was then questioned when a later study demonstrated that the IP₃R1 antisense cDNA also partially decreased the expression of IP₃R2 and IP₃R3 in Jurkat T cells (64). The role of IP₃R1 as a regulator of Ca²⁺ mobilization was further disputed when a previous *in vivo* study concluded that IP₃R1 is not essential for T cell activation and function (65). To establish the *in vivo* role of IP₃R1, bone marrow of IP₃R1⁻/⁻ mice was transplanted into irradiated wild-type mice, since IP₃R1⁻/⁻ mice died shortly after birth (65). Contradictory to the previous *in vitro* results, T cells from mice lacking IP₃R1 were able to mobilize Ca²⁺ from intracellular stores after TCR stimulation, and did not show defective TCR signaling (65). Taken together, the results from these *in vitro* and *in vivo* studies suggest that the IP₃R isoforms may exhibit functional redundancy in T lymphocytes.
Not only are IP3Rs implicated in promoting T cell proliferation through Ca\textsuperscript{2+} release, but IP3Rs have also shown to regulate programmed cell death or apoptosis. As previously mentioned, prolonged high intracellular Ca\textsuperscript{2+} levels can cause cell death through the activation of endonucleases responsible for DNA fragmentation and subsequent apoptosis (55). Previously, it has been shown that IP3R1 deficient Jurkat T cells are resistant to apoptosis induced by dexamethasone (a glucocorticoid that induces DNA damage), TCR stimulation, ionizing radiation and Fas/CD95 activation (64). Therefore, prolonged Ca\textsuperscript{2+} release through the IP3R1 may induce apoptosis. The exact mechanism of how Ca\textsuperscript{2+} regulates apoptotic signals is still unclear; however, it has been proposed that Ca\textsuperscript{2+} may activate Ca\textsuperscript{2+}-dependent proteases and nucleases involved in the apoptotic pathway (64).

From these previous studies on IP3R function, it is clear that IP3R-mediated Ca\textsuperscript{2+} mobilization is not as simplistic as it was once viewed. Functional redundancy of the IP3R isoforms has been demonstrated in the DT40 B cells lacking these channel isoforms. In the DT40 cells, complete inhibition of Ca\textsuperscript{2+} release following B cell antigen receptor stimulation was only accomplished by deletion of all three IP3R isoforms (66). Based on these results, IP3R1, IP3R2, and IP3R3 may function together in coordinating Ca\textsuperscript{2+} mobilization from intracellular Ca\textsuperscript{2+} pools in T lymphocytes. In addition, it remains a puzzle how cytosolic IP\textsubscript{3} levels that rapidly dissipate after 10 min can maintain a long-lasting Ca\textsuperscript{2+} signal for 1 to 2 h. One possibility is that the local IP\textsubscript{3} concentration is high enough to keep IP3R Ca\textsuperscript{2+} channels open (35). An alternative explanation is that IP3Rs may be involved in short-term Ca\textsuperscript{2+} signaling, while RyR Ca\textsuperscript{2+} channels may be responsible for prolonged Ca\textsuperscript{2+} release from the ER (10). Based on the evidence that IP3R
Ca\textsuperscript{2+} channels are inactivated by high, localized IP\textsubscript{3} concentrations, and the increasing data demonstrating a role for RyR Ca\textsuperscript{2+} channels, the latter hypothesis is more plausible.

1.3.2 Ryanodine Receptor Calcium Channels

RyR are the second critical second-messenger operated Ca\textsuperscript{2+} channel responsible for Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores in T lymphocytes. Unlike IP\textsubscript{3}R, the RyR Ca\textsuperscript{2+} channel does not appear to be located in the ER since thapsigargin, an inhibitor of the ER Ca\textsuperscript{2+}-ATPase pump has no effect on Ca\textsuperscript{2+} release by RyR (67). The precise intracellular location of the RyR has not been clearly identified in T cells. Thapsigargin-resistant ER-localized Ca\textsuperscript{2+} pools have been identified; therefore, it is plausible that the RyR Ca\textsuperscript{2+} channels are located in these regions of the ER (68). RyR Ca\textsuperscript{2+} channels are large homotetrameric protein complexes, consisting of monomers of approximately 5000 amino acid residues (~500 kDa) (69). The amino-terminus of the RyR (4000 amino acids) resides in the cytoplasm and contains many regulatory binding sites for nucleotides, calmodulin, and Ca\textsuperscript{2+}, as well as phosphorylation sites (70). Interestingly, the binding partner for the immunosuppressive agent FK506, FK506-binding protein 12, directly associates with RyRs in the amino-terminal region of the channel (70). The carboxyl-terminus of the RyR lies in the cytoplasm, while the adjacent region (1000 amino acids) consists of four transmembrane domains, and the putative channel pore between transmembrane domains 3 and 4 (70). The carboxyl-terminal cytoplasmic tail and adjacent two transmembrane domains of the RyR Ca\textsuperscript{2+} channel share extensive sequence homology to similar regions of the IP\textsubscript{3}R Ca\textsuperscript{2+} channel (71).
Three mammalian RyR Ca\(^{2+}\) channel isoforms have been identified in the SR of skeletal muscle (type 1 RyR), cardiac muscle (type 2 RyR), and the brain (type 3 RyR), as well as in various other tissues through cDNA cloning (70). RyR1, RyR2 and RyR3 display approximately 66-70% amino acid sequence identity to one another (70). Jurkat T cells express the RyR3 isoform, which overall, has 95% amino acid sequence identity to the rabbit brain RyR3 (72). The existence of a functional RyR Ca\(^{2+}\) channel in T lymphocytes was demonstrated by the presence of a ryanodine-sensitive intracellular Ca\(^{2+}\) pool in Jurkat T cells (72). A physiological role for RyR3 was further investigated by knocking-down RyR3 expression in Jurkat T cells through the stable integration of RyR3 antisense ribonucleic acid (RNA) (73). In RyR3 deficient Jurkat T cells, the sustained phase of Ca\(^{2+}\) signaling was impaired following TCR engagement by the agonistic anti-CD3 monoclonal antibody (mAb), OKT3 (73). Although the in vitro studies with Jurkat T cells suggest an important role for RyR3 in regulating intracellular Ca\(^{2+}\) release, splenocytes from RyR3\(-/-\) mice appeared to proliferate normally in response to concanavalin A (Con A), IL-2 and lipopolysaccharide (74).

The endogenous activator of RyR Ca\(^{2+}\) channels is the nicotinamide adenine dinucleotide (NAD\(^{+}\)) metabolite, cyclic adenosine diphosphate ribose (cADPR) (75). The second messenger, cADPR was originally found in sea urchin eggs, where it mobilized intracellular Ca\(^{2+}\) in an IP\(_3\)-independent mechanism via RyR Ca\(^{2+}\) channels (75). The precise mechanism of how cADPR regulates Ca\(^{2+}\) release from RyRs is not clear. cADPR may directly bind to RyRs or may interact with RyRs through a separate cADPR-binding protein (75). Several studies have utilized Jurkat T cells to examine the role of cADPR-induced Ca\(^{2+}\) release. In permeabilized Jurkat T cells, cADPR application induced Ca\(^{2+}\) release.
release from intracellular stores in a dose-dependent manner (67). The cADPR-dependent Ca\(^{2+}\) release was blocked by the chemical analogues of cADPR, 8-amino-cADPR and 8-bromo-cADPR (67). Furthermore, Jurkat T cells stimulated with the OKT3 mAb showed increased levels of cytosolic cADPR that lasted for up to 60 min (76). It was also demonstrated that Jurkat T cells stably expressing RyR3 antisense RNA had decreased Ca\(^{2+}\) release in response to cADPR treatment (73).

The molecular mechanisms for cADPR generation are still unclear in T lymphocytes. In higher eukaryotes, one of the ADP-ribosyl cyclases responsible for converting NAD\(^+\) to cADPR is the cell-surface transmembrane protein, CD38 (77). CD38 may be a potential source of cADPR in T cells since surface expression of CD38 is upregulated following lymphocyte activation (78). The problem with this model is that the enzymatic activity of CD38 is associated with the extracellular domain so it is unknown how the membrane-impermeable cADPR enters T cells (78). It is possible that dimerization or oligomerization after CD38 ligation may trigger internalization of the receptor with attached cADPR (77). The second model for cADPR-controlled Ca\(^{2+}\) release is that the activation of the TCR/CD3 complex stimulates a soluble ADP-ribosyl cyclase to produce cADPR (67). Guse et al. confirmed this hypothesis by demonstrating that TCR stimulation of Jurkat T cells resulted in the activation of a tyrosine-kinase regulated, cytosolic ADP-ribosyl cyclase, in association with a prolonged increase in intracellular cADPR levels (76). Although a soluble ADP-ribosyl cyclase has been identified in T cells, the possible generation of cADPR from CD38 cannot be disproved.

In conclusion, the *in vitro* studies with Jurkat T cells suggest that IP\(_3\) is necessary for the initial Ca\(^{2+}\) release from IP\(_3\)R Ca\(^{2+}\) channels, but not sufficient for sustained Ca\(^{2+}\)
signaling, while cADPR association with RyRCa^{2+} channels is essential for a prolonged second phase of Ca^{2+} release (75). Since the majority of the experiments with cADPR and RyR3 have been conducted in Jurkat T cells, these models need to be confirmed with primary human T lymphocytes.

1.4 Communication between Calcium Store Release and Calcium Influx Pathways

In T lymphocytes, the emptying of intracellular Ca^{2+} stores by Ca^{2+} release through IP_3R and RyR Ca^{2+} channels leads to the opening of store-operated Ca^{2+} channels (SOCs) in the plasma membrane. Through electrophysiological means, the mechanism of store-operated Ca^{2+} entry has been extensively studied in numerous non-excitable cells. To date, the best characterized SOC is the Ca^{2+} release-activated Ca^{2+} (CRAC) channel that is localized to the plasma membrane of T lymphocytes, mast cells and other hematopoietic cells (79). The Ca^{2+} current gated by the CRAC channel, termed I_{CRAC}, is a highly selective Ca^{2+} current with an extremely low Ca^{2+} conductance (79). The dependency for store-depletion to induce I_{CRAC} activation has been well established in T lymphocytes. For instance, I_{CRAC} is activated by the ER Ca^{2+}-ATPase pump inhibitor, thapsigargin, that causes a rise in [Ca^{2+}]_i by preventing ER store-refilling (10). I_{CRAC} is also activated by the membrane permeant Ca^{2+} chelator, N,N,N',N'-tetakis(2-pyridylmethyl)ethylene diamine (TPEN), that directly reduces the intraluminal [Ca^{2+}] in the ER (10). Although it is clear that emptying of intracellular Ca^{2+} stores leads to I_{CRAC} activation in T lymphocytes, the mechanism by which the fall of [Ca^{2+}] within the ER activates I_{CRAC} still remains a mystery. Presently, there are three models linking store-
depletion to CRAC channel activation, however none have received extensive support or have been discounted completely (79).

1.4.1 Diffusible Messenger Model

The first and oldest hypothesis linking store-depletion to $I_{CRAC}$ activation is the diffusible messenger model. This model postulates that a soluble signaling factor is newly synthesized and/or released from the ER following store emptying and subsequently diffuses to the plasma membrane where it activates CRAC channels (Figure 1-3A) (35). An early study by Randriamampita et al. identified a novel soluble mediator, termed the Ca$^{2+}$-influx factor (CIF) that stimulated Ca$^{2+}$ influx in mitogen-activated Jurkat T cells following depletion of intracellular Ca$^{2+}$ stores (80). This model gained further support when cytosolic extracts containing CIF from thapsigargin-treated Jurkat T cells induced Ca$^{2+}$ influx when injected into Xenopus laevis oocytes (81). CIF extracted from the cytosol of Jurkat T cells was proposed to be a small (less than 500 Da), non-protein-like, phosphorylated compound (80). However, the precise identity of CIF from Jurkat T cells is unknown since investigators have been unable to purify this compound.

CIF has also been postulated to induce Ca$^{2+}$ influx through SOCs in cells other than T lymphocytes. For example, a novel SOC in vascular smooth muscle cells was shown to be activated by CIF, and not by a variety of other intracellular second-messengers, including IP$_3$ (82). In the study by Trepakova et al., CIF extracted from pmr1 mutant yeast that lack the PMR1 Ca$^{2+}$-ATPase pump or CIF isolated from human platelets was able to activate the novel store-operated channel in smooth muscle, as well as elicit Ca$^{2+}$ influx in Xenopus oocytes and Jurkat T cells (82). Recently, a study
Figure 1-3: Three proposed models linking ER Ca\textsuperscript{2+} store-depletion to CRAC channel activation.

(A) The diffusible messenger model predicts that a soluble messenger, CIF is either newly synthesized or released from the ER following store emptying and then diffuses to the plasma membrane, activating CRAC channels. (B) The conformational coupling model suggests that either the IP\textsubscript{3}R Ca\textsuperscript{2+} channel or another ER resident protein physically associates with the CRAC channel to induce its opening. (C) The secretion model predicts that activated CRAC channels are inserted into plasma membrane through vesicle fusion following store-depletion. This diagram was adapted from Winslow et al. (19).
examining SOCs in human neutrophils identified that sphingosine 1-phosphate acts as a soluble messenger, linking store-depletion to store-operated Ca\(^{2+}\) entry through the plasma membrane (83). Sphingosine 1-phosphate is a bioactive, small (380 Da) molecule that is generated from the metabolism of sphingolipids by sphingosine kinase (83). In human neutrophils, thapsigargin-induced store emptying induced the synthesis of sphingosine 1-phosphate, which in turn stimulated Ca\(^{2+}\) entry (83). Intriguingly, sphingosine 1-phosphate has many similar biochemical properties to the CIF originally identified in Jurkat T cells. Since sphingosine 1-phosphate is capable of increasing [Ca\(^{2+}\)]\(_i\) in Jurkat T cells (84), it will be interesting to learn whether sphingosine 1-phosphate is newly synthesized in Jurkat T cells following store-depletion and if this molecule can activate \(I_{\text{CRAC}}\) in these cells.

### 1.4.2 Conformational Coupling Model

The conformational coupling model postulates that conformational changes in the IP\(_3\)R Ca\(^{2+}\) channel elicited by store-depletion cause \(I_{\text{CRAC}}\) activation through direct physical interaction between IP\(_3\)R in the ER and CRAC channels in the plasma membrane (Figure 1-3B) (35). In contrast to the other two models, there appears to be a similar number of studies either supporting or refuting the conformational coupling model. This model is based on experiments demonstrating the functional coupling of voltage-gated Ca\(^{2+}\) channels to RyR Ca\(^{2+}\) channels in skeletal muscle (79). In support of the coupling model it has been shown that when a physical barrier is formed between the plasma membrane and the ER through dense polymerization of F-actin in smooth muscle cells, Ca\(^{2+}\) influx is blocked after thapsigargin-induced store-depletion (85). Further
acceptance of this model was gained by the demonstration that the IP3R inhibitor, 2-aminoethoxyborane (2-APB) prevented thapsigargin-induced Ca\textsuperscript{2+} influx and inhibited $I_{\text{CRAC}}$ in Jurkat T cells (79). Transient receptor potential (TRP) Ca\textsuperscript{2+} channels, which are one of the molecular candidates for CRAC channels, have also been shown to directly interact with IP3Rs through coimmunoprecipitation of the TRP family member, TRP3, and IP3R (86). Although the studies involving TRP3 suggest that TRP3 activation requires interaction with IP3R, TRP3 does not exhibit all of the biophysical properties of a CRAC channel (10).

The studies disputing the conformational coupling model involve the examination of store-operated Ca\textsuperscript{2+} entry pathways in the DT40 B cell line lacking all three IP3R isoforms. In the DT40 IP3R deficient cells, thapsigargin-induced Ca\textsuperscript{2+} influx is normal and $I_{\text{CRAC}}$ is still present (66). In addition, treatment of the DT40 IP3R\textsuperscript{--/--} cells with 2-APB also inhibits $I_{\text{CRAC}}$, suggesting that 2-APB does not selectively block IP3Rs (79). Based on these studies, it has been postulated that ER resident proteins, other than the IP3R, may physically couple to the CRAC channel (79). One alternative candidate for the coupling model may be RyR Ca\textsuperscript{2+} channels since it has been shown that the pharmacological inhibitors of RyRs block thapsigargin-induced Ca\textsuperscript{2+} influx in the IP3R deficient DT40 cells (87). RyR Ca\textsuperscript{2+} channels are an attractive candidate for this model, especially because knocking-down RyR3 expression in Jurkat T cells prevents sustained Ca\textsuperscript{2+} influx (73).
1.4.3 Secretion Model

Finally, the secretion model postulates that store-depletion initiates vesicular transport of activated CRAC channels to the plasma membrane followed by insertion of the channels into the membrane via vesicle fusion (Figure 1-3C) (19). Several studies have provided evidence to support the secretion mechanism by demonstrating that inhibitors of the secretion/exocytosis pathway prevent Ca\(^{2+}\) influx in a variety of non-excitable cells. The secretion mechanism is based on the original studies showing that \(I_{\text{CRAC}}\) activation was blocked by the vesicular transport inhibitors, GTP\(\gamma\)S and primaquine (88, 89). In addition, Yao et al. recently established that botulinum neurotoxin A and dominant negative mutants of SNAP-25 (synaptosome-associated protein of MW25), both inhibitors of SNAP-25 function in vesicle fusion, prevented \(I_{\text{CRAC}}\) activation in *Xenopus* oocytes (90). The previously described study that showed cortical actin polymerization blocked store-operated Ca\(^{2+}\) influx in smooth muscle cells may also be in favor of the secretion model (85). The physical barrier formed by actin polymerization may have prevented exocytosis of vesicles containing the activated SOCs (19). Although there are many studies supporting the secretion model, this mechanism remains controversial since \(I_{\text{CRAC}}\) activation is not affected by other clostridial neurotoxins that disrupt vesicle secretion through cleavage of SNAP receptor proteins (10).

At the present time, it is difficult to ascribe a particular mechanism connecting store-depletion to Ca\(^{2+}\) influx in T lymphocytes. The studies that support either the conformational coupling or secretion models seem to argue against the diffusible messenger model (91). Unraveling the mystery behind CRAC channel activation will generate a better understanding of Ca\(^{2+}\) signaling mechanisms in T lymphocytes, and may
lead to the development of specific pharmacological agents that mediate this process (35). Ultimately, discovering the molecular identity of the CRAC channel expressed in T lymphocytes will vastly aid in determining the mechanisms controlling store-operated Ca\(^{2+}\) influx.

1.5 Calcium Influx Pathways in T Cells

Although the Ca\(^{2+}\) channels that regulate stored Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) stores within T lymphocytes are well characterized, the molecular identity of the CRAC channel that modulates Ca\(^{2+}\) influx across the plasma membrane still remains elusive despite the fact that it contributes to the majority of elevated intracellular Ca\(^{2+}\) during T lymphocyte activation. The search for the T lymphocyte CRAC channel, displaying identical biophysical properties to \(I_{\text{CRAC}}\), has been partially hampered since the commonly used heterologous expression systems, such as \(Xenopus\) oocytes, express endogenous SOCs (10). Overexpression of an exogenous channel cDNA may cause the association of transfected channels to endogenous SOCs with variable stoichiometry, leading to the generation of a nonphysiological current that does not resemble \(I_{\text{CRAC}}\) (10). A further challenge in identifying the CRAC channel has been the lack of high-affinity ligands or blockers of this channel (10). There is also the possibility that a single gene product may be unable to form \(I_{\text{CRAC}}\) on its own, and that \(I_{\text{CRAC}}\) may be gated by a heteromultimeric channel complex (19). Several Ca\(^{2+}\) channels that may gate Ca\(^{2+}\) influx across the plasma membrane have been identified in T lymphocytes, including IP\(_3\)R Ca\(^{2+}\) channels, mammalian homologues of the \textit{Drosophila melanogaster} TRP Ca\(^{2+}\) channels,
and L-type VDCCs. Presently, it is still under debate whether one of these Ca\textsuperscript{2+} channels is solely responsible for $I_{CRAC}$.

### 1.5.1 IP\textsubscript{3} Receptor Calcium Channels

Initially, investigators suggested that plasma membrane IP\textsubscript{3}R Ca\textsuperscript{2+} channels, similar to the IP\textsubscript{3}Rs found in the ER, were responsible for Ca\textsuperscript{2+} influx in T lymphocytes. As previously mentioned, IP\textsubscript{3} binding to the IP\textsubscript{3}R Ca\textsuperscript{2+} channel in the ER induces a conformational change, causing the IP\textsubscript{3}R channel to open. It was hypothesized that as Ca\textsuperscript{2+} in the ER is depleted, IP\textsubscript{3}R may change its conformation and this new conformational state would communicate to plasma membrane IP\textsubscript{3}R Ca\textsuperscript{2+} channels, causing the plasma membrane channels to open (71). A study conducted by Khan et al. identified IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} channels in the plasma membrane of Jurkat T cells through patch-clamp recordings, and detected cell surface iodinated IP\textsubscript{3}Rs through immunoblot analysis using an anti-IP\textsubscript{3}R Ab (92). Immunohistochemical staining of Jurkat T cells and human T lymphocytes with the anti-IP\textsubscript{3}R Ab also identified plasma membrane localized IP\textsubscript{3}Rs and capping of IP\textsubscript{3}Rs following Con A stimulation (92). Recently, a study confirmed that Jurkat T cells express IP\textsubscript{3}R Ca\textsuperscript{2+} channels as integral plasma membrane proteins (93). By labeling Jurkat T cells with the membrane-impermeant biotinylating reagent, sulfo-N-hydroxysuccinimide biotin, significant amounts of IP\textsubscript{3}R1, IP\textsubscript{3}R2, and IP\textsubscript{3}R3 were detected in the plasma membrane with isoform-specific Abs (93). However, the IP\textsubscript{3}R isoforms exhibit functional redundancy and are also abundantly expressed in the ER; defining the respective contributions of these channels to Ca\textsuperscript{2+} influx during T cell activation has therefore been difficult (93).
1.5.2 Transient Receptor Potential Calcium Channels

The most well studied molecular candidates for the T lymphocyte CRAC channel are the mammalian homologues of the *Drosophila* TRP Ca\(^{2+}\) channels. Approximately 15 years ago, TRP Ca\(^{2+}\) channels were first identified in mutant flies with impaired vision that responded to continuous light with a “transient receptor potential” (94). Shortly thereafter, the mutated gene was identified as a Ca\(^{2+}\) channel and called *trp* because of the electrical phenotype of the fly mutants (95). In the *Drosophila* retina, TRP Ca\(^{2+}\) channels are activated through a PLC-\(\beta\)-dependent process (36). Once open, TRP Ca\(^{2+}\) channels mediate Ca\(^{2+}\) influx that modulates a light-induced depolarizing current essential for visual transduction (36). Early studies on *Drosophila* TRP Ca\(^{2+}\) channels suggested that TRPs may behave as SOCs through the demonstration that TRP channels open in response to store-depletion (96). The finding that TRP activation may be store-dependent initiated a search for TRP homologues. This led to the discovery of a large superfamily of TRP Ca\(^{2+}\) channel homologues that are ubiquitously expressed in worms, mice and humans (97). Interestingly, during this time, an increasing amount of evidence began to demonstrate that *Drosophila* TRP Ca\(^{2+}\) channels are activated through store-independent mechanisms rather than by store-depletion (98).

Mammalian homologues of the *Drosophila* TRP Ca\(^{2+}\) channels have been categorized into three subfamilies based on sequence and structural homologies (Figure 1-4). The TRP canonical (TRPC) subfamily members display the greatest degree of sequence homology (30-47% over the amino-terminus) to the *Drosophila* TRP Ca\(^{2+}\) channels (99). Members of the TRP vanilloid (TRPV) subfamily are related to the vanilloid receptor 1 (TRPV1), whereas members of the TRP melastatin (TRPM)
Figure 1-4: Phylogenetic tree of the three mammalian TRP subfamilies.

The arrangement of the TRP members in the phylogenetic tree is based on sequence and structural homologies. TRP canonical (TRPC) subfamily members have the highest degree of homology to *Drosophila* TRP. TRP vanilloid (TRPV) subfamily members are most homologous to the vanilloid receptor 1. TRP melastatin (TRPM) subfamily members are most closely related to the tumor suppressor melastatin. The figure was taken from Nilius et al. (97).
subfamily, formerly known as long-TRPs (LTRPs), have the highest sequence similarity to the tumor suppressor melastatin, known as TRPM1 (97). The membrane topology of the TRP Ca\textsuperscript{2+} channels from all three subfamilies closely resembles the structure of VDCCs. Each TRP protein consists of six transmembrane segments with the TRP channel pore formed between transmembrane segments 5 and 6, and the amino- and carboxyl-termini located in the cytoplasm (Figure 1-5) (97). Four TRP proteins are required to form a functional channel unit (100). One notable difference between the basic structure of TRPs and VDCCs is that TRP Ca\textsuperscript{2+} channels lack the conserved positively charged amino acids in the fourth transmembrane segment that contribute to the voltage sensing properties of VDCCs (36). TRPC and TRPV members, but not TRPM, contain ankyrin-like binding repeats in the amino-terminus that are believed to aid in the association with the cytoskeleton (36). Multiple TRP subtypes are expressed in most cells, and consequently, it has been shown that TRP heteromultimers are formed both \textit{in vitro} and \textit{in vivo} (101, 102). In addition, the TRP subtypes are known to respond to a variety of stimuli, including temperature, pain, oxidative stress, hormones and light (36).

Currently, the TRPV6 channel, also known as CaT1 and ECaC2, appears to be the primary TRP gene candidate for the CRAC channel in T lymphocytes. Yue \textit{et al.} first demonstrated that CaT1 exhibited many biophysical properties of the CRAC channel when overexpressed in Chinese hamster ovary-K1 cells (103). Similar to I\textsubscript{CRAC}, CaT1 displayed a high Ca\textsuperscript{2+} selectivity, small 40-picoSiemens (pS) single-channel conductance (a measure of how many ions per second pass through a single channel, where conductance is the inverse of resistance, 1 Amp/1 Volt = 1S), and appeared to be
Figure 1-5: Membrane topology and structure of the three mammalian TRP subfamilies.

The TRPC, TRPV, and TRPM subfamily members each consist of six transmembrane segments with the pore loop region between transmembrane segments 5 and 6. The amino-terminus of TRPC and TRPV contain ankyrin-like repeats (designated as A) that may allow association of the channels with cytoskeletal elements. The carboxyl-terminus of TRPC and TRPM contain a small, highly conserved stretch of 25 amino acids called the TRP domain (designated as T) (97). The endogenous kinase domain (shown in red) in TRPM members regulates the activity of the channel (36). This figure was taken from Venkatachalam et al. (36).
activated by depletion of Ca$^{2+}$ stores (103, 104). Subsequently, CaT1 transcripts were detected in Jurkat T cells and human spleen through reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analysis (105). Further support for CaT1 was achieved when a dominant negative pore-region mutant of CaT1, overexpressed in Jurkat T cells, partially suppressed endogenous $I_{\text{CRAC}}$ following thapsigargin-induced store-depletion (105). Although these studies suggested that CaT1 formed part of or the entire CRAC channel, Voets et al. revealed that rat basophil leukemia and human embryonic kidney cells overexpressing CaT1 displayed many biophysical properties distinct from $I_{\text{CRAC}}$ (106). Central differences between CaT1 and CRAC were that CaT1 is five times more permeant to monovalent cations compared to the CRAC channel, and that the CaT1 pore is blocked by intracellular magnesium ions, whereas in the CRAC channel this gating mechanism is absent (106). Further work on $I_{\text{CRAC}}$ has shown that the CRAC single-channel conductance is only 0.2-pS, and not 40-pS as previously described by Yue et al. (107). It is now accepted that the 40-pS single-channel conductance is attributed to endogenous magnesium-inhibited cation channels (79). These discrepancies between CaT1 and CRAC have lead investigators to reinterpret previous results on CaT1, proposing that CaT1 is not solely responsible for $I_{\text{CRAC}}$ (97). Even though the CaT1 gene product does not appear to exhibit all of the electrophysiological properties associated with $I_{\text{CRAC}}$, it still remains possible that CaT1 may participate with additional TRP subtypes, such as the highly homologous TRPV5 that is also expressed in Jurkat T cells, to form a functional CRAC channel (79). Further work on CaT1 is required to definitely establish whether CaT1 can recapitulate $I_{\text{CRAC}}$ when overexpressed with other TRP subtypes.
TRP subfamily members that are activated by store-independent mechanisms have also been identified in T lymphocytes. The TRPM2 subfamily member, LTRPC2, was recently detected in Jurkat T cells and peripheral blood through RT-PCR (108). In T lymphocytes, LTRPC2 is a non-selective Ca\(^{2+}\) channel that mediates Ca\(^{2+}\) influx in response to the intracellular second messengers, ADPR and NAD\(^+\), but is not activated by store-depletion (108). A recent study by Gamberucci et al. demonstrated that the second messenger, DAG, is capable of generating influx of Ca\(^{2+}\) in Jurkat T cells that was independent of intracellular Ca\(^{2+}\) store-depletion. Since the TRPC subfamily members, TRPC3 and TRPC6 are known to be activated by DAG the expression of TRPCs was examined in Jurkat T cells and human PBTs (109). Although TRPC1, 3, 4 and 6 transcripts were identified through RT-PCR, only the TRPC6 protein was detected in purified plasma membrane fractions, suggesting that TRPC6 is activated by DAG in T lymphocytes (110). Taken together, these recent studies on TRP channel function demonstrate that store-independent Ca\(^{2+}\) influx through LTRPC2 and TRPC6 channels may also participate in sustained Ca\(^{2+}\) signaling during T cell activation. It is still under debate whether LTRPC2 and TRPC6 Ca\(^{2+}\) influx pathways are activated through a TCR-dependent mechanism.

1.5.3 Voltage-Dependent Calcium Channels

There is also evidence to support the existence of voltage-dependent-like Ca\(^{2+}\) channels in the plasma membrane of T lymphocytes. The basis of the VDCC model is that non-excitable cells, such as T lymphocytes, may express a Ca\(^{2+}\) channel that shares common structural features with a VDCC of electrically excitable cells but is not gated
by changes in membrane potential. VDCCs are abundantly expressed in electrically
excitable cells, such as neurons and muscle cells, and activated in response to
depolarization of the plasma membrane (111). Membrane depolarization is a change in
the electrical potential across the plasma membrane that renders the membrane less
polarized (less negative) than the resting potential. In excitable cell types, Ca\(^{2+}\) influx
through VDCCs acts as a second messenger, controlling many intracellular events such
as contraction, secretion, synaptic transmission, and gene expression (112).

Through patch-clamp and pharmacological studies, VDCCs have been classified
into different channel families based on their distinct Ca\(^{2+}\) currents (113). The high
voltage-activated Ca\(^{2+}\) channels that are activated at more positive membrane potentials
include L- (long lasting current), P- (Purkinje cell), Q- (granular cell), N- (neuronal), and
R- (toxin-resistant) type channels (Figure 1-6) (113). The T-type (transient current)
channels are designated as the low voltage-activated Ca\(^{2+}\) channels due to their activation
at negative membrane potentials (112). VDCCs are heteromultimeric protein complexes
consisting of the channel-forming \(\alpha_1\)-subunit and at least three auxiliary subunits called
\(\beta\), \(\alpha_2\delta\), and \(\gamma\), which control the structure and activity of the \(\alpha_1\)-subunit (111) (Figure 1-
7). The primary structure of the \(\alpha_1\)-subunit is composed of four repeated motifs (I to IV),
each consisting of six \(\alpha\)-helical transmembrane segments (S1 to S6) and a loop between
S5 and S6 transmembrane segments that is membrane-associated and forms the channel
pore (112). The S4 transmembrane segments, containing conserved positively charged
amino acids, are termed the voltage sensors and are believed to move outwards upon
membrane depolarization, opening the Ca\(^{2+}\) channel (114). The amino- and carboxyl-
termini of VDCCs reside in the cytoplasm.
### Figure 1-6: Nomenclature, chromosome location and tissue expression of the different channel-forming α1-subunits.

Based on amino acid sequence identity and physiological properties of the α1-subunits of VDCCs, a cohesive nomenclature for the α1-subunits was established (115). High voltage-activated Ca\(^{2+}\) channels include the Cav1 family of α1-subunits that conduct L-type Ca\(^{2+}\) currents and the Cav2 family conduct that either P/Q-, N- or R-type currents. The Cav3 channel family conducts the low voltage-activated Ca\(^{2+}\) currents, which are T-type currents. α1-subunits in the Cav1 family have 75% amino acid identity to the skeletal muscle α1S-subunit (Cav1.1). The Cav1 channel family in its entirety has approximately 40-50% amino acid identity to the Cav2 family, whereas the Cav3 family has less than 25% identity to both Cav1 and Cav2 families (112). The figure was taken from Jurkat-Rott et al. (114).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene</th>
<th>Chromosome</th>
<th>Primary tissues</th>
<th>Calcium current</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(<em>{1.1}) (α(</em>{1.1}))</td>
<td>CACNA1S</td>
<td>1q32</td>
<td>skeletal muscle</td>
<td>L-type</td>
</tr>
<tr>
<td>Ca(<em>{1.2}) (α(</em>{1.2}))</td>
<td>CACNA1C</td>
<td>12p13.3</td>
<td>heart, smooth muscle, brain, heart, pituitary, adrenal</td>
<td>L-type</td>
</tr>
<tr>
<td>Ca(<em>{1.3}) (α(</em>{1.3}))</td>
<td>CACNA1D</td>
<td>3p14.3</td>
<td>brain, pancreas, kidney, ovary, cochlea</td>
<td>L-type</td>
</tr>
<tr>
<td>Ca(<em>{1.4}) (α(</em>{1.4}))</td>
<td>CACNA1F</td>
<td>Xp11.23</td>
<td>retina</td>
<td>L-type</td>
</tr>
<tr>
<td>Ca(<em>{2.1}) (α(</em>{2.1}))</td>
<td>CACNA1A</td>
<td>19p13</td>
<td>brain, cochlea, pituitary</td>
<td>P/Q-type</td>
</tr>
<tr>
<td>Ca(<em>{2.2}) (α(</em>{2.2}))</td>
<td>CACNA1B</td>
<td>9q34</td>
<td>brain, nervous system</td>
<td>N-type</td>
</tr>
<tr>
<td>Ca(<em>{2.3}) (α(</em>{2.3}))</td>
<td>CACNA1E</td>
<td>1q25-31</td>
<td>brain, cochlea, retina, heart, pituitary</td>
<td>R-type</td>
</tr>
<tr>
<td>Ca(<em>{3.1}) (α(</em>{3.1}))</td>
<td>CACNA1G</td>
<td>17q22</td>
<td>brain, nervous system</td>
<td>T-type</td>
</tr>
<tr>
<td>Ca(<em>{3.2}) (α(</em>{3.2}))</td>
<td>CACNA1H</td>
<td>18p13.3</td>
<td>brain, heart, kidney, liver</td>
<td>T-type</td>
</tr>
<tr>
<td>Ca(<em>{3.3}) (α(</em>{3.3}))</td>
<td>CACNA1I</td>
<td>22q12.3-13.2</td>
<td>brain</td>
<td>T-type</td>
</tr>
</tbody>
</table>
VDCCs are oligomeric protein complexes that consist of the 170-250 kDa channel-forming \( \alpha_1 \)-subunit, as well as the auxiliary subunits: 50-78 kDa \( \beta \)-subunit, \( \sim 170 \) kDa \( \alpha_2 \delta \)-subunit, and \( \sim 36 \) kDa \( \gamma \)-subunit (113). The \( \alpha_1 \)-subunit is composed of four motifs (I to IV), which each contain six \( \alpha \)-helical transmembrane segments that are represented as cylinders. The S4 transmembrane segments (red cylinders) in the \( \alpha_1 \)-subunit contain evenly spaced, positively charge amino acids that contribute to the voltage sensing properties of the channel. The \( \alpha_2 \) and \( \delta \) proteins are heavily glycosylated and disulfide-linked (116). The auxiliary subunits modulate the activation and inactivation properties of the \( \alpha_1 \)-subunit, as well as aid in targeting the \( \alpha_1 \)-subunit to the plasma membrane (113).

The figure was adapted from Catterall et al. (112).
Even though the $\alpha_i$-subunits are similar in structure, the $\text{Ca}^{2+}$ currents gated by these channels control many different cellular functions in electrically excitable cells. The L-type $\alpha_{1D}$-subunit (also designated as $\text{Cav}1.3$) in endocrine cells initiates the release of hormones, whereas the L-type $\alpha_{1F}$-subunit ($\text{Cav}1.4$) in retina controls neurotransmitter release (112). Other L-type $\text{Ca}^{2+}$ currents, including the currents gated by $\alpha_{1S}$-subunit ($\text{Cav}1.1$) in skeletal muscle and $\alpha_{1C}$-subunit ($\text{Cav}1.2$) in cardiac muscle, promote excitation-contraction coupling (112). The $\alpha_{1A}$-, $\alpha_{1B}$-, and $\alpha_{1E}$-subunits that gate P/Q-, N- and R-type currents, respectively, are the primary $\text{Ca}^{2+}$ currents in neurons (112).

Initial support for the presence of voltage-dependent-like $\text{Ca}^{2+}$ channels in T lymphocytes came when Densmore et al. identified an electrically responsive current in the plasma membrane of Jurkat T cells through patch-clamp recordings (117, 118). This "voltage-operable" current in Jurkat T cells was activated through the TCR/CD3 complex and $\text{Ca}^{2+}$ store-depletion (117, 118). RT-PCR analysis has also shown that transcripts of the pore-forming $\alpha_{1C}$- and $\alpha_{1S}$-subunits of L-type VDCCs are expressed in Jurkat T cells (119). Savignac et al. demonstrated that murine T cell hybridomas express L-type $\text{Ca}^{2+}$ channel messenger RNA (mRNA) and protein (120). In addition, several pharmacological studies examining the effects of DHPs, a class of synthetic derivatives that specifically modulate L-type VDCC function, have provided further evidence to support the existence of VDCCs in T lymphocytes. Although T lymphocytes are considered to be non-excitable cells, the functional significance of L-type VDCCs and their potential contribution to $I_{\text{CRAC}}$ has not been thoroughly investigated. Furthermore, the hypothesis that voltage-dependent-like $\text{Ca}^{2+}$ channels may control a novel $\text{Ca}^{2+}$ influx pathway in T lymphocytes is an attractive model that has not received a great deal of
attention in recent years compared to TRP Ca\textsuperscript{2+} channels. To address these questions, over the next three chapters, the expression of one channel-forming $\alpha_1$-subunit of L-type VDCCs and its putative role during T cell activation will be evaluated through both molecular and pharmacological analyses.

Despite the importance of Ca\textsuperscript{2+} influx in T lymphocyte activation, the mechanism and regulation of Ca\textsuperscript{2+} entry into T cells is still under debate. A combination of the proposed channel models may be necessary to maintain intracellular Ca\textsuperscript{2+} levels required for T cell activation. It is clear that future investigations, such as elucidating the mechanism coupling store-depletion to CRAC channel activation and the molecular identification of the CRAC channel, are necessary to clarify the mysteries surrounding Ca\textsuperscript{2+} entry in T lymphocytes.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Lines and Culture Conditions

The human retinoblastoma WERI-Rb-1 cell line and the human T cell leukemia line Jurkat clone E6-1 were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and maintained in RPMI (Roswell Park Memorial Institute) 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2 mM glutamine, 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and 1 mM sodium pyruvate. The human cervical carcinoma HeLa cell line (ATCC) was maintained DMEM (Dulbecco’s Modified Eagle Medium) (Invitrogen) supplemented with 10% FBS, 2 mM glutamine, 20 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 U/ml streptomycin (StemCell Technologies Inc., Vancouver, Canada).

2.2 Isolation and Culture of Human Peripheral Blood T Lymphocytes

Whole blood (10-50 ml) was collected from healthy human male and female donors (n=15). Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation at 900 x g for 30 min at 18-20°C over a Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ) gradient. The resulting PBMC layer was washed and resuspended in RPMI supplemented with 10% FBS, 2 mM glutamine, 20 mM HEPES and 1 mM sodium pyruvate. PBMCs were then stimulated for 24 h with 10 µg/ml plate-bound anti-CD3 mAb, OKT3 (ATCC), and resuspended in RPMI containing 5 ng/ml
recombinant human IL-2 (rhIL-2) (Serologicals Corporation, Norcross, GA). After growing for 7 d in rhIL-2, the purity of the human PBTs was analyzed on a fluorescent-activated cell sorter (FACS) Calibur cytometer (BD Biosciences, San Jose, CA) with fluorescein isothiocyanate (FITC)-conjugated OKT3, FITC-conjugated mouse anti-immunoglobulin (Ig)G2a isotype control (Caltag, Burlingame, CA), Cy-Chrome-5-conjugated anti-CD4 mAb (Pharootingen, San Diego, CA), phycoerythrin (PE)-conjugated anti-CD8 mAb (Pharootingen), allophycocyanin (APC)-conjugated anti-CD14 mAb (Pharootingen), FITC-conjugated anti-CD15 mAb (Pharootingen), and PE-conjugated anti-CD19 mAb (Pharootingen). Experiments with the activated human PBTs were conducted with cells from day 8-14 in culture. The cultured human PBTs were used in assays where large numbers of T cells were required, such as measurement of Ca\(^{2+}\) kinetics, immunoblotting, IL-2 assay and IL-2R expression.

2.3 Cell Separations

Whole blood (~20 ml) was collected from healthy human male and female donors (n=3). PBMCs were separated by centrifugation at 900 x g for 30 min at 18-20°C over a Ficoll-Paque PLUS (Amersham Biosciences), washed and incubated with FITC-conjugated OKT3, PE-conjugated anti-CD19 mAb (Pharootingen), or APC-conjugated anti-CD14 mAb (Pharootingen) to selectively label naïve T cells, B cells and monocytes, respectively. The FACS Vantage Sort Enhanced (SE) flow cytometry system (BD Biosciences) was used to sort the labeled cells into individual populations and assess the purity of the sorted cells. Magnetic cell sorting (MACS) CD4 and CD8 microbeads (Miltenyi Biotec, Auburn, CA) were used to positively select and separate human CD4\(^+\)
and CD8\(^+\) T cells from PBTs that were isolated and cultured from whole blood as previously described. The purity of the CD4\(^+\) and CD8\(^+\) T cell populations was analyzed on a FACSCalibur cytometer (BD Biosciences) with FITC-conjugated OKT3, FITC-conjugated mouse anti-IgG\(_{2a}\) isotype control (Caltag), Cy-Chrome-5-conjugated anti-CD4 mAb (Pharmingen), and PE-conjugated anti-CD8 mAb (Pharmingen). The sorted naïve T cells, B cells, monocytes, and CD4\(^+\) and CD8\(^+\) T cells were used in the nested RT-PCR experiments.

For the study involving renal disease patients, whole blood (~10 ml) was obtained from the renal patients (n=3) prior to hemodialysis by Drs. Adeera Levin and Kathryn Tinckman in the Nephrology Division at St. Paul's Hospital, Vancouver, Canada. Whole blood (~10 ml) was also collected from a healthy human female donor (n=1). Human CD3\(^+\) PBTs were separated from whole blood using the Human T cell RosetteSep\(^\text{TM}\) Ab cocktail (StemCell Technologies). In brief, RosetteSep\(^\text{TM}\) Ab cocktail (50 μl per ml blood) containing bispecific Ab complexes to CD16, CD19, CD36 and CD56 and glycophorin A was incubated with whole blood for 20 min at room temperature (RT) to cross-link unwanted cells to red blood cells. Enriched human CD3\(^+\) PBTs were isolated by centrifugation at 900 x g for 30 min at 18-20\(^\circ\)C over a Ficoll-Paque PLUS (Amersham Pharmacia) gradient, removed from the density medium/plasma interface, and washed. The purity of the human CD3\(^+\) PBTs was then analyzed on a FACSCalibur cytometer (BD Biosciences) with FITC-conjugated OKT3, FITC-conjugated mouse anti-IgG\(_{2a}\) isotype control, Cy-Chrome-5-conjugated anti-CD4 mAb, and PE-conjugated anti-CD8 mAb.
2.4 Nested RT-PCR of α_{1F}-Subunit and DNA Sequencing

First strand cDNAs were synthesized with an oligo 2'-deoxy-thymidine (dT) primer using 1 μg of total RNA extracted from WERI-Rb-1 cells, Jurkat T cells, human PBTs, CD4\(^+\) T cells, and CD8\(^+\) T cells with the RNeasy Kit (Qiagen, Alameda, CA). Marathon-ready human retina and human spleen cDNA were purchased from Clontech (Palo Alto, CA) and FirstChoice PCR-Ready human liver cDNA was from Ambion (Austin, TX). RT-PCR fragments spanning exons 28 to 35 of the α_{1F}-subunit cDNA sequence, (GenBank accession number AF067227 from the National Centre for Biotechnology Information database), were generated with sense primer (5'-'GGACCATGGCCCCCATCTATAATTACCG-3') and antisense primer (5'-'CCTGAAGAGCCACCTTGCCGAAC-3'). For nested amplification of the α_{1F}-subunit cDNA sequence, PCR fragments of ~180 base pairs (bp) spanning exons 29 to 30 were generated with sense primer (5'-'GAACCCGCATCAGTATCGTG-3') and antisense primer (5'-'AATAGTGAAGGCCAGTGAAGACC-3'). The housekeeping gene, rig/S15, which encodes a small ribosomal subunit protein that is constitutively expressed in all tissues, was amplified with sense primer (5'-'TTCCGCAAGTTCCACCTACC-3') and antisense primer (5'-'CGGGCCGGCCCATGCTTTACG-3') (121). RT-PCR and nested PCR reactions were performed with Platinum Taq polymerase (Invitrogen) and were conducted in a Whatman Biometra UnoII Thermocycler at 94°C for 1 min, then 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, followed by a 10 min extension at 72°C. PCR fragments were resolved on a 1% agarose gel and visualized by staining with ethidium bromide. The resulting 178 bp fragments were subcloned into
pCR2.1-TOPO vector (Invitrogen) and the nucleotide sequence determined using standard m13R primer at the DNA Sequencing CORE Facility, University of Florida, FL.

Nested RT-PCR was also used to investigate the mRNA expression of the two splice variants of the α_{1F}-subunit in Jurkat T cells, human PBTs, CD4^+ T cells, CD8^+ T cells, and peripheral blood B cells and monocytes. Total RNA was extracted from the cells using the RNeasy Kit (Qiagen). 1 μg of total RNA was used to synthesize first strand cDNAs with an oligo(dT) primer. RT-PCR fragments of the α_{1F}-subunit cDNA sequence were generated as previously described. For nested amplification of the voltage negative splice variant of the α_{1F}-subunit, PCR fragments (~240 bp) spanning exons 29 to 35 were generated with sense primer (5'-CCCAAGAACCAGCATTACAGTATCGTG-3') and an antisense primer (5'-AGCCACCTTGCGACATCTTGGGCTT-3'), which was designed to specifically overlap the exon 30 to 35 splice junction. To amplify the voltage positive splice variant of the α_{1F}-subunit, nested PCR was conducted using the sense primer (5'-CCCAAGAACCAGCATTACAGTATCGTG-3') and the antisense primer (5'-ACTGAGAAGCTTGACGCGTATAAAC-3') to generate a ~380 bp PCR fragment spanning exons 29 to 33. The rig/S15 house-keeping gene served as a loading control. RT-PCR and nested PCR reactions were performed with Advantage-GC 2 polymerase (Clontech) and were conducted in a Whatman Biometra UnoII Thermocycler at 94°C for 1 min, then 40-45 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, followed by a 10 min extension at 72°C. An ethidium bromide stained 1% agarose gel was used to resolve the PCR fragments. The PCR products were subcloned into pCR2.1-TOPO vector (Invitrogen) and sequenced using the m13R primer at the Florida DNA Sequencing CORE Facility.
2.5 Cloning of $\alpha_{1F}$-Subunit cDNA from Human Retina and Spleen Libraries

Marathon-ready human retina and human spleen cDNA libraries (Clontech, Palo Alto, CA) were used to clone the wild type and alternatively spliced $\alpha_{1F}$-subunit cDNA sequences, respectively. The 3'-terminal sequence of the $\alpha_{1F}$-subunit cDNA sequence, corresponding to nucleotides 3328-5813 (nucleotides numbered according to GenBank accession number AF067227), from both libraries was cloned using rapid amplification of cDNA ends (RACE) with gene specific sense primers, adaptor antisense primers, and the Advantage-GC 2 polymerase in the presence of 0.5 M GC Melt (Clontech). The gene specific sense primer for the first PCR was 5'-GGACCATGGCCCCCATGTATAATTACC-3' and for the nested PCR was 5'-CCCAAGAAACCCGCATCAGTATCGTG-3'. The remainder of the $\alpha_{1F}$-subunit cDNA sequence from human retina and spleen libraries was cloned by a series of nested PCR reactions with sense and antisense primers overlapping exon/exon boundaries using the Advantage-GC 2 polymerase. Three individual PCR products were generated from the nested PCR reactions, which correspond to nucleotides 1106-1898, 1750-2635 and 2442-3546 of the $\alpha_{1F}$-subunit cDNA sequence (Figure 2-1). The 5'-terminal sequence of the $\alpha_{1F}$-subunit cDNA (1-1320 bp) was cloned using nested PCR with a 60-oligonucleotide sense primer beginning with the 5'-ATG start codon, 5'-ATGTCGGAATCTGAAGGCGGGAAAGGTGAGAGAATCCTTCCATCCCTTGGA-3', and an antisense primer overlapping an exon/exon boundary. All PCR products were subcloned into pCR2.1-TOPO vector (Invitrogen) and sequenced using standard m13R and T7 primers at the Florida DNA Sequencing CORE Facility. Subsequently, the full-length cDNAs of the retina $\alpha_{1F}$-subunit and voltage negative $\alpha_{1F}$-
Figure 2-1: PCR cloning strategy used to isolate $\alpha_{1F}$-subunit cDNA sequences from human retina and spleen cDNA libraries.

The coding region of the human retina $\alpha_{1F}$-subunit is 5813 bp. Five PCR fragments, represented as black boxes, correspond to different coding regions of the $\alpha_{1F}$-subunit that were amplified from both the human retina and spleen cDNA libraries, using $\alpha_{1F}$-subunit specific primers. The PCR fragments specifically correspond to 1-1320 bp, 1106-1898 bp, 1750-2635 bp and 2442-3546 bp and 3328-5813 bp of the $\alpha_{1F}$-subunit cDNA sequence (GenBank accession number AF067227). Unique, endogenous restriction enzyme sites, including AatII, SphI, SacII and BbsI, were used to ligate the five PCR fragments together to form full-length cDNA sequences for human retina and spleen (voltage negative splice variant) $\alpha_{1F}$-subunits. The location of each restriction enzyme site in the $\alpha_{1F}$-subunit cDNA sequence is indicated by the bp position.
subunit splice isoform amplified from human spleen were constructed through ligation of the individual nested PCR products by a single, common restriction enzyme site in the overlapping regions (Figure 2-1). Once the individual PCR products were ligated together, the full-length cDNAs were subcloned into pCR2.1-TOPO vector.

2.6 Production of Carboxyl-Terminal FLAG-tagged \( \alpha_{1F} \)-Subunit

The coding regions of retina \( \alpha_{1F} \)-subunit previously subcloned in pCR2.1-TOPO vectors (Invitrogen) was amplified by PCR using the NotI containing sense primer (5'-GCAAGCGGCCGATGTAATCCGGAATTCTGAAGGCGGGAA-3') and the SalI containing antisense primer (5'-CGCGTCGACTGAGGGCGTGGACGACGAGCCAT-3'). A PCR fragment of ~5.8 kilobases (kb) with the TAG stop codon removed was generated for the retina \( \alpha_{1F} \)-subunit, using the Advantage-GC 2 polymerase (Clontech). PCR reactions were conducted in a Whatman Biometra UnoII Thermocycler at 94°C for 3 min, then 25 cycles of 94°C for 1 min, 68°C for 1 min, and 72°C for 6 min, followed by a 10 min extension at 72°C. After digestion with NotI and SalI (New England BioLabs), the amplified retina \( \alpha_{1F} \)-subunit was subcloned in-frame with sequences encoding a FLAG-tag into the pIRES-hrGFP-1a mammalian expression vector (pGFP) (Stratagene, La Jolla, CA) (Figure 2-2). The resulting pGFP/Retina \( \alpha_{1F} \)-subunit vector dicistronically expressed the retina \( \alpha_{1F} \)-subunit with a FLAG-tag at the carboxyl-terminus and humanized recombinant green fluorescent protein (hrGFP), which served as a marker for transfected cells. After ligation into the pGFP vector, the nucleotide sequence of the retina \( \alpha_{1F} \)-subunit insert was determined by primer walking at the Florida DNA Sequencing CORE Facility.
Figure 2-2: The pIRES-hrGFP-1a mammalian expression vector.

The pIRES-hrGFP-1a vector (Stratagene) contains a dicistronic expression cassette where the multiple cloning site is followed by an internal ribosomal entry site (IRES) linked to the hrGFP coding sequence. The α₁F-subunit was ligated into the multiple cloning site through NotI and SalI restriction enzyme sites, and the expression of the α₁F-subunit was under the control of the cytomegalovirus (CMV) promoter. The carboxyl-terminus of the α₁F-subunit was also fused in-frame to three contiguous copies of the FLAG epitope (DYKDDDDK). The pIRES-hrGFP-1a vector lacks drug-resistant markers for stable expression in mammalian cells.
2.7 Immunofluorescence Staining

2.5x10⁴ HeLa cells grown on coverslips were transiently transfected with either 2 μg of pGFP or pGFP/Retina α₁F-subunit vectors by using FuGene 6 (Roche). 72 h after transfection, FLAG-tagged protein expression was determined following the methods of Moise et al. (122). In brief, cells were fixed with 2% paraformaldehyde for 20 min and permeabilized with 0.1% saponin in 2% bovine serum albumin (BSA) for 15 min, where indicated. Cells that were not permeabilized were incubated with 15% BSA for 15 min. 2% BSA was used to block cells for 1 h, followed by staining with the mouse M2 anti-FLAG mAb (Sigma) in 2% BSA for 30 min. After mAb incubation, cells were washed thoroughly with 2% BSA, stained in the dark with Alexa Fluor 568 goat anti-mouse Ab (Molecular Probes, Eugene, OR) in 2% BSA for 30 min at RT, and washed with 2% BSA. Cells were then treated with SlowFade Antifade (Molecular Probes), and staining was analyzed by confocal microscopy with a Bio-Rad Radiance Plus on an inverted Zeiss Axiovert with DIC optics and Lasersharp software (Bio-Rad).

2.8 Immunoprecipitation Analysis of α₁F-Subunit

20-30x10⁶ WERI-Rb-1 cells, Jurkat T cells (untreated or OKT3 and 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulated) and human PBTs were washed and detergent-lysed in 500 μl of solubilization buffer containing 50 mM Tris-HCl (pH 7.5), 300 mM NaCl and 0.5% Triton X-100 in the presence of 10 μg/ml soybean trypsin inhibitor, pepstatin, and 40 μg/ml phenylmethanesulfonyl fluoride (PMSF) (Sigma, St. Louise, MO) for 45 min on ice. Protein concentration of the lysates was quantified by
Bicinchonic acid protein assay (Pierce, Rockford, IL). Affinity-purified antibodies against a human retina α₁F-subunit peptide (85-90 amino acids of cytosolic domain I-II interlinker sequence) were raised in rabbit and used to detect α₁F-subunit (provided by Dr. John Mcrory, University of Calgary, Calgary, Canada). Aliquots of the membrane-enriched lysates (1.5 mg of protein) were incubated overnight at 4°C with either 2 μg of α₁F-subunit Ab or affinity-purified rabbit IgG Ab (Sigma) as a control. 30 μl of recombinant protein G sepharose (Amersham Pharmacia) was added for 1 h at 4°C and Ab/α₁F-subunit complexes bound to protein G were then washed 2 x with 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.2 % Nonidet P-40, 1 x with 10 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 2 mM EDTA, 0.2 % Nonidet P-40, and 1 x with 10 mM Tris-HCl (pH 7.5) at 4°C. Samples were denatured by boiling in sodium dodecyl sulfate (SDS) sample buffer, run on 8% SDS-polyacrylamide gel electrophoresis (PAGE) gel and transferred to polyvinylidene fluoride (PVDF) membrane. Western blot analysis was performed with affinity-purified α₁F-subunit rabbit polyclonal Ab.

Simultaneously, a Western blot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was performed as a protein loading control. In brief, 10 μg of each protein lysate was denatured by boiling in SDS sample buffer, loaded on 12% SDS-PAGE gel and transferred to nitrocellulose membrane. Western blot analysis was conducted with mouse anti-GAPDH mAb (Chemicon, Temecula, CA). α₁F-Subunit protein expression in Jurkat T cells stably expressing pSUPER, pSUPER-α₁F-subunit constructs or pEYFP alone were also analyzed through α₁F-subunit protein immunoprecipitation.
2.9 Flow Cytometry Analysis of α_{iF}-Subunit

α_{iF}-Subunit protein expression was determined through flow cytometry by staining 0.1% saponin (Sigma) treated or untreated WERI-Rb-1 cells, Jurkat T cells or human PBTs with affinity-purified α_{iF}-subunit rabbit polyclonal Ab and FITC-conjugated goat anti-rabbit IgG Ab (Jackson ImmunoResearch, West Grove, PA). As a control for Ab specificity, α_{iF}-subunit Ab was preincubated for 15 min at RT with the immunogen (85-90 amino acid peptide of domain I-II interlinker region of human retina α_{iF}-subunit conjugated to the *Caulobacter* RSA protein) in a 1:1 ratio. Ab/immunogen complex was directly added to saponin treated cells and FL1 fluorescence was detected by the addition of FITC-conjugated goat anti-rabbit IgG Ab. As an additional control, cells treated with saponin were also stained with affinity-purified rabbit IgG Ab (Sigma) followed by FITC-conjugated goat anti-rabbit IgG Ab. All data acquisition was performed on a FACSCalibur cytometer (BD Biosciences).

2.10 Real-Time PCR

Quantitative detection of the voltage negative and positive splice variants of the α_{iF}-subunit, and the LTRPC2 Ca^{2+} channel was carried out by real-time PCR with SYBR Green *Taq* Readymix (Sigma) on a LightCycler Instrument (Roche, Indianapolis, IN). For the real-time PCR experiments, the Human T cell RosetteSep™ Ab cocktail (StemCell Technologies) was used to isolate naïve human PBTs from whole blood (20 ml) of healthy human donors (n=3), as previously described. Jurkat T cells or naïve human PBTs at 1x10^6 cells/ml were either untreated or stimulated with 10 μg/ml soluble...
OKT3 and 10 nM TPA (Sigma) for 1 min, 5 min, 10 min, 1 h, and 4 h at 37°C. Following stimulation, total RNA was extracted from cells using the RNeasy Kit (Qiagen) and 1 μg of total RNA was used to synthesize first strand cDNAs with an oligo(dT) primer. For amplification of the voltage negative and positive splice variants of the α1F-subunit, nested PCR was performed on RT-PCR reactions (diluted 1:20 for Jurkat T cells and 1:10 for PBTs) with the previously described splice variant specific primers. The LTRPC2 gene was amplified from cDNA (diluted 1:20 for Jurkat T cells and 1:10 for PBTs) with sense primer (5'-TCTCCGGCGCAGCAACAGCA-3') and antisense primer (5'-CCCTCGCGGCGGTGGACAGT-3') to generate a ~660 bp PCR product (108). The rig/S15 gene amplified using 1:20 diluted Jurkat T cell and 1:10 diluted PBT cDNA, served as a control to normalize expression of the Ca\(^{2+}\) channel mRNA. Real-time PCR was conducted at 95°C for 300 sec, then 30 cycles of 95°C for 5 sec, 55-66°C for 5 sec (depending on the cDNA sequence amplified), and 72°C for 30 sec, followed by a 30 sec cooling to 40°C. PCR products were resolved on a 1% agarose gel and visualized by staining with ethidium bromide to confirm fragment size.

Real-time PCR data analysis was conducted by first calculating the ΔCt value (ΔCt = Ct (cDNA of interest) – Ct (S15 cDNA)) of each sample, where Ct is defined as the cycle number at which fluorescence passes through the fixed threshold, and is inversely proportional to the template starting copy number. The ΔΔCt value is then calculated by subtracting the ΔCt of the untreated control from the ΔCt of the treated sample (ΔΔCt = ΔCt (treated sample) – ΔCt (untreated sample)). Finally, the fold induction is derived from the equation \(2^{-\Delta\Delta Ct}\), where in each experiment the untreated
sample has a fold induction of one. All formulations were derived from Applied Biosystems, Foster City, CA.

2.11 Construction of Plasmids for Stable siRNA Expression

The mammalian expression vector, pSUPER (suppression of endogenous RNA), was used for stable expression of small interfering RNA (siRNA) in Jurkat T cells (provided by Dr. Reuven Agami, Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, Netherlands) (Figure 2-3). Four siRNA target sequences (19 nucleotides in length) against the α1F-subunit mRNA sequence were chosen using Ambion and Oligoengine (Seattle, WA) Web-Based siRNA Target Finder and Design Tools. The 19 nucleotide target sequences began with two adenine nucleotides at the 5’-terminus, contained 40-60% GC nucleotides, and were located in the coding region of the α1F-subunit mRNA at least 100 bp after the start of translation. As a control for successful mRNA knockdown, a siRNA target sequence directed against p53 mRNA was also selected (123). The Blastn Program confirmed that the chosen 19 nucleotide sequences did not cross-react with other mRNA sequences. According to the methods of Brummelkamp et al., the siRNA sequences were each synthesized as forward and reverse 64 bp synthetic DNA oligonucleotides (Sigma), containing the 19 nucleotide sense target sequence, which was separated by a 9 nucleotide non-complementary spacer (tctcttgaa) from the reverse complement of the same 19 nucleotide target sequence (Table 2-1) (123). The 64 bp forward and reverse oligonucleotides were annealed at 70°C, phosphorylated with T4 polynucleotide kinase (Invitrogen), and ligated into the pSUPER vector after digestion with BgIII and HindIII (New England BioLabs Inc., Beverly, MA).
The polymerase III H1-RNA gene promoter in the pSUPER vector directs the synthesis of siRNA transcripts that form short hairpin structures with a 19 bp double stranded region and a short 9 bp loop formed from the spacer region. The siRNA transcript lacks a polyadenosine tail, but contains a termination signal consisting of a row of five thymidines. The hairpin transcript also contains two uridines at the 5'-end of the 9 bp loop, as well as two uridines at the 3'-terminus of the transcript. When the pSUPER vector is expressed, the hairpin loop is cleaved by the endogenous Dicer enzyme and a final siRNA transcript is formed with 5'- and 3'-UU overhangs, which is an optimal siRNA structure for targeted gene silencing. The pSUPER vector also lacks drug-resistant markers for stable expression in mammalian cells, and therefore requires co-transfection with pEYFP.
### pSUPER Constructs

<table>
<thead>
<tr>
<th>pSUPER Constructs</th>
<th>Target mRNA Region</th>
<th>Forward and Reverse 64 bp siRNA Oligonucleotides</th>
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| pSUPER-α₁F-subunit-1 | 288-306 bp | 5'-GATCCCCCTGGGCTGGAAACCTACTCGACttcaagaga  
GTCGAGTACGTTCCAGCCAATTGGAAAAA-3'  
5'-AGCTTTTCCAAAAATGGCTGAACTACTCGAC  
tctctggaGTCGAGTACGTTCCAGCCAATTGGAAAA-3' |
| pSUPER-α₁F-subunit-2 | 489-507 bp | 5'-GATCCCCCTGACATGAAAGGCTCTGttcaagaga  
CAGAGCTTTGATGAAATTCCATCATGAAAGGCTCTG  
tctctggaCAGAGCTTTGATGAAATTCCATCATGAAAGGCTCTG-3' |
| pSUPER-α₁F-subunit-3 | 600-618 bp | 5'-GATCCCCCGAGTCTACTCTCTGAGGAAttcaagaga  
TCCAGGAAATTGACGCTCTCTTTGGAAAA-3'  
5'-AGCTTTTCCAAAAATGCTCATGAAAGGCTCTG  
tctctggaTCCAGGAAATTGACGCTCTCTTTGGAAAA-3' |
| pSUPER-α₁F-subunit-4 | 750-768 bp | 5'-GATCCCCCTTCTTCTTGAGCAGGTAAACttcaagaga  
CAGCATGGCCAGGAAGGGAATTTGGAAAA-3'  
5'-AGCTTTTCCAAAAATGGCTGAACTACTCGAC  
tctctggaCAGCATGGCCAGGAAGGGAATTTGGAAAA-3' |
| pSUPER-p53 | 838-856 bp | 5'-GATCCCCGCTTTGTTGATGAAATCttcaagaga  
GTGAGTTTACCTGGAGTCTTTTTGGAAAA-3'  
5'-AGCTTTTCCAAAAATGGCTGAACTACTCGAC  
tctctggaGTGAGTTTACCTGGAGTCTTTTTGGAAAA-3' |

Table 2-1: pSUPER constructs used for stable expression of siRNA in Jurkat T cells.

siRNA target sequences were generated against four regions of the human retina α₁F-subunit mRNA sequence (GenBank accession number AF067227), and one region of the human p53 mRNA sequence (GenBank accession number AB082923). The target mRNA region corresponds to the number of nucleotides downstream of the translation start site of either the α₁F-subunit or p53 mRNA, where appropriate. For the 64 bp oligonucleotides, the bold, capitalized text refers to the sense and antisense 19 nucleotide siRNA target sequences, whereas the lower cased text corresponds to the 9 nucleotide spacer. The italicized text refers to nucleotides at the 5’- and 3’-termini of each 64 bp oligonucleotide.
If the insertion of siRNA sequences into the pSUPER vector was successful, vectors digested with EcoRI and HindIII (New England BioLabs) contained inserts of 300 bp. For further verification, the inserted siRNAs were sequenced with standard T3 and T7 primers at the Florida DNA Sequencing CORE Facility.

2.12 Generation of Stable Cell Lines Expressing siRNA

1x10^7 Jurkat T cells were washed and resuspended in Opti-MEM (Invitrogen). Cells were co-transfected with a 10:1 ratio of Sea-I (New England BioLabs) linearized pSUPER constructs (2 μg) and non-linearized pEYFP-N1 vector (0.2 μg) (Clontech) by electroporation using a Bio-Rad Gene Pulser Electroporator (Hercules, CA) set at 250 V, 975 μF. As controls, Jurkat T cells were also transfected with either 2 μg pSUPER without a siRNA insert, or 0.2 μg pEYFP-N1 vector alone. The pEYFP vector was used since expression of the amino-terminus of the enhanced yellow fluorescent protein (EYFP) served as a transfection marker and the neomycin resistance cassette served as a selectable marker. Bulk cultures of transfected Jurkat T cells were sorted twice by FACSVantage SE flow cytometer (BD Biosciences) based on EYFP expression, and stable cultures were selected with 2 μg/ml Geneticin (Invitrogen). Clonal cell lines were generated by a third sort of transfected cells into 96-well plates by FACSVantage SE flow cytometer. After the sort, cell lines continued to grow in 2 μg/ml Geneticin. To check for stable integration of pSUPER constructs, genomic DNA was purified from the clonal cell lines using the Genomic DNA Isolation Kit (Sigma). For amplification of the pSUPER construct from genomic DNA, PCR fragments of ~550 bp spanning either side of the 64 bp inserts were generated with sense primer (5'
CACGACGTTGTAAAACGACGG-3') and antisense primer (5'-ACTTTATGCTTCGGCTCCTGATG-3'). PCR reactions were performed with Platinum Taq polymerase (Invitrogen) and were conducted in a Whatman Biometra UnoII Thermocycler at 94°C for 1 min, then 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, followed by a 10 min extension at 72°C. PCR fragments were resolved on a 1% agarose gel and visualized by staining with ethidium bromide. To assess the gene silencing effects of siRNA, p53 and α,β-subunit protein expression were analyzed through immunoprecipitation experiments.

2.13 Immunoprecipitation Analysis of p53

20-30x10^6 Jurkat T cells stably expressing pSUPER, pSUPER-p53 or pEYFP alone were washed and lysed in 500 µl of solubilization buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% NP-40, 5 mM EDTA in the presence of 10 µg/ml soybean trypsin inhibitor, pepstatin, and 40 µg/ml PMSF. Protein concentration of the lysates was quantified by Bicinchonic acid protein assay (Pierce). 6.0 mg aliquots of lysate were incubated overnight at 4°C with 10 µg of mouse anti-human p53 mAb, clone BP53-12 (Upstate Biotechnology, Lake Placid, NY). 30 µl of recombinant protein G sepharose (Amersham Pharmacia) was added for 1 h at 4°C and mAb/p53 complexes bound to protein G were then washed 2 x with 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 2 mM EDTA, 0.2 % Nonidet P-40, 1 x with 10 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 2 mM EDTA, 0.2 % Nonidet P-40, and 1 x with 10 mM Tris-HCl (pH 7.5) at 4°C. Samples were denatured by boiling in SDS sample buffer, run on 12% SDS-PAGE gel and...
transferred to nitrocellulose membrane. Western blot analysis was performed with affinity-purified p53 rabbit polyclonal Ab (Cell Signaling Technology, Beverly, MA). Western blot for GAPDH expression was also performed as a protein loading control, as previously described.

2.14 Measurement of Intracellular Calcium Levels

Intracellular Ca\(^{2+}\) levels were measured using the ratiometric Ca\(^{2+}\) indicator indo-1 acetoxyethyl ester dye (Molecular Probes) according to manufacturer’s recommendations. In brief, Jurkat T cells or human PBTs (donors, n=3) at 1x10\(^7\) cells/ml were loaded with 1 µM indo-1 for 1 h at 37°C in MEM (Minimum Essential Medium) (Invitrogen). For analysis, 100 µl of cell suspension (1x10\(^6\) cells) was added to either 1.9 ml of MEM or Ca\(^{2+}\)-free S-MEM (Invitrogen). Indo-1 loaded T cells were then examined for 10 min time periods following induction at the 2 min mark with either 10-100 µM (+/-) Bay K 8644 (Calbiochem, San Diego, CA), 2 µM ionomycin (Calbiochem) or dimethyl sulfoxide (DMSO) solvent using a FACSVantage SE flow cytometer system (BD Biosciences).

Jurkat T cells and human PBTs loaded with indo-1 were also preincubated with 1-200 µM nifedipine (Calbiochem) or DMSO with or without extracellular Ca\(^{2+}\) in the medium for 10 min. At the 2 min mark, Jurkat T cells were stimulated with 10 µg/ml soluble OKT3, whereas human PBTs required a combination of 2 µg/ml soluble anti-CD28 mAb (Sigma), 10 µg/ml soluble OKT3 and 40 µg/ml soluble rabbit anti-mouse IgG polyclonal Ab (Sigma), which served as a cross-linking Ab, to activate Ca\(^{2+}\) influx.
Anti-CD3 stimulation using the OKT3 mAb alone did not activate Ca\(^{2+}\) influx in PBTs. The change in \([\text{Ca}^{2+}]_i\) was determined by calculating the average ratio of emission signals of indo-1 at 405 nm and 485 nm, representing the ratio of \(\text{Ca}^{2+}\)-bound to \(\text{Ca}^{2+}\)-free indo-1, respectively, using FlowJo software. In all experiments, the amount of DMSO solvent was equal to or less than 0.5% of the total treatment volume. (+/-) Bay K 8644 and nifedipine were both prepared in the dark as a 200 mM stock solution dissolved in DMSO.

2.15 Immunoblot Analysis of Phospho-p44/p42 MAP Kinase

Jurkat T cells or human PBTs were washed, resuspended at 1x10\(^7\) cells/ml in RPMI and incubated for 4 h at 37°C. Cells were then preincubated with or without 2 mM ethylene glycol-bis(2-aminoethyl)ether-N,N,N',N'-tetraacetic acid (EGTA) for 15 min to chelate \(\text{Ca}^{2+}\), followed by 10 min stimulation with either (+/-) Bay K 8644 or 2 \(\mu\)M ionomycin at 37°C. Jurkat T cells were also preincubated with either DMSO, 100 \(\mu\)M or 200 \(\mu\)M nifedipine for 1 h, followed by 10 min stimulation with 100 \(\mu\)M (+/-) Bay K 8644 at 37°C. Additionally, as a positive control for phospho-p44/42 mitogen-activated protein (MAP) kinase activation, Jurkat T cells were stimulated with 10 \(\mu\)g/ml soluble OKT3 for 10 min at 37°C. Following stimulation, cells were lysed in 200 \(\mu\)l of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% NP-40, 5 mM EDTA, 1 mM sodium vandadate, 5 mM sodium fluoride, 1 mM sodium molybdate, and 5 mM \(\beta\) glycerol phosphate, in the presence of 10 \(\mu\)g/ml soybean trypsin inhibitor, pepstatin, and 40 \(\mu\)g/ml PMSF. Cell lysates were denatured by boiling in SDS sample
buffer, run on 12% SDS-PAGE gel and transferred to nitrocellulose membrane. Western blot analysis was performed with phospho-p44/42 MAP kinase rabbit polyclonal Ab (Cell Signaling Technology). After development, the blots were stripped in 62.5 mM Tris-HCl (pH 7.5), 0.2% SDS and 100 mM 2-mercaptoethanol for 30 min at 50°C and then reprobed with Erk1/2 (K-23) polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA) as a protein loading control.

2.16 NFAT-Luciferase Assay

1x10^7 Jurkat T cells were washed and resuspended in Opti-MEM (Invitrogen). Cells were incubated with either 20 μg of pNFAT-TA-Luc or pTA-Luc (Clontech) (Figure 2-4) for 5 min at 4°C and transfected by electroporation using a Bio-Rad Gene Pulser Electroporator set at 280 V, 975 μF. 40-48 h after transfection, cells at 1x10^6 cells/ml were incubated with nifedipine (1-200 μM) or DMSO for 1 h at 37°C, followed by stimulation with 10 μg/ml soluble OKT3 for 6 h at 37°C. NFAT-dependent luciferase activity was assayed on 1x10^5 cells/100 μl using the procedures outlined in the Bright-Glo Luciferase Assay System (Promega, Madison, WI). Luciferase activity was measured in a microplate luminometer.

2.17 IL-2 Assay

1x10^6 Jurkat T cells or human PBTs in 1.0 ml RPMI were incubated with DMSO or nifedipine (1-200 μM) for 1 h at 37°C. Cells were then transferred to a 24-well plate immobilized with 10 μg/ml OKT3, 10 nM TPA was added, and cells were incubated at
Figure 2-4: The pNFAT-TA-Luc vector monitors NFAT-mediated signaling transduction pathways in mammalian cells.

The pNFAT-TA-Luc vector (Clontech) contains three copies of the NFAT consensus sequence located upstream of the TA promoter, which consists of the TATA box from the herpes simplex virus thymidine kinase promoter. Following the promoter sequence is the firefly luciferase reporter gene. Transcription of the luciferase reporter gene is activated once endogenous NFAT transcription factors bind to the NFAT cis-acting enhancer element. To ensure efficient processing of the luciferase transcript in mammalian cells, the SV40 late polyadenylation signal is downstream of the luciferase gene. The pTA-Luc vector (Clontech) is similar to pNFAT-TA-Luc, except for it lacks the NFAT consensus sequences. pTA-Luc is used as a control for monitoring constitutive levels of luciferase activity in transfected cells.
37°C. After 24 h, supernatants were quantified for IL-2 concentration by a standard sandwich Enzyme-Linked Immunosorbent Assay (ELISA) technique (R&D Systems, Minneapolis, MN). To determine whether the Ca²⁺ ionophore, ionomycin, could reverse the inhibitory effect of nifedipine, Jurkat T cells or human PBTs at 1x10⁶ cells/ml were incubated with either DMSO or 1-50 μM nifedipine for 1 h. Cells were then stimulated for 24 h with 10 μg/ml plate-bound OKT3, 10 nM TPA and, where appropriate, 2 μM ionomycin with nifedipine remaining in the medium. The concentration of IL-2 in the supernatants was quantified by sandwich ELISA.

To assess T cell function in renal patients administered DHPs, normal PBTs from a healthy donor and PBTs isolated from renal disease patients at 1x10⁶ cells/ml were stimulated for 24 h at 37°C with 10 μg/ml plate-bound OKT3 and 10 nM TPA. Following the 24 h incubation, the sandwich ELISA was used to quantify IL-2 concentration in the supernatants. To determine the effects of uremic serum on T cell function, normal and uremic serum were separated from whole, coagulated blood of a healthy donor and renal disease patients, respectively, by centrifugation at 900 x g for 30 min. Normal PBTs (from a healthy donor grown in culture for 1 week with 5 ng/ml rhIL-2) at 1x10⁶ cells/ml were incubated with medium containing either 10% FBS alone, or medium with FBS supplemented with 10% normal serum or 10% uremic serum for 1 h at 37°C. With the normal human and uremic serum remaining in the medium, cells were then transferred to a 24-well plate immobilized with 10 μg/ml OKT3, 10 nM TPA was added, and cells were further incubated at 37°C for 24 h. Supernatants were quantified for IL-2 concentration by the sandwich ELISA.
2.18 Flow Cytometry Analysis of IL-2R and CD69

IL-2R and CD69 expression were determined through flow cytometry on a FACSCalibur cytometer (BD Biosciences) by separately staining T cells (from the IL-2 assay) with either human IL-2Rα mAb, clone 7G7/B6 (Upstate Biotechnology) or human CD69 mAb, clone FN50 (Pharmingen), respectively, followed by incubation with FITC-conjugated goat anti-mouse IgG Ab (Jackson ImmunoResearch). Cell viability was assessed by staining dead cells with 2 μg/ml propidium iodide (PI) (Sigma).

2.19 Mice

C57B1/6 female mice bearing a transgenic (Tg) TCRαβ receptor specific for the male antigen H-Y were provided by Dr. Philippe Poussier at Sunnybrook and Women’s College, Health Sciences Centre, Toronto, Canada. Balb/c and C57B1/6 mice (Charles River Laboratories, Wilmington, MA) were housed in the animal facilities at University of British Columbia and were used between 8 to 12 wk of age. All mice studies were approved by the Committee on Animal Care at the University of British Columbia using the guidelines set out by the Canadian Council on Animal Care.

2.20 Mixed Lymphocyte Reaction

Splenocytes from C57Bl/6 (H-2b) mice at 2x10^6 cells were incubated with nifedipine for 1 h at 37°C. C57Bl/6 splenocytes were then stimulated with 2000 Rad-irradiated stimulator splenocytes at 4x10^6 cells from allogeneic Balb/c (H-2^d) or syngeneic C57Bl/6 (H-2^b) mice for 5 to 6 d at 37°C with nifedipine remaining in the
culture medium. Splenocytes were grown in RPMI supplemented with 10% FBS, 2 mM glutamine, 50 nM 2-mercaptoethanol, and 100 U/ml each of penicillin and streptomycin. Proliferation was evaluated by using a flow cytometer-based bead assay (124). In brief, after 5 to 6 days splenocyte cultures were combined with an equal volume of phosphate buffered saline (PBS) containing 2 μg/ml PI and 2x10^6 cells/ml latex beads with a 2 μM diameter (Interfacial Dynamics Corp., Portland, OR). PI positive or dead cells were removed from the analysis on the basis of FL3_{high} staining and forward light scatter gating on a FACSCalibur cytometer (BD Biosciences). Forward light scatter and side light scatter were then used to visualize the latex beads, viable small splenocytes and proliferating blasts. Following data acquisition, gated analysis with the CellQuest software (BD Biosciences) was used to identify the ratios of latex beads to blasts. Using this ratio, the number of proliferating blast cells was then quantified in triplicate for each treatment with Microsoft Excel software.

2.21 In Vivo Proliferation Assay

Thymocytes from C57Bl/6 female mice bearing a Tg TCRαβ receptor that is MHC Class I restricted and specific for the male H-Y antigen were loaded with 5 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes) for 7 min at RT. 20-30x10^6 CFSE loaded Tg thymocytes were intravenous (i.v.) injected into the tail vein of female or male C57Bl/6 recipients, followed by intraperitoneal (i.p.) injection of either vehicle control or 15 mg/kg nifedipine into the C57Bl/6 males (Figure 2-5). Nifedipine was prepared as a 1 mg/ml stock solution, dissolved in PBS containing 5% ethanol and 1% Tween-80 (125). 40 h after the initial i.v. injection, spleens were
Figure 2-5: Experimental design for the in vivo proliferation assay.

(A) Female H-Y-specific TCR-Tg thymocytes were loaded with CFSE and i.v. injected into male C57Bl/6 recipient mice. Following the cell injection, male mice were i.p. injected with 15 mg/kg nifedipine or the vehicle control, and approximately 40 h later spleens were analyzed for transplanted H-Y-specific TCR-Tg CD8+ CFSE+ T cells. (B) Flow cytometry was used to quantify the % total of gated viable, CFSE+, CD8+ and Tg TCRhi cells in the male mice. In the histogram, M1 represents the number of non-dividing cells, M2 (1 cell division), M3 (2 cell divisions), and M4 (3 cell divisions). From this analysis, the proliferation of female H-Y-specific TCRαβ receptor CD8+ T cells was determined in each successive cell division.
harvested and single cell suspensions were prepared. For cell staining, splenocytes were suspended in DMEM and labeled with PE-conjugated anti-CD8α mAb (BD Biosciences) and biotin-conjugated anti-TCRα mAb, clone T3.70 (provided by Dr. Hung-sia Teh, University of British Columbia, Vancouver, Canada), which is specific for Tg TCRα. Cells were then stained with Cy-Chrome conjugated-streptavidin (BD Biosciences), washed and analyzed on a FACSCalibur cytometer (BD Biosciences). Proliferation of female H-Y-specific TCRαβ receptor CD8+ T cells in vivo was quantified by determining the % total of gated viable, CFSE+, CD8+ and Tg TCRhigh cells in successive cell divisions using CellQuest software (Figure 2-5). As a result of CFSE labeling being distributed equally between daughter cells, a halving of cellular fluorescence intensity marked each successive cell division among proliferating cells. The total number of female H-Y-specific TCR-Tg CD8+ CFSE+ T cells that recovered from the spleens was determined by using the flow cytometry-based bead assay as previously described.

2.22 Statistical Analysis

Statistical significance was determined by the Analysis of Variance (ANOVA) test, using two-factorial design without replication with Microsoft Excel software. For all tests, P<0.01 was considered to indicate statistical significance. All error bars shown represent the standard deviation (SD).
CHAPTER 3: MOLECULAR IDENTIFICATION OF L-TYPE VOLTAGE-DEPENDENT CALCIUM CHANNELS IN T LYMPHOCYTES

3.1 Introduction

TCR engagement by peptide-MHC complexes on the surface of APCs initiates the depletion of Ca\(^{2+}\) ions from intracellular Ca\(^{2+}\) stores in T lymphocytes. The rapid rise in \([\text{Ca}^{2+}]_i\), activates the opening of SOCs in the plasma membrane sustaining Ca\(^{2+}\) influx for 1 to 2 h (126). The Ca\(^{2+}\) current gated by SOCs, which is termed the Ca\(^{2+}\)-release activated Ca\(^{2+}\) current (\(I_{\text{CRAC}}\)) in T lymphocytes, has been extensively characterized through patch-clamping Jurkat T cells (127-129) and human PBTs (130). As a result of the electrophysiological studies a biophysical "fingerprint" for \(I_{\text{CRAC}}\) has been generated which has following characteristics; activation by intracellular Ca\(^{2+}\) store-depletion, very small single-channel conductance, high selectivity for Ca\(^{2+}\) over monovalent cations, and \([\text{Ca}^{2+}]_i\)-dependent modulation of channel activity (10, 19, 131). Even though the properties of \(I_{\text{CRAC}}\) have been explicitly defined through electrophysiological means, a candidate protein for the CRAC channel has not been clearly identified.

Finding a protein for \(I_{\text{CRAC}}\) has proven to be very difficult, as many of the potential Ca\(^{2+}\) channel candidates lack one or more of the characteristics of the \(I_{\text{CRAC}}\) "fingerprint". To further complicate matters several different Ca\(^{2+}\) channels together may conduct \(I_{\text{CRAC}}\) during T cell activation lending to the possibility that a single candidate protein may not be solely responsible for \(I_{\text{CRAC}}\) (132). One of the current viewpoints for the \(I_{\text{CRAC}}\) model is that the CRAC channel consists of one or multiple members of the
TRP family of Ca\(^{2+}\) channels originally found in *Drosophila* (99). The most plausible TRP candidate for \(I_{CRAC}\) is CaT1, a TRPV subfamily member, that when overexpressed in Jurkat T cells exhibits many biophysical properties of endogenous \(I_{CRAC}\), but is only partially regulated by store-depletion (105). Although Cui et al. provided evidence supporting that CaT1 comprises all or part of the CRAC channel (105), an earlier study disputed CaT1 forms \(I_{CRAC}\) since there are additional biophysical properties of CaT1 that are distinct from \(I_{CRAC}\) (106).

The reported differences between \(I_{CRAC}\) and CaT1 suggest that other plasma membrane Ca\(^{2+}\) channels expressed in T lymphocytes may also be involved in the generation of \(I_{CRAC}\). Interestingly, many of the characteristics of the \(I_{CRAC}\) “fingerprint” are shared with L-type VDCCs, even though the gating mechanisms for channel activation differ (store-depletion versus membrane depolarization) (128). CRAC channels, like L-type VDCCs have a high selectivity for Ca\(^{2+}\) over monovalent cations, which is dependent on Ca\(^{2+}\) binding to high affinity sites on the channels (133). Furthermore, lowering the extracellular [Ca\(^{2+}\)] to micromolar levels reduces the Ca\(^{2+}\) selectivity of CRAC channels and VDCCs (127, 133). In a study comparing the biophysical properties of CRAC channels to VDCCs, Kerschbaum et al. demonstrated through probing the channels with various organic cations of differing sizes that the predicted diameter of the channel pore is \(~0.6\) nm for both channels (128). Other similarities shared between CRAC channels and VDCCs are comparable sensitivities to external and internal pH (128).

Although VDCCs are typically expressed and function in electrically excitable cell types, several studies have reported the expression of a variety of channel-forming
α₁-subunits and auxiliary β-subunits of L-type VDCCs in human and mouse lymphocytes. For instance, Brereton et al. demonstrated through RT-PCR analysis that the α₁C- and α₁S-subunits of L-type VDCCs are expressed in Jurkat T cells, but whether these channels contribute to TCR-induced Ca²⁺ influx was not addressed (119). Savignac et al. reported that an L-type VDCC transcript is expressed in the 2G12.1 murine T cell hybridoma line where the channel is thought to mediate Ca²⁺-dependent gene transcription of IL-4 (120). Through an oligonucleotide array analysis comparing gene expression profiles in human Th1 and Th2 cells, it was shown that the auxiliary β-subunit of VDCCs had 5.4-fold increased expression level in Th1 compared to Th2 cells (134). In addition, a previous study by Grafton et al. showed that the α₁C-subunit mRNA and protein and β-subunit protein are expressed in various human B and T cell lines (135). Grafton et al. further demonstrated that an Ab raised against an extracellular region of the α₁C-subunit stimulated sustained Ca²⁺ influx when added to the human L3055 B cell line (135). Taken together, the previous studies demonstrate that several α₁-subunits are expressed in T lymphocytes; however, they did not address the functional contribution of each L-type α₁-subunit to Ca²⁺ influx during T cell activation.

The investigation on the role of an L-type α₁-subunit in T lymphocytes was continued here by examining the functional expression of the α₁F-subunit of L-type VDCCs in T lymphocytes. The α₁F-subunit gene, CACNA1F, was originally cloned from human retina (136). In the retina, the α₁F-subunit appears to mediate Ca²⁺ entry into the photoreceptors, promoting tonic neurotransmitter release (137). Through Northern blot analysis it was found that the α₁F-subunit mRNA is not only highly expressed in human retina, but also present in human skeletal muscle, kidney and pancreas at lower levels.
Interestingly, mutations in the CACNA1F gene are responsible for the retinal disorder, incomplete X-linked congenital stationary night blindness (CSNB) (138, 139). Even though the original Northern blot analysis revealed that the α_{1F}-subunit is not exclusively expressed in the retina, there are no detailed reports examining the function of the α_{1F}-subunit in other human tissues. Therefore, the investigation presented here began by determining whether α_{1F}-subunit mRNA and protein are present in human T cells. The examination on α_{1F}-subunit expression revealed that two unique splice isoforms of the retinal channel-forming α_{1F}-subunit are expressed in various human leukocytes, including T lymphocytes, but are not found in normal human retina or in retina of humans with CSNB reported thus far. To determine whether the α_{1F}-subunit splice isoforms regulated Ca^{2+} influx during T cell activation, several experiments were conducted, aimed at examining the expression of the splice isoforms following TCR-induced Ca^{2+} influx in Jurkat T cells and normal human PBTs. The results in this study collectively established that the L-type α_{1F}-subunit is functionally expressed in T lymphocytes, and the expression is partially regulated through the TCR, lending to the possibility that a VDCC may play a role in generating I_{CRAC}. 
3.2 Results

3.2.1 L-Type α_{1F}-Subunit mRNA Transcript is Expressed in T Cells

Through a nested RT-PCR based assay with primers designed to specifically amplify the pore-forming α_{1F}-subunit of an L-type VDCC, a PCR product spanning exons 29 to 30 of the α_{1F}-subunit was detected in human spleen, the human Jurkat T cell leukemia line, and human PBTs (Figure 3-1A) (140). Human retina and WERI-Rb1 retinoblastoma cDNAs were used as positive controls for the PCR assay since the complete α_{1F}-subunit gene, CACNA1F, was previously isolated from human, rat and mouse retina, where it is expressed at high levels (136, 137, 141, 142). In human retina, α_{1F}-subunit mRNA is approximately 5.8 kb in length, consists of 48 exons, and overall the α_{1F}-subunit protein has 55-62% amino acid sequence identity to other L-type Ca^{2+} channel α_{1}-subunits (137).

In the initial studies examining α_{1F}-subunit expression, the α_{1F}-subunit mRNA was not detected in lymphoid tissue (137). As demonstrated here, the α_{1F}-subunit is expressed at low levels in T lymphocytes therefore, α_{1F}-subunit expression in lymphoblastoid tissue may have been overlooked by the lack of a nested RT-PCR-based assay. Using nucleotide sequencing, it was confirmed that the ~180 bp amplified PCR product from Jurkat T cells, human spleen, human PBTs, CD4^+ and CD8^+ T lymphocytes shares 100% nucleotide identity to the L-type VDCC α_{1F}-subunit gene expressed in human retina and WERI-Rb1 retinoblastoma (Figure 3-1B). The α_{1F}-subunit was not expressed ubiquitously in human cells since α_{1F}-subunit expression was not detected in
Figure 3-1: The channel-forming α1F-subunit of L-type VDCCs is expressed in T lymphocytes.

(A) Using a nested RT-PCR reaction, a ~180 bp PCR product corresponding to exons 29 to 30 of the channel-forming α1F-subunit of an L-type VDCC was isolated from human cell lines and tissues, including retina, WERI-Rb1 retinoblastoma, spleen, Jurkat T cells, PBTs, CD4+ and CD8+ T cells, but was not expressed in normal human liver (top panel). The S15 ribosomal subunit PCR served as a loading control (bottom panel). PCR products were resolved on a 1% agarose gel and visualized by staining with ethidium bromide. (B) Nucleotide sequence alignment of the ~180 bp PCR product amplified from retina, WERI-Rb1 retinoblastoma and T lymphocytes using the European Bioinformatics Institute ClustalW alignment program. The PCR product from human T lymphocytes shares 100% nucleotide identity to the L-type α1F-subunit VDCC isolated from human retina.
normal human liver. The expression of the $\alpha_{IF}$-subunit in lymphoid tissue was confirmed by a recent study by McRory et al., demonstrating that $\alpha_{IF}$-subunit mRNA expression is not confined to the retina, but is also present in human spleen, thymus, and bone marrow (143).

3.2.2 Identification of Novel Alternative Splice Variants of the $\alpha_{IF}$-Subunit in T Cells

Since very little is understood about the role of VDCCs during lymphocyte activation, the complete cDNA sequence of the T lymphocyte $\alpha_{IF}$-subunit was sought after for functional analysis of the channel in human T cells. Using the 3'-RACE reaction, a PCR product corresponding to the 3'-terminal sequence of the $\alpha_{IF}$-subunit was isolated from a human spleen cDNA library, which served as an enriched source of human T lymphocytes. In parallel, the complete $\alpha_{IF}$-subunit cDNA sequence from a human retina library was also isolated. It was found that the 3'-terminal cDNA sequence of the $\alpha_{IF}$-subunit from spleen was approximately 430 bp smaller than the cDNA isolated from retina even though identical PCR primers were used in the 3'-RACE reaction (Figure 3-2A). DNA sequence analysis revealed that a novel splice variant of the $\alpha_{IF}$-subunit was expressed in human spleen, which lacked exons 31, 32, 33, 34, and 37 due to alternative splicing (Figure 3-2B). The excision of these exons caused the deletion of transmembrane segments S3, S4, S5 and half of S6 in motif IV of the spleen $\alpha_{IF}$-subunit. As a result, a new membrane topology of the channel protein at the carboxyl-terminus was formed (Figure 3-2C). Removal of transmembrane segment IVS4 (exon 33) through
A

NotI/Spel EcoRI Digest

Precursor Pre-mRNA (Motif IV):

Voltage sensor

DHP site & EF Hand

Mature mRNA (Motif IV):

Alternative Splicing

Splice site

Splice site

B

C

Protein:

Motif I

Motif II

Motif III

Motif IV

Out

Membrane

In

COOH

α1F-Subunit 1284 PSGIWEAVPTPSGLQ8TCNEHAPGLRWDGDIQKHTLCFGPDIPEDQNNR 1333

α1S-Subunit 1281 PLPGKFCRVAQACAGHAPQRRKSHLQCQHTLCPGPHGTQDDQRR 1330
Figure 3-2: Schematic representation of mRNA splice sites and putative protein topology of the voltage negative splice variant of $\alpha_{4}\text{-subunit}$ isolated from human spleen.

(A) Restriction enzyme digests of pCR2.1-TOPO cloning vectors containing either the human retina or spleen 3'-RACE products, which correspond to the 3'terminal cDNA sequence of the voltage negative $\alpha_{4}\text{-subunit}$ splice variant. Digestion of the pCR2.1-TOPO vectors with NotI and SpeI enzymes reveals that the 3'-terminus of $\alpha_{4}\text{-subunit}$ cloned from spleen is significantly smaller than the retina $\alpha_{4}\text{-subunit}$ 3'-terminus. The EcoRI enzyme digest further reveals that the 3'-terminus of $\alpha_{4}\text{-subunit}$ from spleen lacks a single EcoRI restriction site compared to the retina cDNA sequence. (B) Alternative splicing of exons 31, 32, 33, 34 and 37 leads to the deletion of exons encoding transmembrane segments IVS3, S4, S5 and half of S6 of the pore-forming $\alpha_{4}\text{-subunit}$. Exons encoding transmembrane segments are highlighted in bold boxes with the segment number written below the respective box. Introns are represented as lines in between exon boxes. (C) Diagram of putative channel topology of the voltage negative splice variant of the $\alpha_{4}\text{-subunit}$. Motifs I-III are comprised of six transmembrane segments, whereas due to alternative splicing motif IV only contains two transmembrane segments. Removal of exon 37 through splicing leads to a reading frameshift resulting in early termination of the carboxyl-terminus of the channel protein. The reading frameshift causes a portion of the carboxyl-terminus to no longer be homologous to the $\alpha_{4}\text{-subunit}$, but instead to have 40% amino acid homology to the human $\alpha_{5}\text{-subunit}$. 
splicing led to the deletion of a voltage sensor domain, whereas splicing of segment IVS6 (exon 37) resulted in the deletion of a DHP binding site and an EF-hand Ca\textsuperscript{2+}-binding motif. The spleen $\alpha_{1F}$-subunit was termed the voltage negative splice variant since it no longer contained the S4 voltage sensor domain in motif IV (see Appendix A for the nucleotide sequence and Appendix B for the amino acid sequence of the voltage negative variant).

Further sequence analysis of the 3'-terminal sequence demonstrated that the deletion of exon 37 led to the joining of four successive guanines at the exon 36 and 38 junction in the voltage negative splice variant instead of the three guanines that are normally found at the beginning of exon 38 in the retina $\alpha_{1F}$-subunit cDNA sequence. The four consecutive guanines caused a frameshift in the nucleotide and amino acid sequence in the spleen $\alpha_{1F}$-subunit, leading to an early in-frame TAG stop codon (at -4.1 kb downstream from the 5'-ATG start codon) and premature termination of channel protein translation at the carboxyl-terminus. In addition, the four guanines in a row changed the amino acid sequence downstream of the frameshift. Instead of having 95% amino acid identity to the $\alpha_{1F}$-subunit, extraordinarily, the carboxyl-terminus has 40% amino acid identity to the human $\alpha_{1S}$-subunit L-type VDCC found in skeletal muscle (GenBank accession number XP001910) (Figure 3-2C). To my understanding, this is the first reported example of a naturally-occurring chimeric L-type VDCC. In general, this discovery has exciting implications for ion channel biology. A previous study by Brereton et al. also detected the $\alpha_{1S}$-subunit transcript in Jurkat T cells through RT-PCR (119). The $\alpha_{1S}$-subunit transcript identified by Brereton et al. was not identical to the $\alpha_{1F}$-subunit splice variants discovered here since the $\alpha_{1S}$-subunit transcript had nucleotide
identity to the IVS2 to IVS6 regions of the skeletal α1S-subunit and no identity to the α1F-subunit (119). Therefore, it appeared that alternative splicing lead to the formation of a novel α1F-subunit carboxyl-terminus, possibly residing in the cytoplasm. The deletion of exon 37 and the resulting reading frameshift was confirmed by repeatedly isolating and sequencing this unique α1F-subunit cDNA sequence from many different T cell mRNA sources, including human spleen, Jurkat T cells, mouse splenocytes and thymocytes, and naïve human PBTs. The unique 3’-terminal cDNA sequence was not found in the same region of α1F-subunit isolated from human retina.

In addition to the voltage negative splice variant of the α1F-subunit, a second novel splice variant of the α1F-subunit channel was detected in the human spleen cDNA library using the 3’-RACE reaction. Although the second splice variant lacked exons 32 and 37 through alternative splicing, it retained the voltage sensor domain in transmembrane segment IVS4. Therefore, this variant was termed the voltage positive splice variant (Figure 3-3A) (see Appendix A for the nucleotide sequence and Appendix B for the amino acid sequence of the voltage positive variant). Splicing out of the short exon 32, encoding 7 amino acids caused a deletion of part of the extracellular loop between segments IVS3-S4. The deletion of exon 37 in the voltage positive variant also generated a repeat of four guanines in the nucleotide sequence at the exon 36 and 38 junction, resulting in an identical nucleotide and reading frameshift as in the voltage negative splice variant. The voltage positive variant also contained a carboxyl-terminus with 40% amino acid identity to the α1S-subunit, as well as an early stop codon causing premature truncation of the channel protein (Figure 3-3B). Furthermore, one striking difference between the two variants, other than the spliced exons, is that the carboxyl-
Figure 3-3: Schematic representation of mRNA splice sites and putative protein topology of the voltage positive splice variant of α_{1F}-subunit isolated from human spleen.

(A) Alternative splicing of exons 32 and 37 occurs in motif IV of the voltage positive splice variant of the pore-forming α_{1F}-subunit. The voltage sensor domain encoded by exon 33 is not deleted by splicing, and therefore this variant is termed voltage positive. Exons encoding transmembrane segments are highlighted in bold boxes with the segment number written below the respective box. Introns are represented as lines in between exon boxes. (B) Diagram of putative channel topology of the voltage positive splice variant of the α_{1F}-subunit. Alternative splicing of exon 32 leads to the deletion of a portion of the extracellular loop linking segments IVS3 and IVS4, and deletion of exon 37 removes half of the transmembrane segment IVS6 of the pore-forming α_{1F}-subunit. Removal of exon 37 through splicing also leads to a reading frameshift resulting in early termination of the carboxyl-terminus of the channel protein in this splice variant. The reading frameshift causes a portion of the carboxyl-terminus to no longer be homologous to the α_{1F}-subunit, but instead to have 40% amino acid homology to the human α_{1S}-subunit.
terminus of the voltage positive splice variant may reside extracellularly instead of in the cytoplasm as a result of fewer exons being excised in this isoform.

The remaining 5'-cDNA sequences of the voltage negative and positive splice variants amplified from the human spleen library were identical to the retinal αIF-subunit cDNA sequence. Both splice isoforms had 99% nucleotide and 95% amino acid sequence identity to the αIF-subunit from human retina. There were no additional differential splice sites found upstream of exon 31 in the voltage negative and positive splice variants of the αIF-subunit. Therefore, the αIF-subunit splice variants expressed in human spleen contain novel structural features at the carboxyl-terminus which may have a unique impact on the Ca\(^{2+}\) kinetics gated by these channels in T lymphocytes. The full-length cDNA sequences of the retinal αIF-subunit and the voltage negative spliced αIF-subunit were generated from ligation of the individual nested PCR products.

### 3.2.3 Carboxyl-Terminus of the Retina αIF-Subunit Resides in the Cytoplasm

Although the carboxyl-termini of channel-forming L-type αIF-subunits are normally located in the cytoplasm of different cell types, it has not been previously established whether this was also the case for the human retina αIF-subunit. In addition, determining the membrane topology of the retina αIF-subunit would aid in distinguishing the localization of the alternatively spliced carboxyl-termini of the T lymphocyte splice variants. To begin this analysis, the complete cDNA sequence of the retina αIF-subunit was subcloned into the pGFP mammalian expression vector. The pGFP vector not only allowed for the identification of transfected cells due to the dicistronically expressed
hrGFP, but also FLAG-tagged the carboxyl-terminus of the retina \( \alpha_{1F} \)-subunit. The carboxyl-terminal FLAG-tagged \( \alpha_{1F} \)-subunit was used to directly study the topology of the retina \( \alpha_{1F} \)-subunit in transfected cells.

To address whether the retina \( \alpha_{1F} \)-subunit could be overexpressed in mammalian cells, HeLa cells were transiently transfected with either pGFP vector alone or pGFP/Retina \( \alpha_{1F} \)-subunit. After 72 h, the cells were stained for the FLAG-tagged \( \alpha_{1F} \)-subunit. First, it was demonstrated that the anti-FLAG mAb did not non-specifically stain transfected HeLa cells since there was only weak, background staining with the mAb in cells transfected with pGFP vector alone (Figure 3-4A). The staining of pGFP/Retina \( \alpha_{1F} \)-subunit transfected cells treated with or without saponin was then examined. When HeLa cells were saponin treated, the anti-FLAG mAb labeled intracellular membranes as well as the intracellular side of the plasma membrane (Figure 3-4B). Intracellular membrane compartments were stained with the anti-FLAG mAb since it has been previously established that co-expression of the \( \alpha_{1} \)-subunit with the appropriate auxiliary \( \beta \)-subunit is required to localize all of the ectopically expressed \( \alpha_{1} \)-subunit to the plasma membrane (142, 144). In the presence of saponin, it was also observed here that the staining of the FLAG-tagged \( \alpha_{1F} \)-subunit was not cytoplasmic since mAb labeling did not co-localize with hrGFP expression. As expected, HeLa cells transfected with pGFP/Retina \( \alpha_{1F} \)-subunit without saponin treatment showed no staining with the anti-FLAG mAb (Figure 3-4C). These experiments demonstrated for the first time that the retina \( \alpha_{1F} \)-subunit was expressed in mammalian cells with the carboxyl-terminus residing in the cytoplasm. It was also observed that HeLa cells transfected with the retina \( \alpha_{1F} \)-subunit rapidly deteriorated in health compared to cells transfected with the pGFP vector.
Figure 3-4: FLAG-tagged retina α₁F-subunit is expressed in HeLa cells.

(A) HeLa cells were transfected with pGFP vector alone, saponin treated and stained with M2 anti-FLAG mAb, followed by Alexa 568-conjugated anti-mouse Ab (magenta). The pGFP vector emits green fluorescence in transfected cells. HeLa cells were transfected with the pGFP/Retina α₁F-subunit construct and treated (B) or not treated (C) with saponin. Cells were then stained with M2 anti-FLAG mAb and Alexa 568-conjugated anti-mouse Ab (magenta). Carboxyl-terminal FLAG-tagged retina α₁F-subunit was only detected intracellularly in cells permeabilized with saponin and not detected extracellularly in nonpermeabilized cells. The “+” designates saponin treated, permeabilized cells, whereas the “-” designates cells not treated with saponin.
alone. Therefore overexpression of this Ca\textsuperscript{2+} channel may alter Ca\textsuperscript{2+} homeostasis in transfected mammalian cells.

3.2.4 Alternative Splice Variants of α\textsubscript{IF}-Subunit are Differentially Expressed in Human Leukocytes

Through cDNA library cloning, it was established that two unique splice variants of the α\textsubscript{IF}-subunit are expressed in human T lymphocytes. The next step of the investigation was to examine whether the alternative splice variants are differentially expressed in different leukocyte populations found in spleen. Differential expression of the splice variants may provide new insights into the reported Ca\textsuperscript{2+} kinetic differences in physiologically distinct leukocyte populations. For instance, it has been demonstrated that the Ca\textsuperscript{2+} response in human CD4\textsuperscript{+} T cells stimulated by APCs begins after a short delay (145), whereas the interaction of cytotoxic CD8\textsuperscript{+} T cells with target cells results in a rapid increase in intracellular Ca\textsuperscript{2+} that is maximal within 30 to 60 s (146). Fanger et al. observed that the rise in intracellular Ca\textsuperscript{2+} following store-depletion with thapsigargin treatment is significantly lower in mouse Th2 cells compared to Th1 cells (147). It is also well documented that there are differences in Ca\textsuperscript{2+} influx between tolerant versus activated lymphocytes (148).

Using a nested RT-PCR assay with splice variant specific primers (Figures 3-2B and 3-3A depict primer binding sites), the expression of the splice variants in Jurkat T cells and in freshly isolated human PBTs, CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, B cells and monocytes (also isolated from human peripheral blood) was examined. Both the voltage negative and positive splice variants were expressed in Jurkat T cells, and the heterogeneous
population of PBTs, as well as in purified CD4$^+$ and CD8$^+$ T cells (Figure 3-5A). However, voltage negative splice variant expression appeared to be limited to lymphocytes, as this variant was present in B cells, but not found in monocytes (Figure 3-5B). Intriguingly, the voltage positive splice variant was expressed in both peripheral blood B cells and monocytes.

3.2.5 α$_{1F}$-Subunit Protein is Expressed in Human T Cells

To demonstrate that the α$_{1F}$-subunit protein is expressed in T lymphocytes, immunoprecipitations from Triton X-100 membrane solubilized lysates of Jurkat T cells and human PBTs with an Ab specific to the human retina α$_{1F}$-subunit were carried out. The immunoprecipitated protein was then analyzed by Western blotting with the α$_{1F}$-subunit Ab. As illustrated in Figure 3-6A, the α$_{1F}$-subunit Ab detected a single band at ~200 kDa, corresponding to the α$_{1F}$-subunit protein in both Jurkat T cells and in PBTs isolated from two different human donors. The human retinoblastoma WERI-Rb-1 cell line was used as a positive control for α$_{1F}$-subunit expression since the α$_{1F}$-subunit protein is robustly expressed in the retina (137). In a control experiment, it was demonstrated that the α$_{1F}$-subunit Ab specifically immunoprecipitated the α$_{1F}$-subunit protein since a protein of ~200 kDa was not immunoprecipitated using affinity-purified rabbit IgG Abs (Figure 3-6A).

The α$_{1F}$-subunit Ab did not appear to detect two protein bands, representing the alternatively spliced α$_{1F}$-subunit isoforms in either Jurkat T cells or human PBTs. Post-translational carbohydrate modifications, such as N-linked and O-linked glycosylation,
Figure 3-5: Expression of alternatively spliced isoforms of α1f-subunit mRNA in human leukocytes.

(A) Nested RT-PCR detected the voltage negative and positive splice variants of the α1f-subunit in Jurkat T cells, human PBTs, and CD4+ and CD8+ T cells. (B) Peripheral blood B cells expressed both the voltage negative and positive splice variants, whereas peripheral blood monocytes only expressed the voltage positive isoform as determined through nested PCR. The S15 ribosomal subunit PCR served as a control for loading and integrity of cDNA samples. PCR products were analyzed on a 1% ethidium bromide-stained agarose gel. Prior to nested PCR, the purity of the cell populations was assessed by flow cytometry. The human PBTs contained 11.5% CD3+CD4+CD8−, 86.5% CD3+CD4+CD8+, 1.0% CD3+CD4−CD8+, and 1.0% CD3−CD4+CD8+. The CD4+ and CD8+ T cells were >99% pure, whereas B cell and monocyte cell populations were 96% pure. The results are representative of three independent experiments.
A

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<tr>
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L-type α1F-Subunit Calcium Channel

200 kDa

GAPDH

B

- **WERI-Rb-1**
- **Jurkat T Cells**
- **PBTS**

Counts vs. FL1 for each condition.
Figure 3-6: Detection of α_1F-subunit protein in Jurkat T cells and human PBTs.

(A) α_1F-Subunit protein (~200 kDa) was immunoprecipitated using affinity-purified Abs against human α_1F-subunit from 1.5 mg of membrane protein solubilized lysates of WERI-Rb-1 cells, Jurkat T cells and human PBTs isolated from two different donors (top panel). α_1F-subunit protein was not immunoprecipitated with affinity-purified rabbit IgG Ab (Control IP, top panel). The immunoprecipitated protein was analyzed by Western blotting with the α_1F-subunit Ab. The position of the prestained molecular weight marker is indicated on the right-hand side of the α_1F-subunit panel. As a loading control, Western blot analysis was performed on the samples with an Ab directed against GAPDH (bottom panel). The human PBTs from Donor A contained 36.5% CD3⁺CD4⁺CD8⁻, 57.5% CD3⁺CD4⁺CD8⁺, 5.0% CD3⁺CD4⁺CD8⁻, and 1.0% CD3⁺CD4⁺CD8⁻, and from Donor B contained 24.8% CD3⁺CD4⁺CD8⁺, 72.2% CD3⁺CD4⁺CD8⁻, 1.5% CD3⁺CD4⁺CD8⁺, and 1.5% CD3⁺CD4⁺CD8⁻. (B) Flow cytometry analysis detected α_1F-subunit protein expression in WERI-Rb-1 cells, Jurkat T cells and human PBTs. Saponin-treated cells were either stained with α_1F-subunit Ab (bold solid line) or rabbit IgG Ab (thin solid line), or the cells were not saponin-treated and stained with α_1F-subunit Ab (dotted line) (top panel). In the bottom panel the bold solid and thin solid lines are the same as before, but the dotted line is saponin-treated cells stained with α_1F-subunit Ab, which had been preincubated with the antigenic peptide. The human PBTs contained 28.1% CD3⁺CD4⁺CD8⁻, 54.2% CD3⁺CD4⁺CD8⁺, 9.7% CD3⁺CD4⁻CD8⁻, and 8.0% CD3⁺CD4⁻CD8⁻. Results depicted are representative of three independent experiments.
may obscure the size differences between the $\alpha_{1F}$-subunit splice variant proteins. Using the Expert Protein Analysis System (ExPASy) proteomics program, a single putative N-linked glycosylation site at asparagine 230, (NQTE) was identified in both of the $\alpha_{1F}$-subunit splice isoforms, whereas only one O-linked glycosylation site at threonine 1207 was predicted in the voltage positive splice isoform. Due to the additional splice sites in the voltage negative splice variant, there were no predicted O-linked sites in this isoform. In contrast, the ExPASy program identified two putative N-linked glycosylation sites at asparagines 230 (NQTE) and 1459 (NATL), and two O-linked glycosylation sites at threonines 1207 and 1637 in the retinal $\alpha_{1F}$-subunit. These potential differences in glycosylation sites may cause the $\alpha_{1F}$-subunit splice isoforms to have similar molecular weights to one another and the retinal $\alpha_{1F}$-subunit, and therefore may not be resolved as two smaller, individual protein bands on the SDS-PAGE mini-gel.

To further confirm the presence of the $\alpha_{1F}$-subunit protein in T lymphocytes and to demonstrate $\alpha_{1F}$-subunit Ab specificity, a series of flow cytometry experiments with the $\alpha_{1F}$-subunit Ab were conducted. First, it was demonstrated that the $\alpha_{1F}$-subunit Ab bound to its intracellular epitope by labeling either untreated or saponin-treated WERI-Rb-1 cells, Jurkat T cells and PBTs with the $\alpha_{1F}$-subunit Ab. Only cells pretreated with saponin showed $\alpha_{1F}$-subunit Ab labeling as indicated by an increase in mean FL1 fluorescence (Figure 3-6B, top panel). Next, it was determined that the $\alpha_{1F}$-subunit Ab was binding specifically to the $\alpha_{1F}$-subunit protein by preincubating the Ab with the antigenic peptide prior to cell labeling. Preincubation with the antigenic peptide completely abolished $\alpha_{1F}$-subunit Ab binding in all cells tested (Figure 3-6B, bottom panel). In the flow cytometry experiments, the WERI-Rb-1 cells showed a larger increase
in mean FL1 fluorescence compared to Jurkat T cells or PBTs. This correlated with the immunoprecipitation data, which also showed a significantly greater amount of $\alpha_{1F}$-subunit protein expression in WERI-Rb-1 cells compared to T cells.

3.2.6 Comparison of $\alpha_{1F}$-Subunit Alternative Splice Variant mRNA Expression in Resting and Activated T Cells

After establishing that the mRNA and protein of the $\alpha_{1F}$-subunit are expressed in T lymphocytes, it was addressed whether the endogenous expression of the $\alpha_{1F}$-subunit splice isoforms in T cells is regulated by TCR-induced activation. To investigate the effect of activation on $\alpha_{1F}$-subunit splice variant mRNA expression, real-time PCR using splice variant specific primers was performed on Jurkat T cells and naïve human PBTs either untreated or treated with an anti-CD3 mAb, OKT3 and the phorbol ester TPA to induce Ca$^{2+}$ influx (Figure 3-7). T cells were activated for a minimum time period of 1 min to a maximum of 4 h since Ca$^{2+}$ influx is an immediate event following TCR ligation, which is maintained for 1 to 2 h to drive downstream signaling events such as IL-2 expression (126, 149). Therefore, time periods during T cell activation that encompass critical changes in Ca$^{2+}$ influx mediated through plasma membrane Ca$^{2+}$ channels were analyzed.

First, the mRNA expression of the voltage negative splice variant of the $\alpha_{1F}$-subunit in resting and activated Jurkat T cells was investigated. It was observed that treatment with OKT3 and TPA for 1 min and 2 min time periods induced a large 380- and 620-fold increase, respectively, in the mRNA expression of the voltage negative variant (Figure 3-7A). However, slightly longer activation times of 5 and 10 min caused
Figure 3-7: Anti-CD3 stimulation alters mRNA expression levels of α₁F-subunit splice variants and LTRPC2 channel in Jurkat T cells and human PBTs.

Jurkat T cells (A, B, E) and human PBTs (donors, n=3) (C, D, F) were either not treated (NT) or stimulated with 10 μg/ml soluble OKT3 and 10 nM TPA for 1 min, 5 min, 10 min, 1 h and 4 h. Real-time PCR was used to quantify the mRNA abundance of voltage negative and positive splice variants of the α₁F-subunit, as well as the LTRPC2 channel in resting and activated T cells. The S15 ribosomal subunit PCR served as a control for integrity of cDNA samples and to normalize the Ca²⁺ channel mRNA expression. The graphical analysis represents the fold induction or reduction of mRNA expression in the treated samples compared to the untreated sample. The fold induction of the untreated sample equals one in all experiments. The human PBTs contained 55.8% CD3⁺CD4⁺CD8⁻, 34.9% CD3⁺CD4⁺CD8⁺, 8.3% CD3⁺CD4⁺CD8⁺, and 1.0% CD3⁺CD4⁻CD8⁻. Each bar represents the mean and SD of assays from duplicate samples. The results shown here are representative of three independent experiments.
the mRNA expression of this splice variant to return to levels similar to the no treatment (NT) control. When Jurkat T cells were stimulated for 1 h, the voltage negative splice variant underwent a robust 1347-fold increase in mRNA expression, which again returned to the baseline level after 4 h of OKT3 and TPA stimulation. Inducible mRNA expression has been previously observed in T lymphocytes. A regulatory mechanism termed activation-induced splicing is responsible for the 100-fold increase in TNFα mRNA following naïve T lymphocyte activation (150). Activation-induced splicing of TNFα mRNA involves the accumulation of TNFα pre-mRNA in resting T cells, followed by the rapid processing of pre-mRNA into mature TNFα mRNA following TCR-induced activation (150). Since the PCR primers amplified a region in the α1F-subunit mRNA overlapping a ~810 bp intronic sequence, real-time PCR products were analyzed on a 1% agarose gel to determine whether activation-induced splicing may regulate the expression of the voltage negative splice variant. It was found that the expected 240 bp PCR product of the mature mRNA of voltage negative isoform was weakly expressed in untreated Jurkat T cells and present at higher levels following 1 and 2 min of activation (Figure 3-8A). The expected PCR product of the unprocessed pre-mRNA of the voltage negative isoform is ~1050 bp. In the absence of TCR stimulation, there was an additional PCR product present at ~450 bp, and after 5 min of stimulation a ~1050 bp PCR product, possibly corresponding to the pre-mRNA, was observed. The 450 and 1050 bp PCR products were not a result of genomic DNA amplification since prior to cDNA synthesis, total RNA preparations were treated with RNase-free DNase to remove contaminating genomic DNA. Therefore, activation-induced splicing may
Figure 3-8: Activation-induced splicing may control the mRNA expression of the voltage negative splice variant of the $\alpha_{IF}$-subunit in Jurkat T cells.

Jurkat T cells were either untreated or stimulated with 10 $\mu$g/ml soluble OKT3 and 10 nM TPA for 1 min, 2 min, 5 min, 10 min, 1 h and 4 h in duplicate samples. (A) Real-time PCR was used to quantify the mRNA abundance of voltage negative splice variant of the $\alpha_{IF}$-subunit. PCR fragments of ~240 bp (voltage negative mature mRNA), 450 bp, and 1050 bp (voltage negative pre-mRNA) were amplified using splice variant specific primers that spanned exon 29 to the exon 30 and 35 splice junction. (B) Real-time PCR was used to quantify the mRNA abundance of voltage positive splice variant of the $\alpha_{IF}$-subunit. A single PCR fragment of ~380 bp, corresponding to the voltage positive mature mRNA, was detected using splice variant specific primers that spanned exons 29 to 33. The S15 ribosomal subunit PCR served as a loading control. PCR products were analyzed on a 1% ethidium bromide-stained agarose gel. The results are representative of three independent experiments.
partially regulate the mRNA expression of the voltage negative splice variant of the $\alpha_{\text{IF}}$-subunit.

The expression of the voltage positive splice variant of the $\alpha_{\text{IF}}$-subunit in Jurkat T cells was then examined, and it was found that the mRNA expression was also altered following activation, but not to the same extent as the voltage negative variant. A 4-fold increase in the voltage positive variant expression was induced after 1 min of OKT3 and TPA treatment, which quickly returned to baseline levels at 2 and 5 min (Figure 3-7B). At later activation time periods of 10 min, 1 h and 4 h the expression of the voltage positive variant increased 2.4-, 2.8- and 7.6-fold, respectively. Pre-mRNA corresponding to the voltage positive splice variant was not observed in resting or activated Jurkat T cells (Figure 3-8B). Interestingly, the two $\alpha_{\text{IF}}$-subunit splice isoforms appeared to have different expression profiles in Jurkat T cells following TCR-induced activation.

Next, the effect of activation on $\alpha_{\text{IF}}$-subunit splice variant expression in Jurkat T cells to the untransformed naïve human PBTs was directly compared. It was found that the mRNA expression of the voltage negative and positive splice variants of the $\alpha_{\text{IF}}$-subunit was markedly different when comparing activated Jurkat T cells to human PBTs. When PBTs were stimulated with OKT3 and TPA, the mRNA expression of the voltage negative and positive splice variants was not upregulated at the activation time periods tested (Figure 3-7, C and D, respectively). Instead the expression of both of the $\alpha_{\text{IF}}$-subunit splice variants remained relatively constitutive after anti-CD3 stimulation with a consistent yet small decline in expression after 4 h of activation. PCR products representing voltage negative and positive pre-mRNAs were not present in either untreated or stimulated human PBTs.
Finally, the mRNA expression profiles of the α_{iF}-subunit splice variants were compared to one of the recently identified TRP Ca^{2+} channels, LTRPC2, that is expressed in Jurkat T cells, human PBTs and weakly in human spleen (Figure 3-9A). It was previously demonstrated that the LTRPC2 Ca^{2+} channel is a novel mediator of Ca^{2+} influx in immunocytes, including Jurkat T cells and peripheral blood lymphocytes (108). Therefore, it was addressed whether LTRPC2 Ca^{2+} channel expression was differentially regulated in comparison to the α_{iF}-subunit splice variants following TCR-induced activation. As illustrated in Figure 3-7, LTRPC2 mRNA expression in resting and activated Jurkat T cells (Figure 3-7E) and human PBTs (Figure 3-7F) was substantially different compared to the expression of the two α_{iF}-subunit splice variants. It was also found that there were slight differences in LTRPC2 expression between Jurkat T cells and naïve PBTs. In Jurkat T cells, the expression of the LTRPC2 Ca^{2+} channel gradually decreased over increasing activation time, whereas in PBTs there was an immediate 2-fold increase in LTRPC2 expression after 1 min of activation followed by a slow decline in expression of this Ca^{2+} channel.

In addition to the LTRPC2 Ca^{2+} channel, other TRP Ca^{2+} channels, including CaT1 and TRPC6, were reported to contribute to Ca^{2+} influx pathways during T lymphocyte activation (105, 110). Because of their significant roles in mediating Ca^{2+} entrance, it was worthwhile examining the mRNA expression of both CaT1 and TRPC6 Ca^{2+} channels in resting and TCR-stimulated Jurkat T cells and naïve human PBTs through real-time PCR. Surprisingly, after repeated PCR attempts with several primer pairs, including published primer sequences, and different PCR reaction parameters, PCR products of the correct size were not amplified for either CaT1 (Figure 3-9B) or TRPC6
Figure 3-9: mRNA expression of different TRP subfamily members in Jurkat T cells, human PBTs and human spleen.

(A) Using a RT-PCR reaction with human LTRPC2 specific primers, a ~660 bp PCR product corresponding to the LTRPC2 Ca\textsuperscript{2+} channel was isolated from Jurkat T cells, human PBTs and human spleen. (B) Human CaT1 specific primers designed by Cui et al. did not detect the expected PCR fragment of 271 bp from the human T cell mRNAs tested, instead two non-specific PCR fragments of 650 and 300 bp were amplified (105). (C) The predicted 116 bp PCR fragment, corresponding to human TRPC6, was not amplified using TRPC6 specific primers designed by Gamberucci et al. (110). Instead non-specific PCR fragments of ~600 bp and 350 bp that did not correspond to TRPC6 were detected. All PCR products were resolved on an ethidium bromide stained 1% agarose gel. The results shown here are representative of two independent experiments with the abovementioned primer sets.
(Figure 3-9C) from Jurkat T cells, human PBTs and human spleen. Since the amplification of multiple PCR products hinder real-time PCR analysis, the expression of CaT1 and TRPC6 could not be evaluated using this technique.

3.2.7 Comparison of \( \alpha_{1F} \)-Subunit Protein Expression in Resting and Activated Jurkat T Cells

After thoroughly examining the mRNA expression of the \( \alpha_{1F} \)-subunit splice variants in Jurkat T cells, it was then important to determine whether the observed increases in mRNA expression triggered by TCR stimulation lead to augmented \( \alpha_{1F} \)-subunit protein expression. To determine the effects of activation on \( \alpha_{1F} \)-subunit protein levels, Jurkat T cells were either untreated or stimulated with OKT3 and TPA for time periods of 24 h, 48 h and 72 h. In this experiment, longer activation times were used compared to the real-time PCR experiments, to ensure complete protein translation of the \( \alpha_{1F} \)-subunit. Following OKT3/TPA treatment, immunoprecipitations from Triton X-100 membrane solubilized lysates of Jurkat T cells with an Ab specific to the human retina \( \alpha_{1F} \)-subunit were performed. The immunoprecipitated protein was then analyzed by Western blotting with the \( \alpha_{1F} \)-subunit Ab.

As demonstrated in Figure 3-10, the \( \alpha_{1F} \)-subunit protein was expressed at relatively low levels in untreated Jurkat T cells. In the presence of OKT3 and TPA, \( \alpha_{1F} \)-subunit protein expression increased with increasing activation time, and appeared to reach maximal expression levels after 48 h of stimulation. The stability of the \( \alpha_{1F} \)-subunit protein was also examined. Jurkat T cells were stimulated for 24 h, 48 h and 72 h with
Figure 3-10: Activated Jurkat T cells express increased levels of $\alpha_{1F}$-subunit protein.

Jurkat T cells were either NT or stimulated with 10 μg/ml OKT3 and 10 nM TPA for 24 h, 48 h or 72 h. $\alpha_{1F}$-Subunit protein (~200 kDa) was then immunoprecipitated using affinity-purified Abs against human $\alpha_{1F}$-subunit from 1.5 mg of membrane protein solubilized lysates of untreated and activated Jurkat T cells. The immunoprecipitated protein was analyzed by Western blotting with the $\alpha_{1F}$-subunit Ab. Equal protein loading was ensured by quantifying the protein concentration in each sample by the Bicinchonic acid protein assay. Results depicted are representative of two independent experiments.
OKT3 and TPA in the presence of the protein translation inhibitor, 10 μg/ml cycloheximide. It was found that the αtF-subunit protein was weakly expressed at 48 h after activation and cycloheximide treatment, however, at 72 h the αtF-subunit protein could not be detected (data not shown). Overall, in activated Jurkat T cells, increases in αtF-subunit mRNA correlated with enhanced αtF-subunit protein expression. In addition, the expressed αtF-subunit protein was more stable than αtF-subunit mRNA expression in Jurkat T cells.

Finally, it was examined whether TCR-dependent regulation of αtF-subunit mRNA and protein expression was due to a direct association of the αtF-subunit and the TCR/CD3 complex. The possible association between the αtF-subunit and the TCR was investigated by a coimmunoprecipitation assay with the αtF-subunit Ab and the CD3 mAb, OKT3 using Jurkat T cells solubilized with 0.5% Triton X-100. The αtF-subunit protein could not be detected in CD3 immunoprecipitates, and CD3 was not present in αtF-subunit immunoprecipitates under the detergent conditions used (data not shown). Therefore, it appears that the TCR-dependent regulation was not due to physical association of the TCR/CD3 complex with the αtF-subunit channel variants.

3.3 Discussion

Since there are only 10 reported genes encoding α1-subunits, serving many diverse functions, including muscle contraction to initiation of gene transcription, alternative pre-mRNA splicing is one mechanism used to generate tissue specificity and
functional diversity of the channel-forming α₁-subunits (114). In this study, it was established that two novel splice isoforms of the L-type α₁F-subunit are expressed in human T lymphocytes. The truncated α₁F-subunit variants are unlike other alternatively spliced α₁-subunits that have been previously described, and structurally distinct from the α₁F-subunit originally isolated from human retina. Although the functional expression of novel α₁F-subunit isoforms in T lymphocytes has been characterized here, alternative usage of exons has been reported for other α₁-subunits expressed in non-excitable cells. The murine erythroleukemia cell (MELC) line expresses a truncated form of the cardiac α₁C-subunit, which lacks the first four transmembrane segments S1-S4 in motif I (151). When overexpressed as a chimeric channel in *Xenopus* oocytes, the MELC α₁C-subunit forms a functional Ca\(^{2+}\) channel protein (151). In addition, Grafton *et al.* also provided evidence that a similar truncated α₁C-subunit is functionally expressed in both T and B lymphocytes (135, 152). Therefore, it appears that alternative splicing of the α₁-subunits leads to the expression of structurally unique α₁-subunit proteins in non-excitable cell types that have adopted cell-specific functions, while maintaining sequence identity to α₁-subunits present in electrically excitable cells.

Excision of several functional domains in the T lymphocyte α₁F-subunit channel variants by alternative splicing may alter characteristic properties of a VDCC, such as voltage sensing, drug binding and inactivation kinetics. First, it was found that the α₁F-subunit splice isoforms undergo splicing which may render these channels insensitive to membrane depolarization. In the voltage negative splice variant, the splicing out of exon 33 leads to the deletion of the S4 voltage sensor domain in motif IV. Previous studies
using site-directed mutagenesis have revealed that the basic residues in the S4 domains significantly contribute to the voltage sensing properties of voltage-gated ion channels (153, 154). When these basic residues are neutralized the α₁-subunit becomes unresponsive to membrane depolarization (153). Therefore, deletion of the entire IVS4 domain in the voltage negative splice variant and consequential removal of several basic residues would presumably abolish gating charge dependence of this splice isoform even though 3 voltage sensor domains still remain intact.

In the voltage positive splice variant, a voltage sensor domain is not deleted, however, splicing of a small exon that encodes part of the IVS3-S4 extracellular loop may alter the voltage sensing function of this channel isoform. Since the S4 voltage sensor domain moves upon membrane depolarization, it has been proposed that splicing of the IVS3-S4 domain interlinker may prevent S4 movement due to their close proximity (114, 155). The deletion of the short exon encoding part of the IVS3-S4 extracellular loop has also been reported in the α₁C-, α₁D-, and α₁S-subunits of L-type VDCCs (114). For instance, rat osteosarcoma cells express an α₁D-subunit lacking the IVS3-S4 linker that is capable of gating Ca\(^{2+}\) influx in response to the parathyroid hormone, but not to membrane depolarization (156). In conjunction with the previous studies on voltage sensor function, the results presented here indicate that the deletion of either the IVS4 voltage sensor domain or the IVS3-S4 interlinker may prevent the α₁F-subunit isoforms from being gated by membrane depolarization. An alternative gating mechanism may be present in T lymphocytes, such as ER store-depletion or a direct signal from the TCR.
In addition to changes in voltage sensing, alternative splicing may also alter the putative DHP binding properties of the $\alpha_{1F}$-subunit isoforms. It was observed that excision of exon 37 removes 3 amino acids in a portion of the IVS6 transmembrane domain that have been proposed to confer DHP sensitivity and binding to the $\alpha_{1F}$-subunit (137). Previous analysis on the DHP binding site has revealed that DHPs bind to three separate sites on IIIS5, IIIS6 and IVS6 transmembrane domains of $\alpha_1$-subunits that are allosterically linked (157). Site-directed mutagenesis of amino acids that bind DHPs in the IVS6 domain leads to a 100-fold decrease in DHP binding sensitivity (158). It has also been shown that naturally-occurring splice variants of the human $\alpha_{1C}$-subunit have different sensitivities to the inhibitory action of the DHP derivative, (+)-[3H]isradipine (159). Therefore, the lack of at least 3 amino acids required for DHP sensitivity in the $\alpha_{1F}$-subunits splice isoforms may provide one explanation why it is necessary to apply large doses of DHPs, such as nifedipine, to completely block Ca$^{2+}$-influx through L-type VDCCs in T lymphocytes (160).

Along with domain interlinkers and the S6 transmembrane domains, the carboxyl-terminus is also not well conserved within the $\alpha_1$-subunit gene family, implying this region may generate further functional diversity of $\alpha_1$-subunit proteins (114). In this study, it was found that the deletion of exon 37 and the resulting reading frameshift significantly alter the carboxyl-termini of both $\alpha_{1F}$-subunit splice isoforms. First, it was observed that the splicing out of exon 37 leads to the removal of an EF-hand Ca$^{2+}$-binding motif. Next, it was noted that the subsequent reading frameshift prevents the translation of a 4 amino acid cluster (VVAL in $\alpha_{1F}$-subunit, VVTL in $\alpha_{1C}$-subunit) within the $F$ helix of the EF-hand motif that is essential for Ca$^{2+}$-dependent inactivation in high-
voltage activated channels (161). Interestingly, it was recently reported that the retinal 
$\alpha_{1F}$-subunit, unlike other L-type $\alpha_{1}$-subunits, does not display Ca$^{2+}$-dependent 
inactivation (143). The calmodulin-binding isoleucine-glutamine (IQ) motif located in the 
carboxyl-terminus of retinal $\alpha_{1F}$-subunit has also been suggested to confer Ca$^{2+}$-
dependent inactivation; however, it has not been determined whether calmodulin 
mediates Ca$^{2+}$-dependent inactivation through this motif in the $\alpha_{1F}$-subunit (143, 162). It 
should be noted that the IQ motif is absent in both $\alpha_{1F}$-subunit splice isoforms. Due to the 
reported lack of Ca$^{2+}$-dependent inactivation, the removal of the EF-hand and IQ motifs 
through alternative splicing may not effect the biophysical properties of the $\alpha_{1F}$-subunit 
splice isoforms.

It was also observed that the reading frameshift causes a portion of the carboxyl-
termini of the $\alpha_{1F}$-subunit splice isoforms to have 40% amino acid identity to the human 
skeletal muscle $\alpha_{1S}$-subunit L-type VDCC. To my knowledge, this is the first report of 
this form of “splice conversion”. Although the functional significance of the sequence 
identity switching to the $\alpha_{1S}$-subunit is not clear, it may impart important consequences to 
the biological functioning of the channel proteins. Finally, it was observed that the 
reading frameshift also results in early truncation of the carboxyl-termini of both $\alpha_{1F}$-
subunit splice isoforms. There are several studies reporting the expression of truncated L-
type $\alpha_{1}$-subunits. Two-domain splice variants consisting of motifs I and II of the $\alpha_{1C}$-
subunit and motifs I and IV of the $\alpha_{1S}$-subunit exist in cardiac and skeletal muscle, 
respectively (163, 164). However, at the present time, none of these two-domain $\alpha_{1}$-
subunits have been functionally expressed (114). Additionally, a premature stop codon 
mutation located at amino acid 1459 in the carboxyl-termini of the retinal $\alpha_{1F}$-subunit
protein found in CSNB patients does not appear to alter the activation, inactivation, and conductance properties of the α₁F-subunit (143). Based on the experiments with the mutated α₁F-subunit, it is plausible the α₁F-subunit splice isoforms that are truncated at amino acid 1452 may have similar biophysical properties to the retinal α₁F-subunit. In summary, deletion of exon 37 dramatically alters the carboxyl-termini of both α₁F-subunit isoforms. Determining the current gated by the α₁F-subunit isoforms will ultimately provide insight into how alternative splicing effects the biophysical properties of the α₁F-subunit channel.

When the mRNA expression of the α₁F-subunit splice isoforms in different human leukocyte populations was investigated, it was determined that the voltage negative and positive splice variants are expressed in both T and B lymphocytes, whereas monocytes only express the voltage positive variant. It is not surprising that T and B lymphocytes express the two α₁F-subunit splice isoforms since Ca²⁺ influx is induced through antigen receptor ligation and is dependent upon store-depletion in both cell types. However, it remains unclear whether this data supports the hypothesis that the reported Ca²⁺ kinetic differences in distinct lymphocyte populations are due to differential mRNA expression of the α₁F-subunit splice isoforms. Quantitative real-time PCR analysis may reveal more subtle differences in the mRNA expression levels of both splice variants in peripheral blood CD4⁺ and CD8⁺ T cells, as well as B lymphocytes. The demonstration that human monocytes express the voltage positive splice variant is the first direct evidence that an L-type VDCC lacking voltage sensing properties is expressed in monocytes. Interestingly, Willmott et al. reported that store-operated Ca²⁺ influx in the U937 immature human monocytic cell line was mediated through a DHP sensitive L-type VDCC, which was
insensitive to membrane depolarization (165). Therefore, the properties of the voltage positive splice isoform described here suggest that this \( \alpha_{1F} \)-subunit variant may also participate in \( \text{Ca}^{2+} \) influx in human monocytes.

Subsequently, \( \alpha_{1F} \)-subunit protein expression was examined, and it was found that the \( \alpha_{1F} \)-subunit protein is definitively expressed in Jurkat T cells and human PBTs. The identification of the \( \alpha_{1F} \)-subunit protein in T lymphocytes directly contrasts with previous findings on \( \alpha_{1F} \)-subunit protein expression in lymphoid tissues. Through immunohistochemical analysis of human lymph node, thymus, and spleen, McRory et al. showed that T and B lymphocytes did not interact with an \( \alpha_{1F} \)-subunit Ab produced against amino acids 1658-1723 of the human retinal \( \alpha_{1F} \)-subunit protein (GenBank accession number NM_005183) (143). Consistent with our observations, the \( \alpha_{1F} \)-subunit Ab utilized by McRory et al. (directed against a portion of the carboxyl-tail of the retinal \( \alpha_{1F} \)-subunit that is absent in the voltage negative and positive splice isoforms due to a premature stop of protein translation), did not stain T and B lymphocytes positive for the \( \alpha_{1F} \)-subunit protein in the tissues analyzed (143). Although the voltage negative and positive splice variant proteins differ in size by \(~100\) amino acids or 11 kDa, visual resolution of the splice isoforms as individual protein bands on a Western blot was not observed. The individual \( \alpha_{1F} \)-subunit splice isoforms may not have been visualized since one of the splice isoforms may be expressed at too low a level to be detected by immunoblotting. Alternatively, the predicted N-linked and O-linked glycosylation may cause the splice variants to have similar molecular weights, preventing the resolution of the \( \alpha_{1F} \)-subunit splice isoforms as two individual protein bands.
When the endogenous mRNA expression of the \( \alpha_{1F} \)-subunit splice isoforms following anti-CD3 stimulation was examined through real-time PCR, it was found that expression of both isoforms is regulated by TCR-induced activation in Jurkat T cells and to a lesser extent in human PBTs. In T lymphocytes, the mechanisms used to regulate immediate and early activation genes include alterations in the rate of transcription and mRNA stabilization (166). The rapid and large induction of mRNA expression of the voltage negative splice variant in Jurkat T cells activated for 1 and 2 min may be explained by a combinatorial effect of increased transcription rate and mRNA stability, as well as "activation-induced splicing" of this splice isoform. Activation-induced splicing has been previously described as the regulatory mechanism responsible for large increases in TNF\( \alpha \) mRNA following naïve T lymphocyte activation (150). Yang et al. reported that TNF\( \alpha \) pre-mRNA accumulates in naïve mouse CD4\(^+\) T cells and TCR engagement induces splicing of the TNF\( \alpha \) pre-mRNA, resulting in a rapid rise in TNF\( \alpha \) mRNA within 15 min of activation (150). When examining the mRNA expression of the voltage negative splice variant in Jurkat T cells, it was found the expected 240 bp PCR product was amplified, as well as two larger PCR fragments, one of which may correspond to voltage negative variant pre-mRNA. This suggests that activation-induced splicing may play a role in the rapid mRNA expression of this \( \alpha_{1F} \)-subunit splice variant.

In addition to activation-induced splicing, other genes have shown to undergo immediate alternative splicing due to increases in Ca\(^{2+}\) influx. For instance, alternative splicing of the plasma membrane Ca\(^{2+}\)-ATPase pump occurs after only 1 min in response to increased [Ca\(^{2+}\)], and can be suppressed by pretreatment with the membrane permanent Ca\(^{2+}\) chelator, BAPTA-AM, in IMR32 human neuroblastoma cells (167). In this study,
robust increases in the mRNA expression of voltage negative splice variant at 1 and 2 min, and 1 h of TCR stimulation, correlates with increases in \([\text{Ca}^{2+}]_i\). Since it has been previously suggested that all surface TCR/CD3 complexes are recycled within 1 h, the observed increased in mRNA expression after 1 h may be due to increased \([\text{Ca}^{2+}]_i\) as a result of TCR recycling (168). Slight decreases in \([\text{Ca}^{2+}]_i\) may also be responsible for the decline in mRNA expression of the voltage negative variant at 5 and 10 min, as well as at the latest time point of 4 h. In comparison to the voltage negative variant, the voltage positive splice variant undergoes relatively small increases in mRNA expression levels at 1 min, 10 min, 1 h and 4 h of activation in Jurkat T cells. The rapid increases and decreases in mRNA expression of both \(\alpha_{IF}\)-subunit splice isoforms suggest tightly controlled regulatory mechanisms of gene expression (169). In addition, the differences in mRNA expression of the \(\alpha_{IF}\)-subunit splice isoforms indicate that the voltage negative variant may contribute to immediate and sustained \(\text{Ca}^{2+}\) entry, whereas the voltage positive variant may regulate only sustained \(\text{Ca}^{2+}\) influx in activated Jurkat T cells. The observed increase in \(\alpha_{IF}\)-subunit protein expression after 1 to 2 days of activation also supports the hypothesis for a role for both splice variants in contributing to sustained \(\text{Ca}^{2+}\) influx.

In contrast to Jurkat T cells, the mRNA expression of the voltage negative and positive splice variants in human PBTs did not appear to be tightly regulated by TCR-induced activation since the expression of both splice isoforms was not significantly altered following anti-CD3 treatment. The only consistent change was a small decrease in mRNA expression of both splice isoforms following 4 h of activation. The mRNA expression profiles suggest that the \(\alpha_{IF}\)-subunit splice isoforms may contribute to a small,
sustained Ca$^{2+}$ influx in PBTs, which dissipates slowly after 4 h. In addition to the data shown here, there are other reported differences in Ca$^{2+}$ influx pathways when comparing Jurkat T cells to untransformed PBTs. It has been previously demonstrated through electrophysiological means that in Jurkat T cells the number of CRAC channels is between 100 to 400 (129), whereas resting PBTs only express 15 CRAC channels (130). One further difference that was observed between Ca$^{2+}$ channel expression in Jurkat T cells and PBTs was an immediate increase in LTRPC2 Ca$^{2+}$ channel mRNA expression only in PBTs following activation. This suggests that LTRPC2 may significantly contribute to the initial Ca$^{2+}$ response in PBTs but not to the same magnitude in Jurkat T cells. Taken together, the unique mRNA expression profiles of the $\alpha_{1F}$-subunit splice isoforms and the LTRPC2 Ca$^{2+}$ channel illustrate the complexity of the Ca$^{2+}$ response during crucial hours of T cell activation. Furthermore, the identification of novel voltage-dependent-like Ca$^{2+}$ channels suggests that different Ca$^{2+}$ channels may be involved in different phases of the Ca$^{2+}$ influx pathway, and together these channels may generate a current that resembles $I_{\text{CRAC}}$.

In summary, the study on the expression of $\alpha_{1F}$-subunit provided extensive molecular and biochemical evidence for the presence of an L-type Ca$^{2+}$ channel in T lymphocytes. The data presented here provides a foundation for further exploration of the role of the alternatively spliced $\alpha_{1F}$-subunits during T lymphocyte activation. Overexpression of the voltage negative splice variant of the $\alpha_{1F}$-subunit in Jurkat T cells will be conducted to establish a definitive role for this $\alpha_{1F}$-subunit splice isoform during T cell activation. Further elucidation of the role of the L-type Ca$^{2+}$ channel $\alpha_{1F}$-subunit
will provide the basis for a better understanding of the mechanisms controlling Ca\(^{2+}\) influx in T lymphocytes.
CHAPTER 4: DEFINING THE CONTRIBUTION OF L-TYPE CALCIUM CHANNELS TO CALCIUM INFLUX DURING T LYMPHOCYTE ACTIVATION IN VITRO

4.1 Introduction

Pharmacological studies pioneered by the German physiologist Albrecht Fleckenstein in 1964 lead to the discovery of two chemically distinct compounds, verapamil and prenylamine that were found to inhibit cardiac muscle excitation-contraction coupling in a Ca\(^{2+}\)-dependent manner (170). Fleckenstein and his colleagues showed that the effect of these drugs on cardiac muscle mimicked Ca\(^{2+}\) withdrawal, which could be overcome by the addition of high concentrations of extracellular Ca\(^{2+}\) (170). Shortly thereafter, several compounds that were also potent inhibitors of excitation-contraction coupling were synthesized, propelling the emergence of a new class of pharmacodynamic drugs termed “Ca\(^{2+}\) antagonists” (171). Ca\(^{2+}\) antagonists drew immediate attention as a potential therapeutic treatment of cardiovascular diseases because of their cardiodepressant effects. In the mid 1970’s, Ca\(^{2+}\) antagonists, such as verapamil, were first introduced to patients for the treatment of high blood pressure (172), cardiac arrhythmias (173, 174) and angina pectoris (175). Subsequently, researchers elucidated that the mode of action of the Ca\(^{2+}\) antagonists was to selectively bind to L-type VDCCs in the plasma membrane, blocking extracellular Ca\(^{2+}\) influx through these channels, which results in the relaxation of cardiac and vascular smooth
muscle (171, 176). Following these studies Ca\textsuperscript{2+} antagonists were appropriately renamed by some researchers as Ca\textsuperscript{2+} channel blockers (CCBs) (177). Currently, CCBs are still widely prescribed by clinicians for the treatment of several cardiovascular diseases including hypertension, angina and some arrhythmias (157).

From the time of the initial discovery of Ca\textsuperscript{2+} antagonism by Fleckenstein, a continually increasing number of selective second, third and fourth generation CCBs have evolved (178). CCBs are presently classified into three chemically distinct drug groups, including benzothiazepines (i.e. diltiazem), phenylalkylamines (i.e. verapamil), and DHPs (i.e. nifedipine) (Figure 4-1) (157). Although all three classes of CCBs bind and modulate L-type VDCC function, they show marked differences in chemical structure, binding sites to L-type VDCCs, tissue selectivity and clinical activity (157). The physical state of L-type VDCCs are either resting (closed), open (activated) or inactivated (135), and therefore CCB binding to L-type VDCCs is both voltage and channel state dependent (178). Unlike benzothiazepines and phenylalkylamines that require open channels for binding, DHP antagonists bind to the inactivated channel state and stabilize it. DHP agonists, such as (+/-) Bay K 8644 also favor binding to the open state of the channel (Figure 4-1) (135, 157). Diltiazem, verapamil, and nifedipine are all used to treat hypertension since they preferentially bind and inhibit the function of L-type VDCCs in vascular smooth muscle (157). In contrast to DHPs, verapamil and diltiazem also bind to L-type Ca\textsuperscript{2+} channels in cardiac muscle, therefore these drugs are an effective treatment of cardiac arrhythmias (157).

Since the synthesis of the first DHP in 1882 by Hantzsch, the DHP family has grown to contain the largest number of derivatives compared to benzothiazepines and
Figure 4-1: Chemical Structures of L-Type Ca\(^{2+}\) Channel Modulators

(A) First generation CCBs with clinical importance in the treatment of cardiovascular disease are diltiazem (benzothiazepines group) verapamil (phenylalkylamines group) and nifedipine (DHP group). The Ca\(^{2+}\) agonist, (+/-) Bay K 8644 is a member of the DHP group. The structure of DHPs is based upon a core pyridine structure. (B) The orientation of the 4-phenyl ring determines whether the DHP derivative is a Ca\(^{2+}\) channel antagonist or agonist. The “up” position of the 4-phenyl ring confers antagonistic properties, whereas the “down” position confers agonistic properties to the DHP. This figure was taken from Striessnig et al. (179).
phenylalkylamines (180). As a result, DHPs have become the most broadly studied class of CCBs. The specificity of action of DHPs has been confirmed by establishing that the DHP binding sites are located within the channel-forming α₁-subunit of L-type VDCCs. Initially, several groups used photoreactive DHP reagents to covalently label and localize the DHP receptor site within the α₁-subunit (179, 181). The DHP photolabeled α₁-subunits were proteolytically digested, and the resulting labeled peptides were identified by immunoprecipitation with anti-peptide antibodies (157). Studies performed by Nakayama et al. and Striessnig et al. revealed that the photoreactive DHPs bound to transmembrane domain IIIS6 and the domain interlinker connecting IIIS5 and IIIS6 in the skeletal muscle α₁S-subunit (179, 181). Chimeric Ca²⁺ channels were then used to further pinpoint the regions of DHP binding. Tang et al. constructed chimeric α₁-subunits by replacing regions of domains III and IV in the cardiac muscle α₁C-subunit that confer DHP sensitivity with identical portions of the α₁A-subunit that are DHP insensitive (182). This study did not report any DHP binding to domain III, but did demonstrate high-affinity DHP binding to the transmembrane domain IVS6 and to the domain interlinker connecting IVS5 and IVS6 (182). Following these studies, site-directed mutagenesis was used to identify individual amino acids that are the critical determinants for DHP binding. Mutagenesis studies also resolved discrepancies in the different DHP binding sites identified by photoreactive DHPs and channel chimeras (183, 184). Intensive analysis of the DHP binding site has revealed that amino acids in the IIIS5, IIIS6 and IVS6 transmembrane domains of α₁-subunits are allosterically linked and essential for DHP binding and action (177, 185).
Synthetic DHP derivatives are not only utilized as a therapeutic, but they are also powerful research tools used extensively to identify the presence of putative L-type Ca\textsuperscript{2+} channels in the plasma membranes of numerous cell types, including T lymphocytes (157). It has been reported that the DHP L-type Ca\textsuperscript{2+} channel antagonist, nifedipine, is a potent suppressor of T lymphocyte proliferation. Based upon an \textit{in vitro} [\textsuperscript{3}H]-thymidine uptake assay, Birx \textit{et al.} demonstrated that 0.001 µM to 100 µM nifedipine prevented the proliferation of human T lymphocytes in response to the mitogens, phytohemagglutinin (PHA) and Con A (186). In a similar study, human PBMCs stimulated with PHA were unable to proliferate in the presence of 10 µM to 200 µM nifedipine; the addition of IL-2 restored the proliferative response in the nifedipine-treated cells (187). Furthermore, it has been demonstrated through \textit{in vitro} proliferation assays that nifedipine has a dose-dependent inhibitory effect on T lymphocyte proliferation when added in combination with the immunosuppressive agent CsA (188, 189). Ricci \textit{et al.} provided additional evidence that L-type VDCCs are present in lymphocytes by showing that the radiolabeled DHP antagonist (+)-[\textsuperscript{3}H]isradipine bound to human peripheral blood lymphocytes with high affinity and that (+)-[\textsuperscript{3}H]isradipine binding was only weakly displaced by verapamil or diltiazem (190).

The aim of the work presented here was to further investigate the presence of L-type Ca\textsuperscript{2+} channels in T lymphocytes and to understand the contribution of Ca\textsuperscript{2+} influx through L-type VDCCs during T lymphocyte activation and proliferation. After establishing that the channel-forming α\textsubscript{1F}-subunit of L-type Ca\textsuperscript{2+} channels is expressed in T lymphocytes, the next objective was to determine whether L-type Ca\textsuperscript{2+} channels play a critical role in TCR-induced activation. To define the contribution of the α\textsubscript{1F}-subunit
splice isoforms to TCR-induced Ca\(^{2+}\) influx, experiments were conducted to knock-down \(\alpha_{1F}\)-subunit splice variant expression in Jurkat T cells through RNA interference (RNAi). Since the \(\alpha_{1F}\)-subunit and other DHP sensitive \(\alpha_1\)-subunits are expressed in T lymphocytes, it was hypothesized that DHP derivatives would directly bind to these \(\alpha_1\)-subunits and effect Ca\(^{2+}\) influx and Ca\(^{2+}\)-dependent signaling in T cells. Therefore, the effects of (+/-) Bay K 8644 (a DHP agonist that induces L-type Ca\(^{2+}\) channel opening) and nifedipine (a DHP antagonist that blocks L-type Ca\(^{2+}\) channels) on Ca\(^{2+}\) influx and T lymphocyte activation pathways were then examined. Although earlier studies demonstrated that nifedipine could block T cell proliferation, the investigators did not thoroughly explore the effects of nifedipine on the T lymphocyte activation process. Furthermore, while some of the previous reports on nifedipine have examined the non-specific stimulation of T lymphocytes using the mitogenic lectins PHA and Con A, the focus of this study was on the specific activation of T lymphocytes through the TCR/CD3 complex using the anti-CD3 mAb, OKT3. In all experiments where possible the effects of (+/-) Bay K 8644 and nifedipine on the Jurkat T cell leukemia line were compared to T lymphocytes isolated from human peripheral blood to establish if there were discrepancies between T cell lines and untransformed T cells. The results in this study collectively suggest the presence of a DHP sensitive L-type VDCC in the plasma membrane of T lymphocytes that contributes to Ca\(^{2+}\) entry during T cell activation.
4.2 Results

4.2.1 Examination of the Effects of siRNA on α_{iF}-Subunit Expression in Jurkat T Cells

RNAi is a powerful gene silencing technique that uses siRNA duplexes, 19 to 22 nucleotides in length and complementary to the gene of interest, to mediate specific mRNA degradation. To determine the contribution of the α_{iF}-subunit to Ca^{2+} influx following TCR engagement, the pSUPER vector system was used to stably express 19 bp siRNA duplexes specifically targeted to the α_{iF}-subunit in Jurkat T cells (Figure 2-3). The pSUPER constructs used in this study were designed to target the degradation of both α_{iF}-subunit splice isoforms expressed in Jurkat T cells since the siRNA target sequences were located upstream of the spliced regions (Table 2-1). In addition, as a control for siRNA efficacy, Jurkat T cells were transfected with the pSUPER-p53 construct, which has been shown to specifically knock-down endogenous p53 mRNA and protein expression through RNAi in different cell types (123).

After approximately two months growing under selection conditions, bulk cultures of Jurkat T cells co-transfected with the pEYFP vector and either pSUPER (lacking a siRNA insert), pSUPER-α_{iF}-subunit constructs, or pSUPER-p53 were analyzed for stable integration of the pSUPER constructs into the genomic DNA. As illustrated in Figure 4-2A, all of the pSUPER constructs were stably expressed by Jurkat T cells except for pSUPER-α_{iF}-subunit-1. Subsequent transfections of pSUPER-α_{iF}-subunit-1 into Jurkat T cells did not result in stable expression therefore only three
Figure 4-2: Stable expression of siRNAs targeted to the αtF-subunit in Jurkat T cells did not knock-down αtF-subunit protein expression.

(A) Bulk cultures of Jurkat T cells were examined for stable expression of pSUPER constructs. Using a PCR reaction, ~570 bp PCR products, corresponding to pSUPER constructs containing siRNA inserts, were amplified from genomic DNA. The 510 bp PCR product corresponds to the pSUPER construct without a siRNA insert. A 570 bp PCR product was not amplified from Jurkat T cells expressing pSUPER-αtF-subunit-1, indicating a lack of stable integration of this vector. Untransfected refers to Jurkat T cells not transfected with any constructs. Genomic DNA and PCR products were analyzed on a 1% agarose gel stained with ethidium bromide. (B) Knock-down of p53 protein expression was detected in cloned Jurkat T cells expressing the pSUPER-p53 construct by the lack of p53 protein immunoprecipitated with a mAb against human p53. (C and D) Using affinity-purified Abs against human αtF-subunit, the αtF-subunit protein was immunoprecipitated from three different clones of Jurkat T cell lines expressing different pSUPER-αtF-subunit constructs, demonstrating expressed siRNAs did not knock-down αtF-subunit protein levels. As a loading control for all immunoprecipitation experiments, Western blot analysis was performed on the samples with an Ab directed against GAPDH. Results depicted are representative of at least three independent experiments.
pSUPER-α_{IF}-subunit constructs were used in the remaining experiments. Next, the pSUPER expressing cells were cloned and, where appropriate, analyzed for either p53 or α_{IF}-subunit protein levels. As shown in Figure 4-2B, the pSUPER-53 construct effectively knocked-down endogenous p53 protein expression in Jurkat T cells, whereas transfection of cells with pSUPER, or pEYFP alone did not effect the protein levels of p53. Several clonal Jurkat T cell lines, expressing siRNA to different regions of the α_{IF}-subunit, were then analyzed and it was found that none of the constructs reduced the levels of the α_{IF}-subunit protein compared to α_{IF}-subunit expression in cells transfected with pSUPER or pEYFP alone (Figure 4-2, C and D). Additionally, it was observed that the protein levels of the α_{IF}-subunit in cells transfected with pEYFP alone were reduced in one clone (pEYFP alone, Clone 1 in Figure 4-2D). Therefore, it was reasoned that although siRNA is an effective technique at knocking-down protein expression in Jurkat T cells, the siRNA target sequences chosen in this study did not successfully knock-down α_{IF}-subunit protein expression.

4.2.2 Induction of Calcium Influx in Jurkat T Cells and Human PBTs by (+/-) Bay K 8644

Since the pSUPER vector system did not effectively knock-down α_{IF}-subunit expression in Jurkat T cells, a different strategy was required to assess the functional contribution of L-type Ca^{2+} channels to Ca^{2+} influx during T cell activation. As an alternative approach, the effect of the DHP derivative, (+/-) Bay K 8644, on Ca^{2+} influx in human T lymphocytes was tested. It has previously been reported that the treatment of
Jurkat T cells with Bay K 8644 in the range of 0.01-100 μM induces a small rise in intracellular Ca$^{2+}$, indicating the presence of a DHP sensitive Ca$^{2+}$ influx pathway in these cells (191). However, the report did not specify which enantiomer of Bay K 8644 was used. The (-)-enantiomer of Bay K 8644 has strong agonistic properties, whereas the (+)-enantiomer acts as a weak Ca$^{2+}$ channel antagonist. Since the optical isomers of Bay K 8644 induce opposing L-type VDCC activity, the experiment was repeated in this study with (+/-) Bay K 8644, a racemic mixture that has the net effect of enhancing Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels (192). Additionally, a direct comparison of the effects of (+/-) Bay K 8644 treatment on Ca$^{2+}$ influx in Jurkat T cells to the untransformed PBTs was sought after since this has not been previously examined.

When indo-1 loaded Jurkat T cells and human PBTs were treated with (+/-) Bay K 8644, a dose-dependent increase in the mean ratio of indo-1 bound to Ca$^{2+}$ (405 nm)/free indo-1 (485nm) was observed indicating an increase in intracellular Ca$^{2+}$ (Figure 4-3). In both Jurkat T cells and PBTs, 10 μM (+/-) Bay K 8644 generated a small, sustained rise in intracellular Ca$^{2+}$. However, treatment of Jurkat T cells and PBTs with higher concentrations of (+/-) Bay K 8644 caused different responses in Ca$^{2+}$ influx. In Jurkat T cells, 50 μM and 100 μM (+/-) Bay K 8644 induced a sustained increase in cytosolic Ca$^{2+}$ (Figure 4-3A), whereas treatment of human PBTs with either 50 μM or 100 μM (+/-) Bay K 8644 caused a transient Ca$^{2+}$ influx that rapidly declined to below baseline after the 10 min time period (Figure 4-3B). In the absence of extracellular Ca$^{2+}$ in the medium, 100 μM (+/-) Bay K 8644 did not cause a rise in intracellular Ca$^{2+}$ in either Jurkat T cells or human PBTs, indicating that (+/-) Bay K 8644 treatment with extracellular Ca$^{2+}$ specifically allowed Ca$^{2+}$ entry into these cells. Treatment with DMSO
Figure 4-3: (+/-) Bay K 8644 induces Ca\(^{2+}\) influx in a dose-dependent manner in the human Jurkat T cell leukemia line and human PBTs.

Jurkat T cells (A) and human PBTs (donors, \(n=3\)) (B) were loaded with indo-1. The basal concentration of free intracellular Ca\(^{2+}\) was initially measured, after which 10-100 μM (+/-) Bay K 8644 was added to the sample and the analysis was resumed. Red line, DMSO solvent (Control); Yellow, 10 μM (+/-) Bay K 8644; Green, 50 μM (+/-) Bay K 8644; Grey, 100 μM (+/-) Bay K 8644; Purple, 100 μM (+/-) Bay K 8644 with no extracellular Ca\(^{2+}\) in medium. Indo-1 loading was assessed by stimulating Ca\(^{2+}\) influx with 2 μM ionomycin in Jurkat T cells (C) and human PBTs (D). The human PBTs contained 14% CD3\(^+\)CD4\(^+\)CD8\(^-\), 79% CD3\(^+\)CD4\(^-\)CD8\(^+\), 6.5% CD3\(^+\)CD4\(^-\)CD8\(^-\) and 0.5% CD3\(^-\)CD4\(^-\)CD8\(^+\) cells. The results are representative of three independent experiments.
solvent alone did not induce significant Ca\(^{2+}\) entry into T lymphocytes. The effects of the well-characterized lipophilic Ca\(^{2+}\) ionophore, ionomycin, were also examined to ensure efficient loading of the indo-1 dye into T lymphocytes. The addition 2 μM ionomycin induced a rapid and sustained Ca\(^{2+}\) influx in both Jurkat T cells and human PBTs (Figure 4-3, C and D).

### 4.2.3 Nifedipine Inhibits Anti-CD3 Induced Calcium Influx in Jurkat T Cells and Human PBTs

Nifedipine is a blocker of L-type Ca\(^{2+}\) channels and was used to further investigate the role of a DHP sensitive Ca\(^{2+}\) channel in T lymphocytes. Pretreatment of indo-1 loaded Jurkat T cells and human PBTs with nifedipine in the presence of extracellular Ca\(^{2+}\) resulted in a dose-dependent decrease in the mean ratio of indo-1 bound to Ca\(^{2+}\) (405 nm) versus free indo-1 (485nm) following anti-CD3 stimulation (Figure 4-4, A and B). A decrease in the mean indo-1 ratio demonstrates that nifedipine consistently inhibited anti-CD3 induced Ca\(^{2+}\) influx in both Jurkat T cells and PBTs whereas the DMSO solvent control had no effect. It should be noted that an anti-CD3 mAb was used to stimulate Ca\(^{2+}\) influx in Jurkat T cells, whereas experiments conducted with human PBTs required an anti-CD3 mAb as well as an anti-CD28 mAb and a cross-linking Ab to induce Ca\(^{2+}\) influx in these cells.

Although nifedipine clearly inhibited Ca\(^{2+}\) influx in the cells tested, it was important to determine whether this inhibition was a partial or complete blockage of Ca\(^{2+}\) influx. This would help distinguish whether L-type Ca\(^{2+}\) channels are the only channels that mediate Ca\(^{2+}\) influx or if other channels contribute to the Ca\(^{2+}\) response during T
Figure 4-4: Nifedipine blocks Ca\(^{2+}\) influx in the human Jurkat T cell leukemia line and human PBTs.

Jurkat T cells (A) and human PBTs (donors, \(n=3\)) (B) loaded with indo-1 were preincubated with 1-200 μM nifedipine in the presence of extracellular Ca\(^{2+}\). For each sample, after the 10 min treatment with nifedipine baseline Ca\(^{2+}\) measurements were taken, cells were then stimulated at the 2 min mark, and the analysis was immediately resumed. Ca\(^{2+}\) influx was induced with an anti-CD3 mAb in Jurkat T cells and a combination of anti-CD3 mAb, anti-CD28 mAb and a cross-linking Ab in human PBTs. Indo-1 loaded Jurkat T cells (C) and human PBTs (D) were treated with 1-200 μM nifedipine and stimulated in the absence of extracellular Ca\(^{2+}\). Red line, DMSO solvent (Control); Light Blue, 1 μM Nifedipine; Yellow, 10 μM Nifedipine; Green, 50 μM Nifedipine; Grey, 100 μM Nifedipine; Dark Blue, 200 μM Nifedipine. The human PBTs contained 18% CD3\(^{+}\)CD4\(^{+}\)CD8\(^{-}\), 45% CD3\(^{+}\)CD4\(^{+}\)CD8\(^{+}\), 32% CD3\(^{+}\)CD4\(^{+}\)CD8\(^{-}\) and 5% CD3\(^{+}\)CD4\(^{-}\)CD8\(^{-}\) cells. The results depicted are representative of three independent experiments.
lymphocyte activation. To address this question, Ca\(^{2+}\) influx induced in the absence of extracellular Ca\(^{2+}\) was compared to nifedipine-inhibited Ca\(^{2+}\) influx with extracellular Ca\(^{2+}\) present in the medium. In both anti-CD3 stimulated Jurkat T cells and PBTs, a rapid, transient increase in intracellular Ca\(^{2+}\), arising from intracellular Ca\(^{2+}\) stores, was observed when extracellular Ca\(^{2+}\) was absent from the medium (Figure 4-4, C and D, Control, red line). This transient Ca\(^{2+}\) spike was not observed in Jurkat T cells treated with nifedipine in the presence of extracellular Ca\(^{2+}\), indicating that nifedipine only partially blocked Ca\(^{2+}\) influx in these cells. However, in human PBTs, higher concentrations of nifedipine completely abolished Ca\(^{2+}\) influx since the Ca\(^{2+}\) trace with no extracellular Ca\(^{2+}\) was very similar to PBTs treated with 50-200 \(\mu\)M nifedipine when extracellular Ca\(^{2+}\) was present.

Finally, since nifedipine is a lipophilic compound that can accumulate intracellularly, nifedipine blockage of Ca\(^{2+}\) efflux from intracellular Ca\(^{2+}\) stores was investigated (193). To examine this, Jurkat T cells and human PBTs were treated with nifedipine and anti-CD3 stimulated in the absence of extracellular Ca\(^{2+}\) (Figure 4-4, C and D). Nifedipine treatment did not significantly effect the transient rise in cytosolic Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores in Jurkat T cells, whereas in human PBTs 50-200 \(\mu\)M nifedipine reduced the efflux of Ca\(^{2+}\) from intracellular stores in these cells. It should be noted that (+/-) Bay K 8644 and nifedipine are typically used at 1-300 \(\mu\)M on both electrically excitable and non-excitable cell types. The concentration of DHPs used in these experiments therefore replicated the concentration range of Ca\(^{2+}\) channel modulators applied in other studies (165, 192).
4.2.4 (+/-) Bay K 8644 and Nifedipine Modulate Phospho-p44/p42 MAP Kinase Activation in Jurkat T Cells and Human PBTs

The next step in the investigation was to determine whether Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels could activate downstream, Ca\(^{2+}\)-dependent signaling pathways involved in T lymphocyte activation. The expression of the p44/42 MAP kinase, also known as Erk1/2, was examined since a rise in intracellular Ca\(^{2+}\) through Ca\(^{2+}\) ionophores, such as ionomycin and A23187, can induce the activation of Erk1/2 in T lymphocytes (194). The addition of the specific Ca\(^{2+}\) chelator, EGTA, prior to stimulation of Jurkat T cells and human PBTs with ionomycin blocks activation of Erk1/2 demonstrating that one mode of Erk1/2 activation in T lymphocytes is through an increase in intracellular Ca\(^{2+}\) (194). Additionally, DHP derivatives are reported to modulate the MAP kinase pathway in neurons, but this phenomenon has not been examined in T lymphocytes (195).

In Jurkat T cells, 50 μM and 100 μM (+/-) Bay K 8644 stimulation resulted in a rapid and transient phosphorylation of both Erk1 and 2, which was similar to the level of Erk1/2 activated with 2 μM ionomycin (Figure 4-5A). The activation of Erk1/2 with (+/-) Bay K 8644 was blocked by pretreatment with EGTA. In human PBTs, (+/-) Bay K 8644 did not activate Erk1 and only weakly activated Erk2 compared to the ionomycin control (Figure 4-5B). The activation of Erk2 with (+/-) Bay K 8644 was not blocked by pretreatment with EGTA. The treatment of Jurkat T cells and human PBTs with DMSO alone (Control) did not activate Erk1/2. It should be noted that the effects of 100 μM (+/-) Bay K 8644 on naïve human PBTs that were immediately isolated from PBMCs and not
Jurkat T cells (A) and human PBTs (donors, n=3) (B) were preincubated with or without 2 mM EGTA followed by stimulation with either 2 μM ionomycin or (+/-) Bay K 8644 (10-100 μM). Cells incubated with DMSO alone served as control. The human PBTs contained 18% CD3⁺CD4⁺CD8⁻, 69% CD3⁺CD4⁻CD8⁺, 11.5% CD3⁺CD4⁺CD8⁻ and 1.5% CD3⁺CD4⁻CD8⁻ cells. (C) Naive human PBTs and Jurkat T cells were treated with either DMSO or stimulated with 100 μM (+/-) Bay K 8644. Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with a phospho-specific Ab to detect activated Erk1/2 (top panel). The membrane was stripped and reprobed with an Ab directed against Erk1/2 to detect the total amount of kinase loaded in each lane (bottom panel). The results are representative of three independent experiments.

Figure 4-5: (+/-) Bay K 8644 modulates phospho-p44/42 MAP kinase activation in Jurkat T cells and human PBTs.
previously cultured with 10 μg/ml plate-bound OKT3 and rhIL-2 were also examined. Unstimulated naïve human PBTs had the same level of Erk2 activation following (+/-) Bay K 8644 treatment as human PBTs grown for 8 days in culture with OKT3 and rhIL-2 (Figure 4-5C).

Since there is very little information on what aspects of the T lymphocyte activation process are effected by inhibiting Ca\(^{2+}\) influx with nifedipine treatment, the investigation of whether Erk1/2 activation induced by (+/-) Bay K 8644 could be specifically blocked by nifedipine was carried out. Pretreatment of Jurkat T cells with either 100 μM or 200 μM nifedipine, but not the DMSO solvent, significantly inhibited Erk1/2 activation induced by 100 μM (+/-) Bay K 8644 (Figure 4-6). As a positive control for Erk1/2 phosphorylation, Jurkat T cells were stimulated with soluble OKT3. Stimulation of Jurkat T cells through the TCR/CD3 complex induced robust activation of Erk1/2, showing that the Ca\(^{2+}\) influx induced by (+/-) Bay K 8644 supports only partial Erk1/2 activation.

4.2.5 Nifedipine Blocks NFAT-Transcriptional Activity in Jurkat T Cells

The activation of the transcription factor NFAT is dependent upon increased intracellular Ca\(^{2+}\) (196). The investigation of whether the block in Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels by nifedipine alters the transcriptional activity of NFAT was conducted by transiently transfecting an NFAT-luciferase reporter plasmid into Jurkat T cells. Activation of transfected Jurkat T cells induces endogenous NFAT transcription factors to bind to the NFAT cis-acting enhancer element within the construct, and transcribe the reporter gene. First, it was determined that the maximum induction of
Figure 4-6: Nifedipine modulates phospho-p44/42 MAP kinase activation in Jurkat T cells.

Cells were preincubated with either DMSO, 100 µM or 200 µM nifedipine, or NT, preincubated with DMSO, 100 µM or 200 µM nifedipine followed by stimulation with 100 µM (+/-) Bay K 8644. Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with a phospho-specific Ab to detect activated Erk1/2 (top panel). The membrane was stripped and reprobed with an Ab directed against Erk1/2 to detect the total amount of kinase loaded in each lane (bottom panel). The results are representative of three independent experiments.
NFAT when transfected Jurkat T cells were exposed to soluble OKT3 occurred between 5 to 8 h after stimulation (data not shown). Blocking Ca\(^{2+}\) channel activity by pretreatment of Jurkat T cells with nifedipine resulted in inhibition of OKT3-induced NFAT activation in a dose-dependent manner (Figure 4-7). Low concentrations of nifedipine that weakly blocked Ca\(^{2+}\) influx, such as 50 \(\mu\)M nifedipine, significantly reduced the transcriptional activity of NFAT. Higher doses of nifedipine, such as 200 \(\mu\)M almost completely abolished NFAT activity. The DMSO solvent alone did not inhibit NFAT activation in Jurkat T cells. Additionally, OKT3 stimulated Jurkat T cells transiently transfected with a reporter construct that does not contain the NFAT cis-acting enhancer element (Control), did not activate NFAT.

4.2.6 IL-2 Production and IL-2R Expression is Inhibited by Nifedipine in Jurkat T Cells and Human PBTs

Since IL-2 secretion is a definitive indicator of T cell activation, it was assessed whether blocking L-type Ca\(^{2+}\) channels with nifedipine can inhibit IL-2 production in both anti-CD3 stimulated Jurkat T cells (Figure 4-8A) and human PBTs (Figure 4-8B). Nifedipine treatment significantly inhibited IL-2 secretion in both cell types and abolished IL-2 secretion completely with 200 \(\mu\)M nifedipine. This is in agreement with the results showing the inhibitory effects of nifedipine on Ca\(^{2+}\) influx (Figure 4-4) and NFAT activation (Figure 4-7). Treatment with the DMSO solvent did not significantly block IL-2 secretion from either Jurkat T cells or human PBTs.

To ensure the block in IL-2 secretion by nifedipine was not due to cell death induced by drug cytotoxicity, both Jurkat T cells and human PBTs were stained with PI.
Figure 4-7: Inhibition of NFAT transcription by nifedipine in the human Jurkat T cell leukemia line.

Jurkat T cells were either transiently transfected with a NFAT reporter construct (open bars) or with a reporter construct lacking the NFAT cis-acting enhancer element (hatched bars), which monitors for constitutive levels of luciferase activity. After being cultured for 40-48 h, the cells were either untreated (NT), incubated with DMSO (Control) or 1-200 μM nifedipine and were then stimulated with soluble OKT3 (10 μg/ml). Cells were harvested and the NFAT activity was measured by luciferase activity assay. The results are presented as relative NFAT-dependent luciferase activity. Each bar represents the mean and SD of assays from triplicate wells. The results are representative of three independent experiments. P<0.01, relative to DMSO treated Jurkat T cells transfected with the NFAT reporter construct.
Figure 4-8: Nifedipine prevents IL-2 secretion from Jurkat T cells and human PBTs.

Jurkat T cells (A) or human PBTs (donors, n=3) (B) were incubated with 1-200 μM nifedipine and then stimulated with immobilized OKT3 (10 μg/ml) and 10 nM TPA. After cells were stimulated for 24 h, the amount of IL-2 secreted in the supernatant was measured by standard sandwich ELISA. The control is Jurkat T cells or human PBTs treated with the DMSO solvent alone. The human PBTs contained 19% CD3⁺CD4⁺CD8⁻, 72% CD3⁺CD4⁺CD8⁺, 8.0% CD3⁺CD4⁺CD8⁻ and 1.0% CD3⁺CD4⁺CD8⁻ cells. P<0.01, relative to the DMSO control. Viability of Jurkat T cells (C) and human PBTs (D) was assessed by staining with PI. P>0.01, relative to the DMSO control. To demonstrate whether additional Ca²⁺ can overcome the inhibitory effect of nifedipine, Jurkat T cells (E) and human PBTs (donors, n=3) (F) were incubated with 1-50 μM nifedipine. Cells were stimulated with immobilized OKT3 (10 μg/ml), 10 nM TPA and without (open bars) or with 2 μM ionomycin (hatched bars). The human PBTs in this experiment contained 11.5% CD3⁺CD4⁺CD8⁻, 86.5% CD3⁺CD4⁺CD8⁺, 1.0% CD3⁺CD4⁺CD8⁻ and 1.0% CD3⁺CD4⁺CD8⁻ cells. *, P<0.01, as comparing samples with or without ionomycin added. Results depicted are representative of three independent experiments. Each bar represents the mean and SD of assays from triplicate wells.
after culture supernatants were removed for assaying IL-2. The PI negative or viable cell population was then analyzed by flow cytometry. In Jurkat T cells and human PBTs, 1-200 μM nifedipine did not have a statistically significant impact on cell viability compared to the viability of cells stimulated with OKT3/TPA and treated with DMSO (Figure 4-8, C and D).

It was then examined whether the inhibitory effect of nifedipine could be reversed by the addition of Ca\(^{2+}\). Since ionomycin rapidly increases intracellular Ca\(^{2+}\) in T cells, treatment with this ionophore was used to provide additional Ca\(^{2+}\) to the cells. Jurkat T cells (Figure 4-8E) and human PBTs (Figure 4-8F) were treated with nifedipine and ionomycin where indicated and IL-2 secretion was again assayed as an indicator of T cell activation. In both cell types, inhibition of IL-2 secretion by 1 μM and 10 μM nifedipine could be completely overcome by the addition of ionomycin. However, the treatment of T cells with 50 μM nifedipine could only be partially reversed by ionomycin treatment. In all cases, reversing the inhibitory effect of nifedipine with ionomycin was more successful in human PBTs compared to Jurkat T cells.

IL-2R expression was also examined on viable T cells and it was found that only 200 μM nifedipine significantly inhibited receptor expression. In Jurkat T cells (Figure 4-9A), 200 μM nifedipine caused a 55% decrease in the log mean fluorescence intensity of IL-2R expression, whereas in human PBTs pretreated with 200 μM nifedipine (Figure 4-9B) a 70% decrease was observed compared to IL-2R expression of T cells stimulated with OKT3/TPA and treated with DMSO.
Figure 4-9: Decreased IL-2R expression in Jurkat T cells and human PBTs after treatment with nifedipine.

Jurkat T cells (A) or human PBTs (donors, n=3) (B) were preincubated with nifedipine (1-200 μM) for 1 h, and then stimulated with immobilized OKT3 (10 μg/ml) and 10 nM TPA for 24 h with nifedipine remaining in the culture medium. Cells were stained with PI to remove dead cells from analysis and human IL-2Rα mAb followed by FITC conjugated goat anti-mouse Ab. Cells were treated with either DMSO (dotted line), with DMSO and OKT3/TPA (solid line), or with nifedipine and OKT3/TPA (bold solid line). The human PBTs contained 19% CD3⁺CD4⁺CD8⁻, 72% CD3⁺CD4⁺CD8⁺, 8.0% CD3⁺CD4⁺CD8⁻ and 1.0% CD3⁺CD4⁺CD8⁺ cells. The results shown here are representative of three independent experiments.
4.2.7 Nifedipine Suppresses Splenocyte Proliferation

To investigate whether nifedipine could block the proliferation of T lymphocytes, the effects of nifedipine on the proliferative response induced by a mixed lymphocyte reaction (MLR) was assayed. Nifedipine significantly suppressed the proliferation of splenocytes in a dose-dependent fashion (Figure 4-10). Low doses of nifedipine, including 1 µM and 10 µM, weakly inhibited splenocyte proliferation, whereas 100 µM and 200 µM nifedipine completely abrogated proliferation. There was no significant inhibition of splenocyte proliferation by treatment with DMSO solvent alone.

4.3 Discussion

Initially, in an attempt to clearly define the functional contribution of the α_{IF}-subunit variants to Ca^{2+} entry, the gene-silencing technique, RNAi, was employed to downregulate the expression of both splice isoforms in Jurkat T cells. Although there were concerns that the expression of the 200 kDa α_{IF}-subunit membrane-localized protein could not be knocked-down by RNAi, a previous study reported that stable expression of antisense RNA knocked-down protein expression of the type 3 RyR Ca^{2+} channel in Jurkat T cells (73). Nevertheless, the siRNA target sequences chosen in this study were not successful at knocking-down α_{IF}-subunit protein expression in Jurkat T cells. Although RNAi is a potent technique used to suppress gene expression in mammalian cells, it requires the analysis of many different siRNA duplexes in order to successfully knock-down expression of the gene of interest. Future experiments
Figure 4-10: Nifedipine suppresses splenocyte proliferation.

Splenocytes from C57Bl/6 mice were incubated with 1-200 μM nifedipine in triplicate and then stimulated with irradiated Balb/c splenocytes. Five to six days later, each sample was assayed for proliferation as reflected as lymphocyte number determined by flow cytometry. The control is splenocytes treated with DMSO solvent alone. Each bar represents the mean and SD of assays from triplicate wells. P<0.01, relative to the Control.
examining the effects of siRNA should encompass a larger number of siRNA constructs targeted to different regions of the $\alpha_{1F}$-subunit.

As an alternative strategy, the DHP derivatives, (+/-) Bay K 8644 and nifedipine, were used to further investigate the presence of L-type Ca$^{2+}$ channels in T lymphocytes and to assess the contribution of Ca$^{2+}$ influx by L-type Ca$^{2+}$ channels during the T lymphocyte activation process. Previous studies investigating the role of L-type Ca$^{2+}$ channels have used synthetic DHP derivatives to study the function of these channels in the plasma membranes of numerous cell types (157, 165, 192). Through an intracellular Ca$^{2+}$ assay, it was demonstrated here that (+/-) Bay K 8644 exerts an agonistic action on the Ca$^{2+}$ channels of Jurkat T cells and human PBTs. However, treatment with (+/-) Bay K 8644 did not induce maximal Ca$^{2+}$ influx in either cell type since anti-CD3 stimulation of PBTs and Jurkat T cells resulted in a 2- to 4-fold larger increase in intracellular Ca$^{2+}$, respectively. An explanation for this discrepancy may be that the (-)- and (+)-enantiomers of Bay K 8644 may competitively bind to the $\alpha_{1}$-subunits expressed in T cells, dampening the overall agonistic action of the DHP derivative. It was also observed that (+/-) Bay K 8644 treatment resulted in a sustained Ca$^{2+}$ influx in Jurkat T cells and only a transient Ca$^{2+}$ flux was induced in PBTs. Interestingly, previous studies have demonstrated through electrophysiological means that in Jurkat T cells the number of plasma membrane CRAC channels is between 100 to 400 (129), whereas resting PBTs only express 15 CRAC channels (130). If L-type Ca$^{2+}$ channels contribute to $I_{CRAC}$, the discrepancy in (+/-) Bay K 8644 induced Ca$^{2+}$ influx could be explained by the difference in the number of channels expressed by both cell types.
The experiments examining the effects of nifedipine on Ca\textsuperscript{2+} influx showed that nifedipine only partially blocks Ca\textsuperscript{2+} influx in Jurkat T cells and PBTs, and does not significantly affect Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores in Jurkat T cells following stimulation through the TCR/CD3 complex. However, higher concentrations of nifedipine do seem to affect Ca\textsuperscript{2+} release from the ER in human PBTs. There is evidence that high concentrations of DHP antagonists and other CCBs interfere with SR release and uptake of Ca\textsuperscript{2+} in both cardiac and smooth muscle cells (197). Therefore it was concluded that nifedipine is blocking L-type Ca\textsuperscript{2+} channels found in the plasma membrane, with minor inhibitory effects at higher concentrations on Ca\textsuperscript{2+} release from intracellular stores.

Although the specificity of action of DHPs has been confirmed by determining the DHP binding sites on the channel-forming \( \alpha_t \)-subunit of L-type VDCCs (184, 185), there are studies demonstrating the non-specific inhibitory effects of high micromolar concentrations of DHPs on voltage-dependent potassium (K\textsubscript{v}) channels and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (198-200). Even though these observations could be of concern in the present study, Fagni et al. reported that micromolar concentrations of both nifedipine and the stereospecific enantiomers of Bay K 8644 exhibited an inhibitory effect on K\textsuperscript{+} current through K\textsubscript{v} and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (199). This is in contradiction to the results in the present study, since it was shown that (+/-) Bay K 8644 has an agonistic effect while nifedipine antagonizes Ca\textsuperscript{2+} influx in T lymphocytes. In addition, (+/-) Bay K 8644 does not activate K\textsuperscript{+} channel currents (199). Therefore, the overall observed effect of the DHPs in this study was due to modulation of Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels. Minor inhibitory effects of DHPs on K\textsuperscript{+} current may occur at the higher...
micromolar concentrations, but are reportedly absent at the lower range of nifedipine used in this study where inhibition of Ca\textsuperscript{2+} influx is still observed (198).

After confirming that (+/-) Bay K 8644 and nifedipine can modulate Ca\textsuperscript{2+} influx in T lymphocytes, the investigation of whether Ca\textsuperscript{2+} influx through an L-type Ca\textsuperscript{2+} channel can modulate early Ca\textsuperscript{2+}-dependent signaling events, such as MAP kinase activity was conducted. It has been postulated that Ca\textsuperscript{2+} can interact with the MAP kinase signaling pathway in T lymphocytes by activating Lck and calmodulin-kinase, which are upstream of Erk1/2 and responsible for Ca\textsuperscript{2+}-dependent Erk1/2 enzymatic activation (201). It was reported here that (+/-) Bay K 8644 could induce phosphorylation and activation of both Erk1 and Erk2 in Jurkat T cells and weak activation of Erk2 in human PBTs. Furthermore, the activation of Erk1/2 by (+/-) Bay K 8644 could be blocked by pretreatment with nifedipine. These results support the hypothesis that Ca\textsuperscript{2+} influx through an L-type Ca\textsuperscript{2+} channel mediates the MAP kinase signaling pathway during T lymphocyte activation. The quantitative discrepancy between Erk activation in Jurkat T cells and primary T lymphocytes might occur if the human PBTs required a higher sustained level of intracellular Ca\textsuperscript{2+} to induce Erk activity compared to transformed T lymphocytes. The Ca\textsuperscript{2+} ionophore, ionomycin, was observed to induce a 2.5-fold greater increase in intracellular Ca\textsuperscript{2+} in human PBTs compared to Jurkat T cells, which corresponds with a more robust phosphorylation of Erk1/2 by ionomycin in human PBTs. In addition, Ca\textsuperscript{2+} entry induced by (+/-) Bay K 8644 in human PBTs was only transient compared to the sustained Ca\textsuperscript{2+} influx in Jurkat T cells. Therefore the difference in the amount of Ca\textsuperscript{2+} influx could result in (+/-) Bay K 8644 strongly activating Erk1/2 in Jurkat T cells, and only weakly inducing activation of Erk activity in human PBTs.
To examine whether an L-type Ca\(^{2+}\) channel can mediate more downstream Ca\(^{2+}\)-dependent signaling events during T lymphocyte activation, the effects of nifedipine on the transcriptional activity of NFAT, IL-2 secretion and IL-2R expression were studied. It was demonstrated that inhibiting Ca\(^{2+}\) influx with nifedipine could inhibit the activity of NFAT in a dose-dependent manner in Jurkat T cells. Since NFAT regulates the transcription of several cytokine genes, including IL-2, the effects of nifedipine on IL-2 production and IL-2R expression were then examined (196). In both Jurkat T cells and human PBTs, IL-2 production was blocked in the presence of nifedipine. The overall inhibition in IL-2 secretion mediated by nifedipine was confirmed to be due to a block in Ca\(^{2+}\) influx and not cell death since the percentage of viable cells did not significantly change with increasing nifedipine dose. These results are consistent with a previous report showing that inhibition of human T lymphocyte proliferation by 100 μM nifedipine was not due to drug cytotoxicity (186). It was also demonstrated that the block in IL-2 secretion by nifedipine can be reversed by the addition of ionomycin, confirming that low doses of nifedipine are inhibiting L-type Ca\(^{2+}\) channels and not suppressing the function of other channels in T lymphocytes. IL-2R expression was only downregulated with high concentrations of nifedipine. This is consistent with the current understanding that the signaling requirements for expression of the IL-2R are less stringent than those for IL-2 production (202). Thus Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels regulates NFAT activity and IL-2 production but has a lesser effect on IL-2R expression.

In addition to demonstrating that an L-type Ca\(^{2+}\) channel mediates Ca\(^{2+}\) influx during T lymphocyte activation, the investigation of whether an L-type Ca\(^{2+}\) channel would regulate the proliferation of T lymphocytes was addressed. To this end, the
proliferation of mouse splenocytes was assayed through an *in vitro* MLR and found that nifedipine markedly inhibited mouse splenocyte proliferation in a dose-dependent manner. These observations support the hypothesis that Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels is required for sustained Ca\(^{2+}\) influx during T lymphocyte proliferation *in vitro*.

Taken together, the results in this study show that (+/-) Bay K 8644 and nifedipine partially modulate T lymphocyte activation and proliferation. Although evidence has been provided for the presence of a DHP sensitive L-type Ca\(^{2+}\) channel in the plasma membrane of T lymphocytes, additional Ca\(^{2+}\) channels may also contribute to the Ca\(^{2+}\) influx pathway. This conclusion is in agreement with recent studies, describing the involvement of TRP ion channels in regulating Ca\(^{2+}\) influx in T lymphocytes. Cui *et al.* showed that the TRPV subfamily member of ion channels, CaT1, is involved in generating \(I_{\text{CRAC}}\) in Jurkat T cells, which is partially regulated through intracellular Ca\(^{2+}\) store-depletion (105). Although CaT1 plays a significant role in mediating Ca\(^{2+}\) entry, overexpression of a dominant negative pore-region mutant of CaT1 did not completely abolish Ca\(^{2+}\) influx in Jurkat T cells, leading to the possibility that other channels, such as L-type Ca\(^{2+}\) channels are also involved in Ca\(^{2+}\) entry (105). TRP channels that are not modulated by intracellular Ca\(^{2+}\) store-depletion have also been found in human T lymphocytes. For instance, Sano *et al.* reported that the LTRPC2 protein is abundantly expressed in human peripheral blood and Jurkat T cells and mediates Ca\(^{2+}\) influx in response to elevated levels of pyrimidine nucleotides, ADPR, and NAD\(^{+}\) (108). TRPC6 mRNA and protein is also expressed in Jurkat T cells and PBTs and Ca\(^{2+}\) influx through this channel is activated by DAG (110). In conjunction with the results from this study and the recent discovery of TRP protein expression in T lymphocytes, it is highly
probable Ca\textsuperscript{2+} entrance into T lymphocytes is mediated through multiple Ca\textsuperscript{2+} channels, including both TRP and L-type Ca\textsuperscript{2+} channels. Given that the amplitude and duration of Ca\textsuperscript{2+} signals in T lymphocytes are very diverse, a number of different channels may be necessary to coordinate the different Ca\textsuperscript{2+} responses required for T lymphocyte activation, proliferation and death.
CHAPTER 5: DETERMINING THE ROLE OF L-TYPE CALCIUM CHANNELS IN T LYMPHOCYTES IN VIVO

5.1 Introduction

In 2003, a study conducted by the World Health Organization reported that the cardiovascular disease, hypertension, is estimated to cause 4.5% of the global disease burden (203). In the United States, approximately 50 million American adults are affected by hypertension (204). Recent surveys on antihypertensive medications documented that the short-acting first generation DHP, nifedipine (clinically known as Procardia™), and the long-acting third generation DHP, amlodipine (Norvasc™), are two of the most commonly prescribed drugs for the treatment of hypertension (205, 206). In patients with cardiovascular disease, nifedipine and amlodipine block the action of L-type VDCCs that are responsible for initiating the contraction of cardiac and vascular smooth muscles (157). Nifedipine and amlodipine are also frequently administered for other clinical ailments including angina, congestive heart failure, ischemia, migraine headaches and Raynaud’s syndrome (205). Although DHP derivatives adequately control the symptoms of several diseases, there are many well-documented side effects associated with these medications, such as dizziness, lightheadedness, heat sensation, nausea, heartburn, palpation, nasal congestion and sore throat (205). Another troublesome side effect of both second and third generation DHP derivatives is peripheral edema (178).
In addition to these adverse reactions brought on by DHP administration, there are concerns that DHP derivatives might also affect the immune competence of patients receiving DHPs as an anti-hypertensive therapy. These concerns were initiated by the early demonstration that several clinically administered DHPs have marked inhibitory effects on T cell function in vitro (186, 187). Subsequently, the DHP Ca$^{2+}$ channel antagonist, nifedipine, was also found to augment both early and late Ca$^{2+}$-dependent signaling events during T lymphocyte activation and proliferation in vitro (140). In conjunction with these studies, several investigators have shown that channel-forming $\alpha_1$-subunits of L-type Ca$^{2+}$ channels, which contain DHP binding sites, are expressed in both human and mouse T lymphocytes (119, 120, 140). The findings in the previous studies collectively raise the question of whether long-term treatment with DHPs may act as an immunosuppressant, even though the serum concentrations of DHPs in patients (see Table 5-1) are 10 to 100-fold lower than the minimal inhibitory dose of nifedipine used in vitro.

The concern that DHP administration may compromise the immune system of patients is heightened by the fact that a few studies have reported that CCBs have immunosuppressive effects in humans. It has been shown that administration of one 10 mg oral dose of nifedipine to healthy humans transiently downregulated immune responses in these individuals (207). In this study, Morgano et al. demonstrated that four hours after oral administration of nifedipine, PHA-induced peripheral blood lymphocyte proliferation was significantly reduced relative to the proliferation of lymphocytes isolated from the same individual prior to drug administration (207). The defect in proliferation was attributed to impaired production of IL-2 by lymphocytes shortly after
Nifedipine and amlodipine are two structurally similar DHP derivatives that are commonly prescribed for the treatment of hypertension. The clinical name for nifedipine is Procardia™, whereas amlodipine is clinically known as Norvasc™. The serum concentration and mean plasma half-life of both nifedipine and amlodipine were measured in several volunteers by high performance liquid chromatography and the average values were determined. Higher doses of nifedipine are typically administered to hypertensive patients compared to the amlodipine dose due to the short plasma half-life of nifedipine.

Table 5-1: Summary of pharmacokinetic parameters of nifedipine and amlodipine in humans.

<table>
<thead>
<tr>
<th>1,4-Dihydropyridine</th>
<th>Chemical Structure</th>
<th>Serum Concentration</th>
<th>Mean Plasma Half-Life (Single dose-studies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nifedipine (Procardia™)</td>
<td><img src="image" alt="Nifedipine Structure" /></td>
<td>~80-170 nM (20 mg oral dose over 7 days)</td>
<td>~6 h (40 mg oral dose)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(208)</td>
<td>(209)</td>
</tr>
<tr>
<td>Amlodipine (Norvasc™)</td>
<td><img src="image" alt="Amlodipine Structure" /></td>
<td>~14 nM (5 mg oral dose over 7 days)</td>
<td>35 h (15 mg oral dose)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(210)</td>
<td>(211)</td>
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nifedipine treatment (207). Administration of the DHP derivative, nilvadipine, for a six month period has also shown to adversely affect several immunological parameters of hypertensive patients (212). The patients treated with nilvadipine not only showed a reduced frequency in various PBT subsets including CD4+CD8−, CD4+CD45RA− and CD4+CD45RA+ T cells, but also had decreased concentrations of soluble IL-2R in the peripheral blood (212). In addition to the studies detailing the inhibitory effects of DHPs in patients, a single study has shown that the first generation phenylalkylamine, verapamil, also diminished the immune response of a patient receiving this drug (213). Administration of verapamil caused the patient to experience repeated and prolonged viral infections that was associated with suppressed lymphocyte proliferation in response to mitogen stimulation (213). Although the immunosuppressive properties of CCBs are not well defined, these previous studies suggest that CCBs may bind and modulate the function of L-type Ca^{2+} channels in circulating lymphocytes, which may directly affect the function of T cells, and therefore the immune competence of these patients.

The investigation presented here began by exploring the effects of nifedipine treatment on the CD8+ T cell response to a defined histocompatibility antigen in vivo. DHP inhibition of T cell function in vivo would provide additional support that functional L-type Ca^{2+} channels are expressed in T lymphocytes, and that DHPs may have immunosuppressive effects in patients. The in vivo proliferation assay quantified the response of female T cells proliferating to the male-specific minor histocompatibility (H) antigen encoded on the Y chromosome (H-Y antigen) in male recipient mice treated with nifedipine. Only the female CD8+ T cell response was measured in this assay since the female mice were bearing a Tg TCRζβ receptor that is MHC Class I restricted and
specific for the male H-Y antigen. The CD8+ T cell proliferative response was quantified by loading the female H-Y specific TCR-Tg T cells with the fluorescent dye, CFSE, prior to female cell injection and nifedipine treatment of the male mice. CFSE labeling marked each successive cell division among proliferating female T cells since the fluorescence intensity of the dye is halved and distributed equally in the daughter cells. As a result of this analysis, the first direct evidence that nifedipine can inhibit T cell proliferation \textit{in vivo} was demonstrated.

Since millions of people are administered DHPs for the treatment of cardiovascular diseases and other clinical disorders, it was important to further investigate the potential immunosuppressive risks associated with long-term DHP administration. The investigation was continued by examining T cell function in renal disease patients that had received DHP treatment. Renal disease patients from St. Paul’s Hospital in Vancouver, B.C. were chosen for this analysis since there is a high prevalence of hypertension in these patients, and a large cohort receiving either nifedipine or amlodipine treatment. Additionally, at St. Paul’s Hospital, the demographic and medication data, as well as the infection rate is well recorded for renal disease patients with chronic kidney disease, on hemodialysis, on peritoneal dialysis and post-renal transplant. Taken together, the results from this study provide further support for the hypothesis that long-term treatment with DHPs may affect the immune competence of hypertensive patients.
5.2 Results

5.2.1 Nifedipine Inhibits T Cell Proliferation in Mice

Since nifedipine clearly inhibited MLR induced splenocyte proliferation \textit{in vitro} (140), it was evaluated whether nifedipine could block the proliferation of an antigen specific T cell response \textit{in vivo}. To address this question the proliferative response of female H-Y-specific TCR-Tg CD8$^+$ CFSE$^+$ thymocytes transferred into male C57Bl/6 mice was examined. In male recipients, H-Y-specific CD8$^+$ CFSE$^+$ T cells become activated upon TCR engagement, eliciting a small persistent Ca$^{2+}$ influx and clonal expansion that proceeds for several days (214). This clonal expansion can be easily monitored in cells derived from recipient spleens (214).

This study is the first reported investigation examining the effects of nifedipine on T cell proliferation \textit{in vivo}, therefore preliminary experiments were conducted to establish an effective treatment concentration of nifedipine in mice. Male mice injected with CFSE-labeled female H-Y-specific TCR-Tg T cells were treated with either the vehicle control (PBS containing 5% ethanol and 1% Tween-80) or nifedipine in concentrations ranging from 10, 15, 20, to 50 mg/kg. These nifedipine concentrations were chosen since previous investigations examining the \textit{in vivo} pharmacokinetics of nifedipine and other DHP derivatives in mice used similar concentration ranges (125, 215, 216). In this study, the preliminary experiments showed that 10 mg/kg nifedipine had no inhibitory effects on T cell proliferation, whereas 15 mg/kg nifedipine retarded proliferation of H-Y-specific CD8$^+$ CFSE$^+$ T cells (data not shown). Male mice treated either 20 or 50 mg/kg nifedipine demonstrated significant morbidity; in fact 50 mg/kg caused lethality in all
mice shortly after the i.p. nifedipine injection. As a result of this preliminary analysis, a nifedipine concentration of 15 mg/kg was used in the remaining in vivo proliferation assays.

The in vivo anti-proliferative effects of nifedipine were confirmed by treating male mice with either four doses of 15 mg/kg nifedipine or vehicle control after the i.v. injection of 20-30x10^6 female H-Y-specific TCR-Tg CFSE^+ T cells. Four doses of nifedipine were administered to the male mice at 6 h intervals over a period of 40 h since it was previously determined that the half-life of nifedipine in mouse peripheral blood is very short. Larkin et al. demonstrated that the half-life of a single i.p. dose of 6 mg/kg nifedipine is 11 min and 60 mg/kg nifedipine is 30 min in mouse blood (215). As illustrated in Figure 5-1A, treating male mice with four doses of 15 mg/kg nifedipine inhibited, but did not completely abrogate H-Y-specific CD8^+ T cell proliferation. Nifedipine treatment resulted in an increased number of H-Y-specific CD8^+ T cells not dividing or undergoing only 1 cell division, and significantly fewer cells transiting to 2 divisions compared to the vehicle control. The proliferative response of H-Y-specific CD8^+ T cells in female C57BL/6 mice was also assayed as a control for no proliferation. As expected, the H-Y-specific CD8^+ T cells present in the spleen did not proliferate in the female mice due to the absence of the male H-Y antigen (Figure 5-1A, Control, open bar).

The total number of H-Y-specific TCR-Tg CD8^+ CFSE^+ T cells present in the spleen of female control, vehicle, and 15 mg/kg nifedipine treated male mice was also determined. It was found that significantly fewer proliferating T cells reached the spleen in nifedipine treated mice compared to the vehicle control (Figure 5-1B). Additionally,
Figure 5-1: T cell proliferative response to H-Y male antigen is decreased in mice following repeated nifedipine treatment.

CFSE loaded thymocytes from C57Bl/6 female mice with Tg TCR αβ H-Y receptor were i.v. injected into female (Control, n=1) or male recipients. Male mice received one i.p. dose of vehicle (n=5) or 15 mg/kg nifedipine (n=5) 1 h after the cell injection followed by three i.p. injections of either vehicle or nifedipine at 6 h intervals. Splenocytes were harvested 40 h after the initial i.v. injection and proliferation of CFSE⁺, CD8⁺ and Tg TCR₀high splenocytes was quantified by flow cytometry. (A) Bars represent % of total cells exhibiting a discrete CFSE (FL1) intensity reflecting an equal number of cell divisions: no divisions (open bar), 1 division (solid bar), and 2 divisions (hatched bar). *, P<0.01, as comparing cell divisions between vehicle control and nifedipine treated male mice. (B) Total number of viable, H-Y-specific TCR-Tg CD8⁺ CFSE⁺ T cells present in the spleen of female control, vehicle, and nifedipine treated male recipients. *, P<0.01, when comparing total T cell number in vehicle control to nifedipine treated male mice. Each bar represents the mean and SD of assays from five mice. The results depicted are representative of two independent experiments.
the number of proliferating H-Y-specific CD8\(^+\) T cells in the spleen of nifedipine treated mice was similar to the number of non-proliferating T cells found in the spleen of the female mouse. It should be noted that in each of the following experiments there was a variable number of T cells recovered in the female controls and male treated mice. These inconsistencies were probably due to slight differences in the number of female H-Y-specific TCR-Tg CFSE\(^+\) T cells initially i.v. injected into recipient animals.

5.2.2 Increasing the Number of Nifedipine Doses Augments Anti-Proliferative Effect

Initial experiments examining the anti-proliferative effects of nifedipine established that four doses of 15 mg/kg nifedipine considerably slowed down T cell proliferation \textit{in vivo}. The investigation on the anti-proliferative effects of nifedipine was continued by determining the minimal number of 15 mg/kg doses of nifedipine that would significantly inhibit T cell proliferation in mice. Beginning 1 h after the i.v. injection of the H-Y-specific TCR-Tg T cells, male mice were injected i.p. with 1, 2, or 3 doses of 15 mg/kg nifedipine at 6 h intervals. Mice treated with either 1 or 2 doses of 15 mg/kg nifedipine showed no profound changes in the percentage of proliferating H-Y-specific CD8\(^+\) T cells in each cell division compared to the vehicle control (Figure 5-2A). However, treatment of the mice with 3 doses of 15 mg/kg nifedipine resulted in an increase in the number of proliferating T cells that had divided once, and a decrease in the number of cells that had undergone 2 divisions compared to the vehicle control (Figure 5-2A). When comparing the degree of inhibition by either 3 or 4 doses of 15 mg/kg nifedipine, it was found that 4 doses of 15 mg/kg nifedipine inhibited T cell proliferation to a greater extent than 3 doses. In summary, these experiments
CFSE loaded thymocytes from female mice with Tg TCRαβ H-Y receptor were i.v. injected into female (Control, \( n=1 \)) or male recipients. Male mice received 3 i.p. doses of vehicle (\( n=2 \)) or 1, 2 or 3 doses of 15 mg/kg nifedipine (\( n=2 \)). The first dose of either vehicle or nifedipine was administered 1 h after the cell injection followed by subsequent doses at 6 h intervals. Splenocytes were harvested 40 h after the initial i.v. injection and proliferation of CFSE\(^+\), CD8\(^+\) and Tg TCR\(^{high}\) splenocytes was quantified. (A) Bars represent % of total cells exhibiting no divisions (open bar), 1 division (solid bar), 2 divisions (hatched bar), and 3 divisions (dotted bar). (B) Total number of viable, H-Y-specific TCR-Tg CD8\(^+\) CFSE\(^+\) T cells isolated from spleen of female control, vehicle, and nifedipine treated male recipients. Each bar represents the mean and SD of assays from two mice.
demonstrated that a minimal number of 3 to 4 doses of 15 mg/kg nifedipine were necessary to reproducibly inhibit T cell proliferation \textit{in vivo}.

The total number of H-Y-specific TCR-Tg CD8$^+$ CFSE$^+$ T cells that reached the spleen of both female and male treated mice was also evaluated. In comparison to the vehicle control, only mice treated with 3 doses of 15 mg/kg nifedipine showed a decrease in the number of proliferating T cells that could be harvested from the spleen (Figure 5-2B). In general, a decrease in T cell proliferation consistently correlated with fewer H-Y-specific CD8$^+$ T cells present in the spleen of nifedipine treated male mice.

\textbf{5.2.3 Anti-Proliferative Effect of Nifedipine is Dependent on the Timing of Administration}

Although the previous experiments demonstrated that 3 to 4 doses of 15 mg/kg nifedipine were necessary to inhibit T cell proliferation \textit{in vivo}, it was important to determine whether the timing of the dose was more crucial to the anti-proliferative effect of nifedipine than the number of doses administered. To address this question, male mice were i.v. injected with H-Y-specific TCR-Tg T cells and then treated with one dose of 15 mg/kg nifedipine 21 h after the cell injection. It should be noted that in all of the previous experiments the first dose of 15 mg/kg nifedipine was administered only 1 h after the H-Y-specific TCR-Tg T cells were injected. In this experiment, nifedipine was injected at 21 h since the H-Y-specific TCR-Tg T cells require approximately 24 h to home to peripheral lymphoid organs and proliferate in response to the male H-Y antigen (214).

Even though the half-life of nifedipine is short-lived in mouse blood, treating male mice with one dose of 15 mg/kg nifedipine at a later time point inhibited H-Y-
specific CD8$^+$ T cell proliferation to a similar extent as four doses of 15 mg/kg nifedipine (Figure 5-3A). A single dose of nifedipine resulted in an increased number of H-Y-specific CD8$^+$ T cells undergoing only 1-2 cell divisions and significantly fewer T cells transiting to 3 divisions compared to the vehicle control. Treatment with only one dose of 15 mg/kg nifedipine also resulted in a decrease in the absolute number of female H-Y-specific TCR-Tg CD8$^+$ CFSE$^+$ T cells that were isolated from the spleen (Figure 5-3B). In the previous experiment, male mice treated with one dose of 15 mg/kg nifedipine only 1 h after the i.v. cell injection showed no decrease in T cell proliferation or the total number of H-Y-specific CD8$^+$ T cells in the spleen (Figure 5-2). Therefore timing of nifedipine administration is critical for the anti-proliferative behavior of the drug.

5.2.4 Nifedipine Specifically Blocks T Cell Proliferation

The previously observed decrease in T cell proliferation brought on by nifedipine treatment may have been caused by nifedipine inducing a non-specific change in the mice that lead to retarded T cell proliferation. To address this concern, the specificity of nifedipine was then investigated by determining whether 3 doses of 15 mg/kg nifedipine with the final dose administered 1 h (elimination of nifedipine half-life in mice) prior to i.v. injection of the H-Y-specific thymocytes would lead to a difference in the T cell proliferation profile compared to vehicle control (215). It was important to wait for the drug concentration to diminish so that there was no effective drug remaining in the bloodstream once the female T cells were i.v. injected. When examining the proliferative response of the H-Y-specific CD8$^+$ T cells, it was found that the T cells from the nifedipine treated mice proliferated to the same extent as the vehicle control, suggesting
Figure 5-3: One later dose of nifedipine suppresses T cell proliferation in response to the H-Y male antigen in mice.

CFSE loaded thymocytes from female mice with Tg TCRαβ H-Y receptor were i.v. injected into female (Control, n=1) or male recipients. After 20-24 h, male mice received one i.p. dose of vehicle (n=4) or 15 mg/kg nifedipine (n=4). Splenocytes were harvested 40 h after the initial i.v. injection and proliferation of CFSE\(^*\), CD8\(^+\) and Tg TCR\(^{\text{high}}\) splenocytes was quantified. (A) Bars represent % of total cells exhibiting no divisions (open bar), 1 division (solid bar), 2 divisions (hatched bar), and 3 divisions (dotted bar). *, P<0.01, as comparing cell divisions between vehicle control and nifedipine treated male mice. (B) Total number of viable, H-Y-specific TCR-Tg CD8\(^+\) CFSE\(^*\) T cells present in the spleen of female control, vehicle, and nifedipine treated male recipients. Each bar represents the mean and SD of assays from four mice. Results are representative of three separate experiments.
that nifedipine is specifically blocking L-type Ca\(^{2+}\) channels, and not causing a non-specific effect (Figure 5-4). The absolute number of H-Y-specific TCR-Tg CD8\(^+\) CFSE\(^+\) T cells present in the spleen was not determined in this experiment since there was no change in proliferation in the vehicle versus nifedipine treated mice.

5.2.5 Nifedipine Reduces T Cell Recovery Through an Antigen-Dependent Mechanism

The final step in this investigation was to assess whether nifedipine treatment was directly inhibiting antigen-dependent T cell recovery in spleens of the male mice. In all previous experiments, a significant reduction in T cell proliferation by nifedipine administration was associated with a decrease in the total number of H-Y-specific CD8\(^+\) T cells that reached the spleen. It was important to determine whether the decrease in total T cell numbers in the spleen was simply due to reduced T cell proliferation or to specific inhibition of antigen-dependent T cell recovery by nifedipine. This question was addressed by examining the effects of nifedipine treatment on antigen-independent recovery of the H-Y-specific CD8\(^+\) T cells in the spleens of female C57Bl/6 mice. In this experiment, female mice were treated with either 1 dose of vehicle or 15 mg/kg nifedipine approximately 20 h after the i.v. injection of the H-Y-specific TCR-Tg CFSE\(^+\) thymocytes. In the female recipients, there was no significant difference observed between the percentage of non-dividing T cells (Figure 5-5A) or the total number of H-Y-specific CD8\(^+\) T cells that reached the spleens (Figure 5-5B) when comparing vehicle to nifedipine treated mice. The results from this experiment demonstrate that H-Y-specific CD8\(^+\) T cell recovery from the spleen in an antigen-independent manner was not effected.
Figure 5-4: Anti-proliferative effect of nifedipine is not due to non-specific drug toxicity.

Male mice received 3 i.p. doses of vehicle or 15 mg/kg nifedipine at 6 h intervals. 1 h after the last dose of either vehicle or nifedipine, CFSE loaded thymocytes from female mice with Tg TCRαβ H-Y receptor were i.v. injected into female (Control, n=1) or male (n=4) recipients. Splenocytes were harvested 40 h after the initial i.v. injection and proliferation of CFSE⁺, CD8⁺ and Tg TCRhigh⁺ splenocytes was quantified. Bars represent % of total cells exhibiting no divisions (open bar), 1 division (solid bar), and 2 divisions (hatched bar). P>0.01, as comparing cell divisions between vehicle control and nifedipine treated male mice. Each bar represents the mean and SD of assays from four mice.
Figure 5-5: Nifedipine treatment does not suppress antigen-independent T cell recovery in female mice.

CFSE loaded thymocytes from female mice with Tg TCRαβ H-Y receptor were i.v. injected into female recipients. After 20-24 h, female mice received one i.p. dose of vehicle (n=4) or 15 mg/kg nifedipine (n=4). Splenocytes were harvested 40 h after the initial i.v. injection and proliferation of CFSE⁺, CD8⁺ and Tg TCR⁹⁺ spleocytes was quantified. (A) Bars represent % of total cells exhibiting no divisions (open bar). The % of total cells in 1, 2, and 3 divisions are not shown since the values are negligible. P>0.01, as comparing cell divisions between vehicle control and nifedipine treated mice. (B) Total number of viable, H-Y-specific TCR-Tg CD8⁺ CFSE⁺ T cells isolated from the spleen of vehicle and nifedipine treated female recipients. P>0.01, when comparing total T cell number in vehicle control to nifedipine treated female mice. Each bar represents the mean and SD of assays from four mice.
by nifedipine treatment. Instead, the data presented in Figure 5-5 confirms that nifedipine is inhibiting T cell recovery in the male mice through an antigen-dependent mechanism.

5.2.6 Examination of Human PBT Function from Renal Disease Patients Following DHP Administration

Thus far it has been established that CCBs, such as nifedipine partially block L-type Ca\(^{2+}\) channel function during T cell activation and proliferation *in vitro* and *in vivo*. The documented inhibitory effects of nifedipine raise the question of whether long-term administration of CCBs to patients for the treatment of cardiovascular diseases has deleterious side effects on circulating T lymphocytes. To further explore this question, PBT function from renal disease patients administered either first generation (i.e. nifedipine) or third generation (i.e. amlodipine) DHP derivatives for the treatment of hypertension was evaluated. Table 5-2 summarizes the DHP dosage, creatinine levels and T cell characteristics of the healthy female volunteer and three renal disease patients on hemodialysis and DHP therapy used in this study. Although a small cohort of renal disease patients were analyzed, previous studies examining the effects of DHPs on immune responses have used a similar number human subjects (207, 213). In the renal disease patients examined here, abnormal kidney function causes the levels of creatinine (a breakdown product of muscle creatine) to increase in the blood due to decreased excretion of creatinine in the urine (217). It should be noted that the sex, age and duration of DHP therapy of the renal disease patients was not available.

The quantity of IL-2 secreted by anti-CD3 stimulated PBTs isolated from the healthy volunteer and the renal disease patients was compared in order to determine
<table>
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<th>Patient B</th>
<th>Patient C</th>
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<tr>
<td>Medication:</td>
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<td>60 mg nifedipine, twice daily</td>
<td>10 mg amlodipine, twice daily</td>
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<td>Creatinine level:</td>
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<td>1012</td>
<td>900</td>
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<tr>
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<td>51</td>
<td>27</td>
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Table 5-2: Summary of the DHP dosage, creatinine levels and T cell characteristics of the human subjects used in the study.

The participants of the study were one healthy human female donor receiving no DHP therapy, and one renal disease patient administered nifedipine (patient A) and two renal disease patients receiving amlodipine (patients B and C) for the treatment of hypertension. T cell populations from all four individuals differed in the number of T cells isolated from peripheral blood, the percentage of CD3$^+$ T cells, as well as the percentage of CD4$^+$ and CD8$^+$ T cells in the peripheral blood of each individual. Peripheral blood creatinine levels are also shown as an indicator of kidney function.
whether DHP administration affected T cell function. The production of IL-2 was used as an indicator of T cell function since IL-2 gene expression is dependent upon extracellular Ca\(^{2+}\) influx (126), and blockage of L-type Ca\(^{2+}\) channels by nifedipine in vitro prevents IL-2 secretion from human PBTs (140). As illustrated in Figure 5-6A, IL-2 secreted by PBTs from patient A receiving nifedipine and patient B administered amlodipine was impaired. However, the PBTs from patient C (also receiving a similar dose of amlodipine as patient B) showed no reduction in the amount of IL-2 secreted compared to IL-2 produced by the normal PBT control. The data presented in Figure 5-6A was normalized for the number of CD3\(^+\) T cells since each population of isolated PBTs had significantly different CD3\(^+\) T cell numbers (Table 5-2).

Since CD3\(^+\)CD4\(^+\)CD8\(^-\) T cells produce the majority of IL-2 compared to other lymphocytes, the same data was then normalized for the number of CD3\(^+\)CD4\(^+\)CD8\(^-\) T cells. Under these new parameters, all three patients administered DHPs showed a subtle defect in IL-2 secretion compared to the normal PBT control (Figure 5-6B). Finally, the surface expression of the IL-2R and the early T cell activation marker, CD69 were also analyzed on the viable (PI negative) human PBTs after culture supernatants were removed for the IL-2 assay. In comparison to the expression on normal PBTs, IL-2R and CD69 receptor expression on the renal patients’ PBTs was not downregulated following anti-CD3 stimulation (data not shown).
Human PBTs isolated from a healthy donor and three renal disease patients (patients A, B and C) were stimulated with immobilized OKT3 (10 µg/ml) and 10 nM TPA. After 24 h, IL-2 secreted in the supernatants was measured by standard sandwich ELISA techniques. The results are normalized for either the quantity of IL-2 secreted by CD3⁺ T cells (A) or by CD3⁺CD4⁺CD8⁻ T cells alone (B). The normal control is PBTs isolated from a healthy human female not administered DHPs. The DHP administered to each renal patient is indicated above the bars. Results depicted are representative of three independent experiments. Each bar represents the mean and SD of assays from triplicate wells.
5.2.7 Uremic Serum from Renal Disease Patients Inhibits IL-2 Secretion from Normal Human PBTs

One concern with the observed immunosuppressive effects of DHP administration was that uremic serum in renal disease patients may be partially responsible for the defect in IL-2 secretion, and therefore may mask any potential inhibitory effects of the DHP derivatives. This concern was warranted since an early report by Donati et al. demonstrated that 20% uremic serum from renal disease patients receiving hemodialysis inhibited PHA-induced proliferation of PBMCs and purified T cells, which coincided with downregulation of IL-2 production (217). The inhibitory effects of uremic serum were assessed in this study by treating normal human PBTs with either 10% normal serum from a healthy individual or 10% uremic serum from the three renal disease patients previously described. The PBTs were then stimulated with OKT3 and TPA, and IL-2 secretion was measured. A serum concentration of 10% was used in this analysis since a preliminary experiment demonstrated that both 15% and 20% normal serum completely abolished IL-2 secretion from normal human PBTs.

As shown in Figure 5-7, treatment of normal PBTs with 10% normal and uremic serum slightly inhibited IL-2 secretion compared to preincubation of PBTs with medium alone. In addition, it was observed that only uremic serum from the two patients administered amlodipine reduced IL-2 secretion compared to treatment with normal serum or uremic serum from the patient administered nifedipine, whereas CD3^+CD4^+CD8^- T cells from all three renal patients showed impairment in IL-2 secretion. IL-2R and CD69 surface expression were not downregulated on normal, viable PBTs preincubated with either normal or uremic serum following anti-CD3 stimulation.
Figure 5-7: Uremic serum from renal disease patients reduces IL-2 secretion from human PBTs.

Human PBTs (donor, n=1) were incubated with 10% normal serum from a healthy donor or 10% uremic serum isolated from renal disease patients A, B and C. PBTs were stimulated with immobilized OKT3 (10 μg/ml) and 10 nM TPA, and IL-2 secreted in the supernatants was measured by standard sandwich ELISA techniques. The control is normal human PBTs treated with medium alone. The DHP administered to each renal patient is indicated above the bars. The human PBTs contained 10% \( \text{CD}^+\text{CD}^+\text{CD}^+\), 85% \( \text{CD}^+\text{CD}^+\text{CD}^+\), 4.0% \( \text{CD}^+\text{CD}^+\text{CD}^+\) and 1.0% \( \text{CD}^+\text{CD}^+\text{CD}^+\) cells. Similar data was obtained in two independent experiments. Each bar represents the mean and SD of assays from triplicate wells.
compared to the media controls (data not shown). These results are in agreement with the study by Donati et al. that showed no alteration in IL-2R surface expression following normal or uremic serum treatment of PBMCs stimulated in the presence of PHA (217).

5.3 Discussion

Sustained Ca\textsuperscript{2+} influx for 1 to 2 h through plasma membrane Ca\textsuperscript{2+} channels is necessary to promote and maintain T cell proliferation in response to peptide-MHC complexes (10). To investigate whether L-type VDCCs contribute to sustained Ca\textsuperscript{2+} influx during T cell proliferation \textit{in vivo}, the effects of nifedipine on H-Y-specific TCR-Tg CD8\textsuperscript{+} T cell proliferation in male recipient mice were examined. Through a series of experiments it was demonstrated that one dose of 15 mg/kg nifedipine is sufficient to slow down the proliferation of T cells \textit{in vivo}, even though the half-life of a single 15 mg/kg dose of nifedipine is less than 30 min in mouse blood (215). T cell proliferation was effectively inhibited by a single dose of nifedipine only when drug administration occurred at approximately 24 h after the H-Y-specific TCR-Tg thymocyte injection. Since the bulk of the H-Y-specific TCR-Tg T cells have reached the spleen and are beginning to proliferate in response to the male H-Y antigen at 24 h (214), nifedipine treatment at this time point may block L-type Ca\textsuperscript{2+} channels involved in sustained Ca\textsuperscript{2+} mobilization, which is crucial for the initiation IL-2 gene transcription and T cell proliferation (10). The specificity of nifedipine in the \textit{in vivo} proliferation assay was also addressed by demonstrating that treatment of the male mice with nifedipine prior to H-Y-
specific thymocyte injection did not interfere with the proliferation of the responding H-Y-specific CD8+ T cells.

Overall, the observed anti-proliferative effects of nifedipine in mice paralleled the inhibitory action of nifedipine on mouse splenocyte proliferation in vitro. Low doses of nifedipine (1-10 μM) used in the in vitro MLR and the single dose of 15 mg/kg nifedipine in vivo only lead to a partial block of T cell proliferation. Even following the application of multiple doses of nifedipine, the proliferation of H-Y-specific CD8+ T cells was not completely abolished. Therefore, the in vivo data also supports the hypothesis that in addition to L-type Ca2+ channels, other Ca2+ channels, such as TRP Ca2+ channels, may contribute to the Ca2+ influx pathway involved in T cell proliferation in vivo. In summary, the data collectively demonstrates that blocking the function of L-type Ca2+ channels by nifedipine treatment significantly effects T cell proliferation both in vitro and in vivo.

Nifedipine treatment not only caused a decrease in T cell proliferation, but it also resulted in fewer H-Y-specific CD8+ T cells reaching the spleen compared to mice administered vehicle alone. Even though there are several plausible explanations for the reduced T cell numbers in nifedipine treated mice, the H-Y antigen model had its limitations and therefore, there was no clear method in determining how nifedipine treatment reduced the total number of H-Y-specific CD8+ T cells recovered from the spleen of male mice. One possible explanation is that the decrease in T cell proliferation led to fewer cells existing in the nifedipine treated male mice, and consequently fewer cells reaching the spleens of these animals. In an attempt to understand how nifedipine reduced total H-Y-specific T cell numbers, it was found that nifedipine treatment of
female mice did not reduce the total number of H-Y-specific CD8\(^+\) T cells present in the spleen. This data suggested that nifedipine exerts its inhibitory action through an antigen-dependent mechanism, such as antigen-dependent cell death or T cell homing to the spleen. The homing and extravasation of naïve and effector T cells to secondary lymphoid organs, such as the peripheral and mesenteric lymph nodes and the spleen, is a complex, multiple step process involving the interaction between homing receptors on T cells and corresponding ligands on endothelial cells (218). Recent studies have established that the selective homing receptor-ligand interactions induce Ca\(^{2+}\)-dependent signaling at different stages in the T cell homing process. Consequently, treatment of male mice with nifedipine may disrupt Ca\(^{2+}\) mobilization triggered by ligated homing receptors in the H-Y-specific CD8\(^+\) T cells, directly effecting Ca\(^{2+}\)-dependent signaling in these cells.

In addition to demonstrating the anti-proliferative effects of nifedipine in mice, the effects of nifedipine and amlodipine administration on circulating T lymphocytes in renal disease patients was examined. It was found that CD3^+CD4^+CD8^- T cells from renal patients secreted less IL-2 compared to CD3^+CD4^+CD8^- T cells isolated from a healthy individual when stimulated \textit{in vitro}, suggesting that DHP therapy may act an immunosuppressant. However, conflicting data has demonstrated that renal disease patients not receiving DHPs also have impaired T cell responses (217). In the 1980's, studies showed an altered number of T cell subsets (219), and decreased lymphocyte responses to mitogenic stimulation in patients with end-stage renal disease (220). Uremic serum in these patients is partially responsible for the dampened immune responses (217). The inhibitory effects of uremic serum were confirmed in this study, by
demonstrating that treatment of normal PBTs with uremic serum from patients receiving amlodipine resulted in decreased IL-2 production. It should be noted that uremic serum from the patient administered nifedipine did not inhibit IL-2 secretion from PBTs to a greater extent than treatment with normal serum. Although uremic serum may contribute to impaired immune responses in renal disease patients, exacerbation of this defect by DHP administration cannot be ruled out.

The present findings reported here should encourage further research into determining whether patients receiving DHPs are immunosuppressed. Future experiments examining T cell function of patients administered DHPs should be expanded to include a larger cohort of healthy controls as well as renal disease patients. By comparing T cell function in renal disease patients with or without DHP administration, the additive side effects of DHPs could be evaluated. Furthermore, it would be beneficial to examine T cell responses in renal patients immediately before and after DHP administration in order to pinpoint the potential immunosuppressive effects of DHPs. Examining whether the incidence of infection and cancer is higher in patients receiving DHPs would also lend support that these patients have compromised immune responses. In fact, recent studies have reported that patients receiving CCBs, including nifedipine, for the treatment of cardiovascular diseases may have increased risk of cancer (221, 222). Given the profound effects DHPs have on T cell responses \textit{in vitro} and \textit{in vivo}, the possibility that long-term treatment with DHPs may act as an immunosuppressant cannot be overlooked.
CHAPTER 6: DISCUSSION

6.1 General Conclusions

From the results presented here, evidence was provided that human T lymphocytes, although non-excitable, express voltage-dependent-like Ca\(^{2+}\) channels that share some of the structural properties of conventional L-type VDCCs found in electrically excitable cells. A novel feature of both of the \(\alpha_{1F}\)-subunit splice isoforms is the presence of exonic sequence with amino acid identity to the human skeletal muscle \(\alpha_{1S}\)-subunit. This is the first reported example of “splice conversion” of an L-type \(\alpha_{1}\)-subunit. The cDNA cloning of one of the alternatively spliced \(\alpha_{1F}\)-subunit isoforms is the first direct evidence that a full-length \(\alpha_{1}\)-subunit mRNA of L-type VDCCs is expressed in T lymphocytes. Even though it was demonstrated that the mRNA and protein of \(\alpha_{1F}\)-subunit exist in T lymphocytes, the functional role of L-type VDCCs in non-excitable cells still remains under debate. It is well established that in electrically excitable cells, such as neurons and muscle cells, L-type VDCCs are activated in response to membrane depolarization, which is a large shift in electrical potential across the plasma membrane to a less negative state. In resting T cells, the membrane potential across the plasma membrane is reported to be in the range of -50 to -70 mV (223). This membrane potential is primarily generated by \(K^+\) diffusion potential through \(K_v\), and \(Ca^{2+}\)-activated \(K^+\) channels, and the sodium-\(K^+\) ATPase-pump (224). Unlike excitable cells, only small depolarizations and hyperpolarizations of the plasma membrane are measured in T cells when activated with the mitogens, Con A and PHA (223). Furthermore, depolarizing the
plasma membrane with high concentrations of K\(^+\) does not stimulate or inhibit T cell mitogenesis (223). Therefore, it is generally considered that membrane potential changes do not play a role in initiating signal transduction pathways during T cell activation (223).

Based on these observations, many investigators have questioned how an L-type VDCC that is typically gated by membrane depolarization is capable of functioning in T lymphocytes when these cells do not exhibit large changes in membrane potential. One plausible explanation is that voltage-dependent-like Ca\(^{2+}\) channels expressed in T lymphocytes and other non-excitable cells may not be gated by changes in membrane potential. Interestingly, this hypothesis is supported by the identification of the \(\alpha_{1f}\)-subunit splice isoforms that contain unique exon usages distinct from the \(\alpha_{1f}\)-subunit isolated from human retina that may render these channel variants insensitive to changes in membrane depolarization. The deletion of the IVS4 voltage sensor domain in the voltage negative variant, and the IVS3-S4 interlinker in the voltage positive variant cause the splice isoforms to lack important voltage sensing domains. Instead of being activated by depolarization, the \(\alpha_{1f}\)-subunit splice isoforms may be gated by an alternative mechanism, such as ER store-depletion or a direct signal from the TCR. In support of this hypothesis, it was demonstrated that the expression of the \(\alpha_{1f}\)-subunit splice isoforms is increased following TCR engagement in Jurkat T cells and to a lesser extent in human PBTs, suggesting that at least in Jurkat T cells, Ca\(^{2+}\) influx through \(\alpha_{1f}\)-subunit splice isoforms may be regulated through the TCR/CD3 complex. In summary, it appears that through alternative splicing, T lymphocytes have adopted structurally unique \(\alpha_{1f}\)-subunit proteins that in all probability are not gated by membrane depolarization.
The presence of an L-type channel-forming α1-subunit in T lymphocytes and its contribution to Ca\(^{2+}\) influx during the T lymphocyte activation process was further established by the results from the *in vitro* study with the DHP derivatives, (+/-) Bay K 8644 and nifedipine. Both (+/-) Bay K 8644 and nifedipine modulated early and late Ca\(^{2+}\)-dependent signaling events in Jurkat T cells and human PBTs, strengthening the hypothesis that a DHP sensitive L-type VDCC is present in the plasma membrane of T lymphocytes. Furthermore, nifedipine inhibition of T cell proliferation *in vivo* provided additional support that functional L-type Ca\(^{2+}\) channels are expressed in T lymphocytes. The mode of action of (+/-) Bay K 8644 and nifedipine may have been through binding to, and mediating the function of the alternatively spliced α1F-subunit isoforms. However, several studies have demonstrated that partial transcripts of two other channel-forming α1-subunits of L-type VDCCs, the α1C-subunit and α1S-subunit, exist in T lymphocytes (119, 135). The α1C-subunit and α1S-subunit also contain amino acids in the IIIIS5, IIIIS6 and IVS6 transmembrane domains that are capable of selectively binding to DHP derivatives. Due to the possible expression of more than one α1-subunit protein in T lymphocytes, it is plausible that (+/-) Bay K 8644 and nifedipine were mediating Ca\(^{2+}\) influx and Ca\(^{2+}\)-dependent T cell responses not only through the α1F-subunit splice isoforms, but also through α1C-subunit and α1S-subunit. Regardless if (+/-) Bay K 8644 and nifedipine were binding to more than one α1-subunit subtype, it is clear that DHP sensitive L-type Ca\(^{2+}\) channels play a significant role in contributing to Ca\(^{2+}\) entry into T lymphocytes.

Although the molecular and pharmacological studies point to a role for L-type VDCCs in mediating Ca\(^{2+}\) influx pathways in T cells, at the present time, it is unclear
whether the α₁F-subunits splice isoforms or other α₁-subunits present in T cells contribute to $I_{\text{CRAC}}$. It has been well established through patch-clamp recordings that the sole Ca$^{2+}$ current induced by TCR engagement and by passive store-depletion in T lymphocytes is $I_{\text{CRAC}}$ (10, 79). As previously mentioned, many of the biophysical characteristics of $I_{\text{CRAC}}$ are closely shared with L-type VDCCs. Similar to L-type VDCCs, CRAC channels exhibit a high selectivity for Ca$^{2+}$ over monovalent cations, that can be ascribed to Ca$^{2+}$ binding with micromolar affinity to sites within these channels (133). CRAC channels and VDCCs both exhibit a loss in Ca$^{2+}$ selectivity when the external [Ca$^{2+}$] is lowered to micromolar levels (127, 133). Kerschbaum et al. also demonstrated through probing CRAC channels with various organic monovalent cations of differing sizes that the physical diameter of the channel pore is ~0.6 nm for both CRAC channels and L-type VDCCs (128). Even though CRAC channels and L-type VDCCs differ in their gating mechanisms and single-channel conductance (VDCC in excitable cells is 300 times larger than the CRAC channel), they do share many biophysical characteristics, indicating that these channels may have a similar structure (128). Based on this analogous biophysical “fingerprint”, and the possibility that the α₁F-subunit splice isoforms may not be gated by depolarization, the argument that L-type VDCCs may be responsible for conducting part of $I_{\text{CRAC}}$ is plausible.

Over the past several decades, the continual discovery of novel channels proteins that mediate either Ca$^{2+}$ release from intracellular stores or Ca$^{2+}$ influx across the plasma membrane is constantly reshaping the proposed models for Ca$^{2+}$ mobilization in T lymphocytes. For example, it was initially reported that IP$_3$ binding to the IP$_3$R Ca$^{2+}$ channel was the central mechanism for Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores.
However, the identification of RyR3 Ca\(^{2+}\) channel in T lymphocytes, together with the second messenger, cADPR, has led investigators to reinterpret earlier models. Presently, it is believed that IP\(_3\) is necessary for the initial Ca\(^{2+}\) release from IP\(_3\)R Ca\(^{2+}\) channels, while cADPR association with RyR Ca\(^{2+}\) channels is essential for a prolonged second phase of Ca\(^{2+}\) release (75). The discovery of novel Ca\(^{2+}\) channels that mediate Ca\(^{2+}\) entry across the plasma membrane is also redefining Ca\(^{2+}\) influx models. The data obtained from the \textit{in vitro} and \textit{in vivo} DHP studies demonstrated that (+/-) Bay K 8644 did not maximally activate Ca\(^{2+}\) influx, while TCR-induced Ca\(^{2+}\) influx was not completely inhibited by nifedipine in Jurkat T cells and human PBTs. These results suggest that, although L-type VDCCs contribute to Ca\(^{2+}\) influx, additional Ca\(^{2+}\) channels also mediate Ca\(^{2+}\) entry during T cell activation. When the TRP Ca\(^{2+}\) channel, CaT1, was originally identified in T lymphocytes, it was proposed that this single channel protein was responsible for generating \(I_{\text{CRAC}}\). Further work by Cui \textit{et al.} revealed that CaT1 was not solely responsible for \(I_{\text{CRAC}}\) since overexpression of a dominant negative CaT1 pore mutant did not completely abolish endogenous CRAC channel activity in Jurkat T cells (105). Since CaT1 does not recapitulate all the properties of \(I_{\text{CRAC}}\), it has been suggested that CaT1 multimerizes with the TRPV5 channel protein and together, the CaT1/TRPV5 complex forms endogenous \(I_{\text{CRAC}}\) (105). In addition to L-type VDCCs and TRP Ca\(^{2+}\) channels, other Ca\(^{2+}\) channels that are store-independent, such as LTRPC2 and TRPC6, also may shape the Ca\(^{2+}\) signal during sustained T cell activation. Since Ca\(^{2+}\) signaling lasts for 1 to 2 h in stimulated T cells, distinct Ca\(^{2+}\) channels may be involved in different phases of the response, thus illustrating the complexity of Ca\(^{2+}\) mobilization.
Based on these studies and the recent data presented on the α₁F-subunit splice isoforms, a model of immediate and sustained Ca²⁺ signaling in T lymphocytes has been composed (Figure 6-1). The immediate phase of Ca²⁺ mobilization involves the Ca²⁺ channels that are activated shortly after the engagement of the TCR/CD3 complex by peptide-MHC. During this phase, the emptying of intracellular Ca²⁺ stores by Ca²⁺ release through IP₃R Ca²⁺ channels causes the opening of the CRAC channel tetrameric complex, potentially comprised of CaT1 and TRPV5. The voltage negative splice variant of the α₁F-subunit may be involved in this phase since the mRNA expression of this channel isoform rapidly increases following TCR engagement. The mRNA expression of LTRPC2 was also shown to increase promptly after anti-CD3 treatment, so this channel may also contribute to the early phase of Ca²⁺ signaling. Finally, DAG production may activate the TRPC6 channel immediately following ligation of the TCR. During the sustained phase of Ca²⁺ mobilization, RyR Ca²⁺ channels are essential for a prolonged release of Ca²⁺ from intracellular stores. The mRNA expression profiles of the voltage negative and positive splice variants in Jurkat T cells indicate that both channel isoforms may participate in Ca²⁺ influx in this second phase. The observed increase in α₁F-subunit protein expression after 1 to 2 days of activation also supports the hypothesis for a role for both splice variants in contributing to sustained Ca²⁺ influx. In summary, together these Ca²⁺ channels coordinate a stable and dynamic Ca²⁺ signal that is necessary to promote T cell activation and clonal expansion.
During T cell activation, intracellular Ca\(^{2+}\) mobilization can be divided into immediate and sustained phases of Ca\(^{2+}\) signaling. In the early phase, it appears that the IP\(_3\)R Ca\(^{2+}\) channel is responsible for Ca\(^{2+}\) release from the ER, whereas CaT1, TRPC6, LTRPC2 and possibly the voltage negative splice variant of the \(\alpha\)IF subunit may regulate Ca\(^{2+}\) influx pathways. RyR Ca\(^{2+}\) channels participate in Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores in the sustained phase of Ca\(^{2+}\) signaling. CaT1 continues to provide Ca\(^{2+}\) influx during this later phase of the Ca\(^{2+}\) response. Both \(\alpha\)IF subunit splice isoforms may also contribute to Ca\(^{2+}\) delivery across the plasma membrane. This figure was adapted from Lewis et al. (10).
6.2 Future Directions

The ultimate goal of the research presented here is to understand the physiological role of L-type VDCCs in the Ca\textsuperscript{2+} entry process during T lymphocyte activation. Through this research it has been established that novel channel-forming \(\alpha_{1F}\)-subunits of the L-type VDCC are expressed in T lymphocytes. Since it was determined that the alternatively spliced \(\alpha_{1F}\)-subunits lack several exons compared to the known \(\alpha_{1F}\)-subunit from human retina, it is important to demonstrate that the T lymphocyte \(\alpha_{1F}\)-subunits form functional Ca\textsuperscript{2+} channels, and definitely determine whether these channel isoforms contribute to Ca\textsuperscript{2+} influx pathways during T cell activation.

One method that is routinely used to establish whether a Ca\textsuperscript{2+} channel is functional is to overexpress the cloned channel in a heterologous expression system, such as *Xenopus* oocytes. The Ca\textsuperscript{2+} currents gated by the overexpressed channel can then be measured through patch-clamp recordings. These experiments can be performed with the voltage negative splice variant of the \(\alpha_{1F}\)-subunit and the human retina \(\alpha_{1F}\)-subunit since the full-length cDNA sequences of both channels have been cloned. By comparing the Ca\textsuperscript{2+} currents gated by the different Ca\textsuperscript{2+} channels through patch-clamping, it will be established whether the voltage negative splice variant of the \(\alpha_{1F}\)-subunit is actually insensitive to membrane depolarization. It may also be possible to determine whether the Ca\textsuperscript{2+} current gated by the voltage negative isoform can recapitulate the biophysical properties of \(I_{\text{CRAC}}\). If it is not feasible to measure the current gated by the cloned \(\alpha_{1F}\)-subunits in *Xenopus* oocytes, alternative approaches will be considered. For instance, the voltage negative splice variant may be overexpressed in Jurkat T cells. Ca\textsuperscript{2+} influx in
untransfected and transfected Jurkat T cells can be compared when the cells are loaded
with the Ca\(^{2+}\) sensitive dye, indo-1, and activated with various stimuli, such as anti-CD3
mAb, thapsigargin (to deplete ER stores), or high concentrations of K\(^{+}\) (to induce
membrane depolarization). These experiments will determine whether the exons that
have been removed through alternative splicing in the voltage negative splice variant are
required for a functioning Ca\(^{2+}\) channel.

In addition to overexpressing the cloned voltage negative variant in T
lymphocytes, experiments will also be conducted to produce \(\alpha_{1F}\)-subunit knock-out mice
that are deficient in both \(\alpha_{1F}\)-subunit splice isoforms. The generation of \(\alpha_{1F}\)-subunit
knock-out mice will allow the determination of whether the lack of \(\alpha_{1F}\)-subunit
expression leads to deficiencies in Ca\(^{2+}\) influx in the T cells of these animals. The \(\alpha_{1F}\)-
subunit knock-out mice will be generated by injecting embryonic stem cells (129
background) with the pPNT vector, containing 2 kb of the 5’-untranslated region and
exon 1 of the CACNA1F gene from human retina in the short arm, and 6 kb of exons 7-
14 in the long arm. This will lead to an out-of-frame CACNA1F gene sequence due to
neomycin insertion in exons 2-6, converting an intracellular loop of the channel to an
extracellular loop, disrupting \(\alpha_{1F}\)-subunit channel function. Splenocytes and thymocytes
from these knock-out animals will be assayed for T cell function, including Ca\(^{2+}\) influx,
NFAT activation and IL-2 secretion. It will also be assessed whether T lymphocytes from
the knock-out mice proliferate in a MLR. These assays will determine whether the \(\alpha_{1F}\)-
subunit splice isoforms directly contribute to Ca\(^{2+}\) influx during T cell activation and
proliferation. Furthermore, these experiments should clearly address the function of the
\(\alpha_{1F}\)-subunit channel isoforms in T lymphocytes.
Although substantial efforts have been made over the past few years to elucidate the mechanisms controlling Ca\(^{2+}\) influx pathways in T lymphocytes, identifying the precise Ca\(^{2+}\) channels that coordinate Ca\(^{2+}\) entry remains a central goal for investigators. The identification of novel voltage-dependent-like Ca\(^{2+}\) channels in T lymphocytes, along with TRP Ca\(^{2+}\) channels, implies that Ca\(^{2+}\) influx may be gated by several distinct Ca\(^{2+}\) channels during the T cell activation process. An important long-range goal will be establishing the contribution of each Ca\(^{2+}\) channel that shape the immediate and sustained Ca\(^{2+}\) signals. This is especially imperative in light of the fact that T lymphocytes from severe-combined immunodeficiency patients have a principal defect in transmembrane Ca\(^{2+}\) influx (225). Although the patients' T cells are currently being used as a tool to understand Ca\(^{2+}\)-dependent signaling pathways, elucidation of the Ca\(^{2+}\) channels mediating Ca\(^{2+}\) influx is paramount to providing an effective treatment to these individuals. In conclusion, understanding the mode of Ca\(^{2+}\) entry will eventually lead to the development of novel therapeutic agents that could mediate T cell activation or inactivation states during an immune response.
REFERENCES


97. Nilius, B. 2003. From TRPs to SOCs, CCEs, and CRACs: consensus and controversies. *Cell Calcium* 33:293.


### APPENDIX A

ClustalW cDNA sequence alignment of the human retina $\alpha_{1F}$-subunit (GenBank accession number AF067227) with the voltage negative splice isoform (Variant1) and the voltage positive splice isoform (Variant2) of the $\alpha_{1F}$-subunit isolated from human spleen. The asterisks represent identically aligned nucleotides, whereas dashes represent spliced exons.

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Variant2 AGAGTCTGCGCGCCCTCCGCGAGCCACCCCGGTCCCCTCCGCGACGTGCGCTGCGGCA 1380

Retina CTGAAAGTCCATGCTCTACTGGGCTGTGCTGTTGCTCTTCCTCAACACGTTGACC 1440
Variant1 CTGAAAGTCCATGCTCTACTGGGCTGTGCTGTTGCTCTTCCTCAACACGTTGACC 1440
Variant2 CTGAAAGTCCATGCTCTACTGGGCTGTGCTGTTGCTCTTCCTCAACACGTTGACC 1440

Retina ATCGCCTCTGAGCACCACGGGCAGCCTGTGTGGCTCACCCAGATCCAGGAGTATGCCAAC 1500
Variant1 ATCGCCTCTGAGCACCACGGGCAGCCTGTGTGGCTCACCCAGATCCAGGAGTATGCCAAC 1500
Variant2 ATCGCCTCTGAGCACCACGGGCAGCCTGTGTGGCTCACCCAGATCCAGGAGTATGCCAAC 1500

Retina AAAGTGTTGCTCTGTCTGTTCACGGTGGAGATGCTTCTCAAATTGTACGGTCTGGGCCCC 1560
Variant1 AAAGTGTTGCTCTGTCTGTTCACGGTGGAGATGCTTCTCAAATTGTACGGTCTGGGCCCC 1560
Variant2 AAAGTGTTGCTCTGTCTGTTCACGGTGGAGATGCTTCTCAAATTGTACGGTCTGGGCCCC 1560

Retina TCTGCCTATGTGTCTTCCTTCTTCAACCGCTTTGACTGCTTTGTGGTCTGTGGGGGCATC 1620
Variant1 TCTGCCTATGTGTCTTCCTTCTTCAACCGCTTTGACTGCTTTGTGGTCTGTGGGGGCATC 1620
Variant2 TCTGCCTATGTGTCTTCCTTCTTCAACCGCTTTGACTGCTTTGTGGTCTGTGGGGGCATC 1620

Retina CTAGAGACCACCTTGGTGGAGGTGGGCGCCATGCAGCCCTTGGGCATCTCAGTGCTCCGA 1680
Variant1 CTAGAGACCACCTTGGTGGAGGTGGGCGCCATGCAGCCCTTGGGCATCTCAGTGCTCCGA 1680
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Retina GTGGCATCCCTGCTCAATTCAATGAAATCCATCGCATCCTTGCTGCTTCTCCTCTTCCTC 1800
Variant1 GTGGCATCCCTGCTCAATTCAATGAAATCCATCGCATCCTTGCTGCTTCTCCTCTTCCTC 1800
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Retina CAGACCCACACCAAGCGAAGCACCTTTGACACGTTCCCCCAGGCCCTCCTCACTGTCTTT 1860
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Retina CAGATCCTGACAGGTGAGGACTGGAACGTGGTCATGTATGATGGTATCATGGCATATGGT 1920
Variant1 CAGGTCCTGACAGGTGAGGACTGGAACGTGGTCATGTATGATGGTATCATGGCATATGGT 1920
Variant2 CAGGTCCTGACAGGTGAGGACTGGAACGTGGTCATGTATGATGGTATCATGGCATATGGT 1920

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Variant1 GGCCCCTTCTTCCCAGGAATGTTGGTGTGCATCTATTTCATCATTCTCTTCATCTGTGGC 1980
Variant2 GGCCCCTTCTTCCCAGGAATGTTGGTGTGCATCTATTTCATCATTCTCTTCATCTGTGGC 1980

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Variant1 TTTCATCATTATCTTCTCCCTCCTCTCATGTCATTTTCATCATTCTCTTCATCTGTGGC 2040
Variant2 TTTCATCATTATCTTCTCCCTCCTCTCATGTCATTTTCATCATTCTCTTCATCTGTGGC 2040
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Variant1: AACATGGTCTCAGCCTCTCAGTATTGAGATGGTCTAAATGACGCTTTCAAG
Variant2: AACATGGTCTCAGCCTCTCAGTATTGAGATGGTCTAAATGACGCTTTCAAG

Retina: CCCAAGCATTACTTCACTGATGCCTGGGAACACGTTTGACGCTCTTATTGTGGTGGGCAGC
Variant1: CCCAAG
Variant2: CCCAAGCATTACTTCACTGATGCCTGGGAACACGTTTGACGCTCTTATTGTGGTGGGCAGC

Retina: ATAGTGGATATTGCCGTCACTGAAGTCAATAATGGTGGCCACCTTGGCGAGAGCTCTGAG
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Variant2: ATAGTGGATATTGCCGTCACTGAAGTCAATAATGGTGGCCACCTTGGCGAGAGCTCTGAG

Retina: GACAGCTCCCGCATTTCCATTACCTTCTTTCGCCTCTTCCGAGTTATGCGGCTGGTCAAG
Variant1: GACAGCTCCCGCATTTCCATTACCTTCTTTCGCCTCTTCCGAGTTATGCGGCTGGTCAAG
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Variant1: GCCTTGCCCTATGTGGCTCTTCTCATCGCAATGATATTCTTCATCTATGCCGTCATTGGC
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Variant1: ATGCAGATGTTCGGCAAGGTGGCTCTTCAGGATGGCACACAGATAAACCGAAACAACAAC
Variant2: ATGCAGATGTTCGGCAAGGTGGCTCTTCAGGATGGCACACAGATAAACCGAAACAACAAC

Retina: TTCCAGACCTTTCCACAGGCTGTGCTGCTTCTGTTCAGGTGTGCCACTGGTGAGGCATGG
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Retina: CAGGAGATAATGCTTGCCAGCCTTCCCGGAAATCGGTGTGATCCTGAGTCTGACTTTTAC
Variant1: CAGGAGATAATGCTTGCCAGCCTTCCCGGAAATCGGTGTGATCCTGAGTCTGACTTTTAC
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Retina: CTTGGTGAAGAGTTTACCTGTGGTAGCAATTTTGCCATCGCCTATTTCATCAGCTTCTTC
Variant1: CTTGGTGAAGAGTTTACCTGTGGTAGCAATTTTGCCATCGCCTATTTCATCAGCTTCTTC
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Variant1: ATGCTCTGTGCCTTCCTGATCATAAATCTCTTTGTGGCTGTGATCATGGACAACTTTGAT
Variant2: ATGCTCTGTGCCTTCCTGATCATAAATCTCTTTGTGGCTGTGATCATGGACAACTTTGAT

Retina: TATCTCACCAGGAGATTTGCTCAATCTGCGCCCATACCCATACCTTTGAGAAATTTCAAGAGAT
Variant1: TATCTCACCAGGAGATTTGCTCAATCTGCGCCCATACCCATACCTTTGAGAAATTTCAAGAGAT
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Variant1 CAGGGACCCAGCTCTCTCTATAGCGACGAGGAGTCCATCCTCTCCCGCTTCGATGAGGAG 5272
Variant2 CAGGGACCCAGCTCTCTCTATAGCGACGAGGAGTCCATCCTCTCCCGCTTCGATGAGGAG 5551

Retina GACTTGGGAGACGAGATGGCCTGCGTCCACGCCCTCTGAATTCCCACCCCTCCCCAACTG 5760
Variant1 GACTTGGGAGACGAGATGGCCTGCGTCCACGCCCTCTGAATTCCCACCCCTCCCCAACTG 5332
Variant2 GACTTGGGAGACGAGATGGCCTGCGTCCACGCCCTCTGAATTCCCACCCCTCCCCAACTG 5611

Retina CTCAATAAACCTCCCTGCCCCCTCCCCAGCAGGAGGCAGGCATGGACCACA 5813
Variant1 CTCAATAAACCTCCCTGCCCCCTCCCCAGCAGGAGGCAGGCATGGACCACA 5385
Variant2 CTCAATAAACCTCCCTGCCCCCTCCCCAGCAGGAGGCAGGCATGGACCACA 5664

*****************************************************
APPENDIX B

ClustalW amino acid sequence alignment of the human retina $\alpha_{1F}$-subunit (GenBank accession number AF067227) with the voltage negative splice isoform (Variant1) and the voltage positive splice isoform (Variant2) of the $\alpha_{1F}$-subunit isolated from human spleen.

Bech-Hansen et al. predicted the transmembrane domains in the human retina $\alpha_{1F}$-subunit (226). The transmembrane sequences are underlined and highlighted in red, and the putative EF-hand Ca$^{2+}$ binding motif in the carboxyl-terminus is underlined and highlighted in blue. The asterisks represent identically aligned amino acids.

| IS1  | Retina     | MSEEGGKGERILPSLTQGASIVEMK | PFDIILLLTIFANVCVALG | GYIPFPEDDSNANTANH 60 |
|      | Variant1   | MSEEGGKGERILPSLTQGASIVEMK | PFDIILLLTIFANVCVALG | GYIPFPEDDSNANTANH 60 |
|      | Variant2   | MSEEGGKGERILPSLTQGASIVEMK | PFDIILLLTIFANVCVALG | GYIPFPEDDSNANTANH 60 |

| IS2  | Retina     | NLEQVETFLVPTVTVKIVAYGLVLHP | 8NGWNLLDFIVVVGLF5V5L | EQQPG 120 |
|      | Variant1   | NLEQVETFLVPTVTVKIVAYGLVLHP | 8NGWNLLDFIVVVGLF5V5L | EQQPG 120 |
|      | Variant2   | NLEQVETFLVPTVTVKIVAYGLVLHP | 8NGWNLLDFIVVVGLF5V5L | EQQPG 120 |

| IS3  | Retina     | RPGDAPHTGGKPGGFDVKAARFLVRPLRLVGS | 9P5LHLV5NIS5M5KL5V5L | H1ALLVL 180 |
|      | Variant1   | RPGDAPHTGGKPGGFDVKAARFLVRPLRLVGS | 9P5LHLV5NIS5M5KL5V5L | H1ALLVL 180 |
|      | Variant2   | RPGDAPHTGGKPGGFDVKAARFLVRPLRLVGS | 9P5LHLV5NIS5M5KL5V5L | H1ALLVL 180 |

| IS4  | Retina     | PVIIYIAGLELFGLRMHKTFCYFLGSDM5EAEEDP5PC5ASSG5GR5ACTL5NQ5TCP5CR55RWP5G 240 |
|      | Variant1   | PVIIYIAGLELFGLRMHKTFCYFLGSDM5EAEEDP5PC5ASSG5GR5ACTL5NQ5TCP5CR55RWP5G 240 |
|      | Variant2   | PVIIYIAGLELFGLRMHKTFCYFLGSDM5EAEEDP5PC5ASSG5GR5ACTL5NQ5TCP5CR55RWP5G 240 |

| IS5  | Retina     | PNGGTTNXFDNFFAMLFVTQCVTMEGWTDVLYWNQDAMG5EL | 5PW5VFVS5LVIFS5G5F5V5NL | 30 |
|      | Variant1   | PNGGTTNXFDNFFAMLFVTQCVTMEGWTDVLYWNQDAMG5EL | 5PW5VFVS5LVIFS5G5F5V5NL | 30 |
|      | Variant2   | PNGGTTNXFDNFFAMLFVTQCVTMEGWTDVLYWNQDAMG5EL | 5PW5VFVS5LVIFS5G5F5V5NL | 30 |

| IS6  | Retina     | LVGLVLS | GESKREKAKARGDFQKQREKQMQMEDLRGMYLDWITQAEELMDPSADNL 360 |
|      | Variant1   | LVGLVLS | GESKREKAKARGDFQKQREKQMQMEDLRGMYLDWITQAEELMDPSADNL 360 |
|      | Variant2   | LVGLVLS | GESKREKAKARGDFQKQREKQMQMEDLRGMYLDWITQAEELMDPSADNL 360 |

<p>| Retina | GSSM5E5GRAGHRP5Q5LAE5LTN5RR5RGRLRFHSTR5THS5TSS5HAS5L5PAS5TD5G5MT5ET5Q5D5E 420 |
| Variant1 | GSSM5E5GRAGHRP5Q5LAE5LTN5RR5RGRLRFHSTR5THS5TSS5HAS5L5PAS5TD5G5MT5ET5Q5D5E 420 |
| Variant2 | GSSM5E5GRAGHRP5Q5LAE5LTN5RR5RGRLRFHSTR5THS5TSS5HAS5L5PAS5TD5G5MT5ET5Q5D5E 420 |</p>
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| Variant 2 | GPFFPGMLVCIYFNILFICGNYILLNVFLAILAVCNLASGDATKDGKEGKSNEDKLPQE | 720 |

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Variant1: QEYQNCLEKDNQRQCVEYALKQPRKRIPKNHPQYRWSVATNSAIFYLMPILLILMTTV 1140
Variant2: QEYQNCLEKDNQRQCVEYALKQPRKRIPKNHPQYRWSVATNSAIFYLMPILLILMTTV 1140

IVS2
Retina: ALAMQHEYQTAPFNAMYDIKMYGPLTQELMIIAFKPKHYFTDAWNTF DALIVVGS 1200
Variant1: ALAMQHEYQTAPFNAMYDIKMYGPLTQELMIIAFKPK 1182
Variant2: ALAMQHEYQTAPFNAMYDIKMYGPLTQELMIIAFKPKHYFTDAWNTF DALIVVGS 1200

IVS3
Retina: IVDIAVTEVNNNGHLESESSRISITFLLRFLRVMLVLLLSKEGIRIUTLWTFIKSFQ 1260
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Variant2: IVDIAVTEVNNNGHLESESSRISITFLLRFLRVMLVLLLSKEGIRIUTLWTFIKSFQ 1260

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Retina: IVDIAVTEVNNNGHLESESSRISITFLLRFLRVMLVLLLSKEGIRIUTLWTFIKSFQ 1260
Variant1: IVDIAVTEVNNNGHLESESSRISITFLLRFLRVMLVLLLSKEGIRIUTLWTFIKSFQ 1260
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Variant1: ALPYVALLAIIPIATYAVIGMQRMFPGKVALQDQCHNRRNNNFFQTFPQAVLLFLRGCATGEAW 1320
Variant2: ALPYVALLAIIPIATYAVIGMQRMFPGKVALQDQCHNRRNNNFFQTFPQAVLLFLRGCATGEAW 1320

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Variant2: QEIMLASLPGNRCDPESDFFGPEEFTCGSNFAISFFMLCAPFLNHFLVAVIMDNFD 1380

EF-hand motif
Retina: YLTRDWSILGPHHLEKFRIWSEYDPGAKGRKHLDDVALLRRRIQPLPGFGKLC PHRVAC 1440
Variant1: YLTRDWSILGPHHLEKFRIWSEYDPGAKGRKHLDDVALLRRRIQPLPGFGKLC PHRVAC 1440
Variant2: YLTRDWSILGPHHLEKFRIWSEYDPGAKGRKHLDDVALLRRRIQPLPGFGKLC PHRVAC 1440

Retina: KRLVAMNHPLNSDGTVTFINATLFALVRTSLKIKTEGNLEQANQELRIVIKIIWKR MKQL 1500
Variant1: KRLVAMNHPLNSDGTVTFINATLFALVRTSLKIKTEGNLEQANQELRIVIKIIWKR MKQL 1500
Variant2: KRLVAMNHPLNSDGTVTFINATLFALVRTSLKIKTEGNLEQANQELRIVIKIIWKR MKQL 1500

Retina: LDEVIPPPDEEEVTGVKAFYATFLQIQYFRFKRRRKEKGELLGANDAPSTALLQAGL RSLQ 1560
Variant1: LDEVIPPPDEEEVTGVKAFYATFLQIQYFRFKRRRKEKGELLGANDAPSTALLQAGL RSLQ 1560
Variant2: LDEVIPPPDEEEVTGVKAFYATFLQIQYFRFKRRRKEKGELLGANDAPSTALLQAGL RSLQ 1560

Retina: DLGP3EMQRALTCDEEEEEEGEQEVEEDEKDLETNKATMWSQSARRSGISVSLPGD 1620
Variant1: DLGP3EMQRALTCDEEEEEEGEQEVEEDEKDLETNKATMWSQSARRSGISVSLPGD 1620
Variant2: DLGP3EMQRALTCDEEEEEEGEQEVEEDEKDLETNKATMWSQSARRSGISVSLPGD 1620

Retina: RLPDSLGFPSDDDRGPTPTSSQPSVPSVQAGSNTHRRSGCALIFTPEEGNSQPKGQNK 1680
Variant1: RLPDSLGFPSDDDRGPTPTSSQPSVPSVQAGSNTHRRSGCALIFTPEEGNSQPKGQNK 1680
Variant2: RLPDSLGFPSDDDRGPTPTSSQPSVPSVQAGSNTHRRSGCALIFTPEEGNSQPKGQNK 1680

207
Retina  QDEDEVPDRLSYLDQAGTPPCSVLLPPHMRAQRYMDGHLVPRRLLPPTAGRKSFTI 1740
Variant1  
Variant2  

Retina  QCLQRQGSCEDLPITYHRGRNSGPNRAGGNGSWATPPQRGRLLYAPLLLVEEAGEGYL 1800
Variant1  
Variant2  

Retina  GRSSGPLRTFTCLHPGTHSDPSHGKRGSADSLVEAVLISEGLGFLARDFPRFVALKQEI 1860
Variant1  
Variant2  

Retina  ADACRLTLEDMDNAASDLIAQGTSLESSILLESRDFDEEDLGDEMACVHAL 1912
Variant1  
Variant2  

208