Identification and Functional Characterization of Voltage-dependent Calcium Channels in T Lymphocytes*

Maya F. Kotturi†‡§, Douglas A. Carlow‡, Junnella C. Lee‡§, Hermann J. Ziltener‡§, and Wilfred A. Jeffreys‡§**

From the †Biomedical Research Centre, the §Biotechnology Laboratory and Department of Microbiology and Immunology, the ‡Departments of Medical Genetics and Zoology, and the ¶Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

In T lymphocytes, sustained calcium (Ca²⁺) influx through Ca²⁺ channels localized in the plasma membrane is critical for T cell activation and proliferation. Previous studies indicated that voltage-dependent Ca²⁺ channels (VDCCs) play a role in Ca²⁺ mobilization during T lymphocyte activation. However, the role of VDCCs in otherwise nonexcitable cells is still poorly understood. We used RT-PCR to identify a transcript encoding the pore-forming α₁v subunit of an L-type Ca²⁺ channel in T lymphocytes. Its identity was confirmed by DNA sequencing. To further investigate the contribution of Ca²⁺ influx through VDCCs, we assessed the effects of the 1,4-dihydropyridine L-type Ca²⁺ channel agonist, (+/−) Bay K 8644, and antagonist, nifedipine, on the human Jurkat T cell leukemia line, human peripheral blood T lymphocytes and mouse splenocytes. We found that treatment of T lymphocytes with (+/−) Bay K 8644 increased intracellular Ca²⁺ and induced the activation of phospholipase C-δ1, whereas nifedipine blocked Ca²⁺ influx, the activity of Erk1/2 and nuclear factor of activated T cells (NFAT), interleukin-2 (IL-2) production, and IL-2 receptor expression. Nifedipine also significantly suppressed splenocyte proliferation in an in vitro mixed lymphocyte reaction and the proliferation of male antigen (H-Y)-primed male mice in vivo. Taken together these novel findings indicate that an L-type Ca²⁺ channel plays a significant role in the Ca²⁺ influx pathways mediating T lymphocyte activation and proliferation in vitro and in vivo.

In T lymphocytes, calcium (Ca²⁺) plays a fundamental role as a second messenger in regulating numerous cellular functions, including activation, proliferation, and death (1, 2). The events surrounding Ca²⁺ mobilization in T lymphocytes are tightly regulated through membrane receptors, signaling molecules, and ion channels. Intracellular Ca²⁺ release is initiated through the recognition of antigen/major histocompatibility complex by the T cell receptor (TCR)/CD3 complex during T lymphocyte activation (3). Following ligation of the TCR, non-receptor tyrosine kinases phosphorylate and activate phospholipase C-γ1, which cleaves phosphatidylinositol 4,5-bisphosphate from plasma membrane phospholipids to generate diacylglycerol and inositol 1,4,5-trisphosphate (IP₃) (4). Elevated levels of IP₃ in the cytosol lead to the release of Ca²⁺ from intracellular stores in the endoplasmic reticulum (ER) and a sustained Ca²⁺ influx from the extracellular space (5, 6). A sustained Ca²⁺ signal ranging from a concentration of ~200 nM to >1 μM for up to 48 h is necessary to activate nuclear factor of activated T cells (NFAT), a transcription factor that regulates the expression of various cytokine genes including interleukin-2 (II-2) (7).

Although the mechanisms of Ca²⁺ release from the intracellular stores within T lymphocytes are well characterized, the Ca²⁺ entry pathway from extracellular sources into T lymphocytes still remains elusive despite the fact that it contributes to the majority of elevated intracellular Ca²⁺ during T lymphocyte activation (8). Several models for Ca²⁺ channels in the plasma membrane of T lymphocytes have been proposed, including IP₃ receptor (IP₃R) Ca²⁺ channels, mammalian homologues of transient receptor potential (TRP) Ca²⁺ channels, and L-type voltage-dependent Ca²⁺ channels (VDCCs). Initially, investigators suggested that a plasma membrane IP₃R Ca²⁺ channel, similar to the IP₃R found in the ER, was responsible for Ca²⁺ influx in T lymphocytes (9). A recent study confirmed that T lymphocytes express three isoforms of the IP₃R Ca²⁺ channel as integral plasma membrane proteins (10). However, the IP₃R isoforms exhibit functional redundancy; defining the respective contributions of these channels to Ca²⁺ influx during T cell activation has therefore been difficult (11). Studies based on electrophysiology of T lymphocytes lead to a second model for Ca²⁺ entry across the plasma membrane through Ca²⁺-release-activated Ca²⁺ (CRAC) channels (12, 13). Although the molecular identity of the CRAC channel is still unclear, potential gene candidates include the TRP gene superfamily of ion channels (14–16). Currently, CaT1 appears to be the primary TRP gene candidate for the CRAC channel, but

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** To whom correspondence should be addressed: The Biomedical Research Centre, University of British Columbia, 2222 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada. Tel.: 604-822-6961; Fax: 604-822-6780; E-mail: wil@interchange.ubc.ca.

1 The abbreviations used are: TCR, T cell receptor; IP₃, inositol 1,4,5-trisphosphate; ER, endoplasmic reticulum; NFAT, nuclear factor of activated T cells; IL-2, interleukin-2; IP₃R, IP₃ receptor; TRP, transient receptor potential; VDCCs, voltage-dependent Ca²⁺ channels; CRAC channel, Ca²⁺-release-activated Ca²⁺ (CRAC) channel; IP₃RC; CRAC current; DHP, 1,4-dihydropyridine; PHA, phytohemagglutinin; ConA, concanavalin A; PBMCs, peripheral blood mononuclear cells; Tg, transgenic; mAb, monoclonal antibody; rIL-2, recombinant human IL-2; PBTs, peripheral blood T lymphocytes; MAP, mitogen-activated protein; Erk1/2, extracellular-regulated kinase 1/2; PPA, 12-O-tetradecanoylphorbol 13-acetate; IL-2R, IL-2 receptor; PI, propidium iodide; CFSE, 5- and 6-carboxyfluorescein diacetate, succinimidyl ester; MLR, mixed lymphocyte reaction; K⁺, potassium; NT, no treatment.
the CaT1 gene product does not seem to exhibit all of the electrophysiological properties associated with CRAC current (ICRAC) (15, 17).

There is also evidence to support the existence of voltage-dependent Ca2+ channels in the plasma membrane of T lymphocytes. VDCCs are heteromultimeric proteins whose conformations are sensitive to changes in the electrical potential across the plasma membrane (18). The basis of the VDCC model is that nonexcitable cells, such as T lymphocytes, may express a Ca2+-channel that shares common structural features with a VDCC of electrically excitable cells but is voltage-gated by changes in membrane potential. Initial support for the presence of voltage-dependent Ca2+-channels in T lymphocytes came when Denmore et al. (19, 20) identified an electrically responsive current in the plasma membrane of Jurkat T lymphocytes. This "voltage-operable" current had different electrophysiological properties than ICRAC, but was activated through the TCR/CD3 complex and Ca2+-store depletion (19, 20). RT-PCR analysis has also shown that transcripts of the pore-forming α1C- and α1S-subunits of L-type VDCCs are expressed in Jurkat T cells (21). In addition, Savignac et al. (22) demonstrated that murine T cell hybridomas express L-type Ca2+-channel mRNA and protein.

Several pharmacological studies have provided further evidence to support the expression of VDCCs in T lymphocytes. For instance, it has been reported that the synthetic 1,4-dihydropyridine (DHP) L-type Ca2+-channel antagonist, nifedipine, is a potent suppressor of T lymphocyte proliferation. Based upon an in vitro [3H]thymidine uptake assay, Birx et al. (23) demonstrated that 0.001–100 μM nifedipine prevented the proliferation of human T lymphocytes in response to the mitogens, phytohemagglutinin (PHA), and concanavalin A (ConA). In a similar study, human peripheral blood mononuclear cells (PBMCs) stimulated with PHA were unable to proliferate in the presence of 10–200 μM nifedipine; the addition of IL-2 restored the proliferative response in the nifedipine-treated cells (24). Furthermore, it has been demonstrated through in vitro proliferation assays that nifedipine has a dose-dependent inhibitory effect on T lymphocyte proliferation when added in combination with the immunosuppressive agent cyclosporin A (25, 26).

The aim of this work was to determine the contribution of Ca2+ influx through VDCCs during T lymphocyte activation and proliferation. We began this investigation by using a PCR assay to demonstrate for the first time that the pore-forming α1P-subunit L-type Ca2+-channel transcript is expressed in human T lymphocytes. After confirming the presence of a VDCC in T lymphocytes, we then determined that L-type Ca2+-channels play a critical role in TCR-induced activation. We show that both (+/-) Bay K 8644 (a DHP agonist that induces L-type Ca2+-channel opening) and nifedipine (a DHP antagonist that blocks L-type Ca2+-channels) can modulate early and late signaling events during T lymphocyte activation and proliferation. The results in this study collectively suggest the presence of a DHP-sensitive L-type VDCC in the plasma membrane of T lymphocytes.

**EXPERIMENTAL PROCEDURES**

**Mice—**C57Bl/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 C57Bl/6 mice (Charles River Laboratories, Wilmington, MA) were housed in the animal facilities at University of British Columbia and were used between 8 and 12 weeks 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.

**Cell Line and Culture Conditions—**The human T cell leukemia line Jurkat clone E6–1 was obtained from American Type Culture Collection (ATCC, Manassas, VA) maintained in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 20 mM HEPES, and 1 mM sodium pyruvate.

**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). PBMCs were separated by centrifugation at 900 × g for 30 min at 18–20 °C over a Ficoll-Paque PLUS (Amersham Biosciences) 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 monoclonal antibody (mAb), OKT3 (ATCC), and resuspended in RPMI 1640 containing 5% fetal calf serum (FCS) (Hyclone) and 2 mM glutamine (Hyclone). For activation, the human peripheral blood T lymphocytes (PBMCs) were analyzed using a FACSCalibur cytometer (BD Biosciences) with FITC-conjugated OKT3, FITC-conjugated mouse anti-IgG2a isotype control (Caltag, Burlingame, CA), Cy5- and Cy7-conjugated anti-CD4 mAb (BD Pharmingen), PC-conjugated anti-CD14 mAb (BD Pharmingen), FITC-conjugated anti-CD15 mAb (BD Pharmingen), and PE-conjugated anti-CD19 mAb (BD Pharmingen). Experiments with the activated human PBMCs were conducted with cells from day 8–14 in culture.

**Nested RT-PCR and DNA Sequencing—**First strand cDNAs were synthesized with an oligo(dT) primer using 1 μg of total RNA extracted from Weri-Rhi retinoblastoma, Jurkat T cells, PBMCs, CD4+ T cells, and CD8+ T cells with the RNeasy Kit (Qiagen, Mississauga, Ontario). MACS CD4 and CD8 microbeads (Miltenyi Biotec, Auburn, CA) were used to positively select and separate human CD4+ and CD8+ T cells from PBMCs, respectively. Marathon-ready human retina and human spleen cDNA were purchased from Clontech and FirstChoice PCR-Ready human liver cDNA was from Ambion (Austin, TX). RT-PCR fragments (~770 bp) spanning exons 28–35 of the L-type Ca2+-channel α1P-subunit gene, CACNA1F, were generated with sense primer (5'-GGACCATGGCCGCACTTCAAATACTCAGC-3') and antisense primer (5'-CGTGAAGACGACGCTTGCCGAC-3'). For nested amplification of the CACNA1F gene, PCR fragments (~180 bp) were generated with sense primer (5'-GAAAGGAGGTCAGTGCTGC-3') and antisense primer (5'-AATACTGTAAGGGCCTAGTGAGACC-3'). The housekeeping gene, rps15, which encodes a small ribosomal subunit protein, was amplified with sense primer (5'-TTCGGACAGTTCACTACC-3') and antisense primer (5'-CGGCCGCGCCATCTC-3') (27). 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 s, 60 °C for 30 s 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 PCR product were subcloned into Invitrogen's vector and the nucleotide sequence determined using standard m13R primer at the DNA Sequencing CORE Facility, University of Florida.

**Measurement of Intracellular Ca2+ Levels—**Intracellular Ca2+ levels were measured using the ratiometric Ca2+ indicator indo-1 aceto-methyl ester dye (Molecular Probes, Eugene, OR) according to manufacturer's recommendations. In brief, Jurkat T cells or human PBMCs (donors, n = 3) at 1 × 10⁶ cells/ml were loaded with 1 μM indo-1 for 1 h at 37 °C in MEM. For analysis, 100 μl of cell suspension (1 × 10⁶ cells) was added to each 1.9 ml of MEM containing 10 μM calcium chloride (CaCl2). Measurement of [Ca2+]i was performed using the fluorimeter, and the nucleotide sequence determined using standard m13R primer at the DNA Sequencing CORE Facility, University of Florida.

**Measurement of Intracellular Ca2+ Levels—**Intracellular Ca2+ levels were measured using the ratiometric Ca2+ indicator indo-1 aceto-methyl ester dye (Molecular Probes, Eugene, OR) according to manufacturer's recommendations. In brief, Jurkat T cells or human PBMCs (donors, n = 3) at 1 × 10⁶ cells/ml were loaded with 1 μM CaCl2. For analysis, 100 μl of cell suspension (1 × 10⁶ cells) was added to each 1.9 ml of MEM containing 10 μM calcium chloride (CaCl2). Measurement of [Ca2+]i was performed using the fluorimeter, and the nucleotide sequence determined using standard m13R primer at the DNA Sequencing CORE Facility, University of Florida.
Immunoblot Analysis of Phospho-p44/42 MAP Kinase—Jurkat T cells or human PBTs were washed, resuspended at 1 × 10^6 cells/ml in RPMI 1640 and incubated for 4 h at 37 °C. Cells were then preincubated with or without 2 mM EGTA for 15 min to chelate Ca^{2+}, followed by 10 min stimulation with either (+/-) Bay K 8644 or 2 μM ionomycin at 37 °C. Jurkat T cells were also preincubated with either Me_{6}SO, 100 μM or 200 μM nifedipine for 1 h, followed by 10 min stimulation with 100 μM (+/-) Bay K 8644 at 37 °C. Additionally, as a positive control for phospho-p44/42 MAP kinase activation, Jurkat T cells were stimulated with 10 μg/ml soluble OKT3 for 10 min at 37 °C. Following stimulation, cells were lysed in 200 μl of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM EDTA, 1 mM sodium sandovalate, 5 mM sodium fluoride, 1 mM sodium molybdate, 5 mM β-glycerolphosphate, in the presence of 10 μg/ml soybean trypsin inhibitor, pepstatin, and 40 μg/ml phenylmethylsulfonyl fluoride. 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, Beverly, MA). 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 extracelular regulated kinase1/2 (Erk1/2) (K-23) polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA) as a protein-loading control.

NFAT Luciferase Assay—1 × 10^6 Jurkat T cells were washed and reopted in Opti-MEM. Cells were incubated with either 20 μg of pHNFAT-TA-Luc or pTA-Luc (Clontech) for 5 min at 4 °C and transfected by electroporation using a BioRad Gene Pulser Electroporator set at 280 V, 975 μF, 40–48 h after transfection, cells at 1 × 10^6 cells/ml were incubated with nifedipine (1–200 μM) or Me_{6}SO 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 1 × 10^6 cells/100 l using the procedures outlined in the Bright-Glo Luciferase Assay System (Promega, Madison, WI). Luciferase activity was measured in a microplate luminometer.

IL-2 Assay and IL-2 Receptor Expression—Jurkat T cells or human PBTs at 1 × 10^6 cells/ml were incubated with Me_{6}SO or nifedipine (1–200 μM) for 24 h at 37 °C. Cells were then transferred to a 24-well plate immobilized with 10 μg/ml OKT3, 10 mM 12-O-tetradecanoylphor- bol 13-acetate (TPA) (Sigma) was added, and cells were incubated at 37 °C. After 24 h, supernatants were quantified for IL-2 concentration by a standard sandwich ELISA technique (R&D Systems, Minneapolis, MN).

To determine whether the Ca^{2+} ionophore, ionomycin, could reverse the inhibitory effect of nifedipine, Jurkat T cells or human PBTs at 1 × 10^6 cells/ml were incubated with either Me_{6}SO or 1–50 μM nifedipine for 1 h. Cells were then stimulated for 24 h with 10 μg/ml plate-bound OKT3, 10 mM TPA and, where appropriate, 2 μM ionomycin. The concentration of IL-2 in the supernatants was quantified by sandwich ELISA.

IL-2 receptor (IL-2R) expression was determined through flow cytometry by directly staining cells with human IL-2Rα mAb, clone 7G7/B6 (Upstate Biotechnology, Lake Placid, NY) and FITC-conjugated goat anti-mouse IgG Ab (Jackson Immunoresearch, West Grove, PA). Cell viability was assessed by staining dead cells with 2 μg/ml propidium iodide (Pi) (Sigma).

Mixed Lymphocyte Reaction—Splenocytes from C57Bl/6 (H-2b) mice were mixed with C57Bl/4 (H-2b) splenocytes, 1:1 ratio. The mixture of splenocytes was incubated for 10 min at 37 °C and then washed twice with RPMI 1640 supplemented with 1% FBS, 2 mM glutamine, 50 nM 2-mercaptoethanol, and 100 units/ml of each of penicillin and streptomycin. Proliferation was evaluated by using a flow cytometer-based bead assay as previously described (28).

In Vivo Proliferation Assay—Thymocytes from C57Bl/6 female mice bearing a Tg TCRαβ receptor specific for the male antigen were loaded with 5 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes) for 7 min at room temperature. 20–30 × 10^5 CFSE-loaded Tg thymocytes were i.v. injected into the tail vein of female or male C57Bl/6 recipients, followed by one intraperitoneal injection of either vehicle control or 15 mg/kg nifedipine into the C57Bl/6 males 20–24 h later. Nifedipine was prepared as a 1 mg/ml stock solution, dissolved in PBS containing 5% ethanol and 1% Tween-80. 40 h after the initial i.v. injection, spleens were harvested and single cell suspensions were prepared. For cell staining, spleenocytes were suspended in Dulbecco’s modified Eagle’s medium and labeled with PE-conjugated anti-CD45 mAb (BD Pharmingen) and biotin-conjugated anti-TCRαβ mAb, clone T3.70 (provided by Dr. Hung-sia Teh, UBC, Vancouver, Canada), which is specific for Tg TCRαβ. Cells were then stained with Cyochrome-conjugated-streptavidin, 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 percent total of gated viable, CFSE-, CD8+, and Tg TCRαβ+ cells in successive cell divisions using CellQuest software (BD Biosciences). 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.

Statistical Analysis—Statistical significance was determined by the ANOVA test, using a two-factorial design without replication. For all tests, p < 0.01 was considered to indicate statistical significance. All standard errors shown represent the S.D.

RESULTS

L-Type Ca^{2+} Channel Transcript Is Expressed in T Lymphocytes—Nifedipine has been shown to antagonize Ca^{2+} influx through L-type Ca^{2+} channels. Since previous studies reported that nifedipine interfered with T lymphocyte proliferation we sought to demonstrate the expression of an L-type Ca^{2+} channel in T lymphocytes. Using a nested RT-PCR-based assay a PCR product specific to the pore-forming α_{1D}-subunit of an L-type VDCC was amplified from T lymphocytes (Fig. 1A). Human retina and Weri-Rb1 retinoblastoma cDNAs were used as positive controls for the PCR assay since the α_{1D}-subunit gene, CACNA1F, was first cloned from human retina and is highly expressed in this tissue (29, 30). In the initial studies examining α_{1D}-subunit gene expression, the α_{1D}-subunit mRNA was not detected in lymphoblastoid tissue (29). The expression may have been overlooked by the lack of a nested RT-PCR-based assay. Using nucleotide sequencing, we were able to confirm that the ~180 bp amplified PCR product from Jurkat T cells, human spleen, PBTs, CD4+, and CD8+ T lymphocytes shares 100% nucleotide identity to the L-type Ca^{2+} channel α_{1D}-subunit gene expressed in human retina and Weri-Rb1 retinoblastoma (Fig. 1B). The α_{1D}-subunit is not expressed ubiquitously in human cells since we were not able to detect α_{1D}-subunit expression in normal human liver.

Induction of Ca^{2+} Influx in Jurkat T Cell Leukemia Line and Human PBTs by L-Type Ca^{2+} Channel Agonist, (+/-) Bay K 8644—To demonstrate that L-type Ca^{2+} channels contribute to Ca^{2+} entry, we tested the effect of the DHP derivative, (+/-) Bay K 8644, on Ca^{2+} influx in human T lymphocytes. 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 (31). However, the report did not specify which enantiomer of Bay K 8644 was used. Since levo- and dextro-rotatory enantiomers of Bay K 8644 can 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 (32). Additionally, we wanted to directly compare the effects of (+/-) Bay K 8644 treatment on Ca^{2+} influx in Jurkat T cells to the untransformed PBTs 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 (485 nm) was observed indicating an increase in intracellular Ca^{2+} (Fig. 2). In both Jurkat T cells and PBTs, 10 μM (+/-) Bay K 8644 induced a small, sustained rise in intracellular Ca^{2+}. However, treatment of Jurkat T cells and PBTs with higher concentrations of (+/-) Bay K 8644 induced different responses in Ca^{2+} influx. In Jurkat T cells, 50 and 100 μM (+/-) Bay K 8644 induced a sustained increase in cytosolic Ca^{2+} (Fig. 2A). Interestingly, treatment of human PBTs with either 50 or 100
A transient Ca\(^{2+}\) influx that rapidly declined to below baseline after the 10 min time period (Fig. 2B). In the absence of extracellular Ca\(^{2+}\) in the medium, 100 \(\mu M\) Bay 8644 did not cause a rise in intracellular Ca\(^{2+}\) in either Jurkat T cells or human PB T cells, indicating that Bay 8644 treatment with extracellular Ca\(^{2+}\) specifically allowed Ca\(^{2+}\) entrance into these cells. Furthermore, the solvent alone did not induce significant Ca\(^{2+}\) entry into T lymphocytes. We also examined the effects of the well-characterized lipophilic Ca\(^{2+}\) ionophore, ionomycin, to ensure efficient loading of the indo-1 dye into T lymphocytes. The addition of ionomycin induced a rapid and sustained Ca\(^{2+}\) influx in both Jurkat T cells and human PB T cells (Fig. 2, C and D).

L-type Ca\(^{2+}\) Channel Antagonist, Nifedipine Inhibits Anti-CD3-induced Ca\(^{2+}\) Influx in Jurkat T Cell Leukemia Line and Human PB T Cells—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. While some of the previous reports on nifedipine have examined the nonspecific stimulation of T lymphocytes using the mitogenic lectins PHA and ConA, we focused on the specific activation of T lymphocytes through the TCR/CD3 complex using the anti-CD3 mAb, OKT3. Pretreatment of indo-1-loaded Jurkat T cells and human PB T cells 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 (485 nm) following anti-CD3 stimulation (Fig. 3, 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 PB T cells whereas the Me\(_{2}\)SO solvent control had no effect.

Although nifedipine clearly inhibited Ca\(^{2+}\) influx in the cells tested, we wanted 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 lymphocyte activation. To address this question, we compared nifedipine-inhibited Ca\(^{2+}\) influx with extracellular Ca\(^{2+}\) to Ca\(^{2+}\) influx induced in the absence of extracellular Ca\(^{2+}\). In both anti-CD3 stimulated Jurkat T cells and PB T cells, 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 (Fig. 3, 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 PB T cells, 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 μM nifedipine when extracellular Ca\textsuperscript{2+} was present. We also determined whether nifedipine blocked Ca\textsuperscript{2+} efflux from intracellular Ca\textsuperscript{2+} stores. To investigate this, Jurkat T cells and human PBTs were treated with nifedipine and anti-CD3 stimulated in the absence of extracellular Ca\textsuperscript{2+} (Fig. 3, C and D). Nifedipine treatment did not significantly effect the transient rise in cytosolic Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores in Jurkat T cells, whereas in human PBTs 50–200 μM nifedipine reduced the efflux of Ca\textsuperscript{2+} from intracellular stores in these cells.

It should be noted that (+/-) Bay K 8644 and nifedipine are typically used at 1–300 μM on both electrically excitable and nonexcitable cell types. The concentration of DHPs used in these experiments therefore replicated the concentration range of Ca\textsuperscript{2+} channel modulators applied in other studies (32, 33).

**Fig. 2.** (+/-) Bay K 8644 induces a Ca\textsuperscript{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\textsuperscript{2+} was initially measured, after which 10–100 μM (+/-) Bay K 8644 was added to the sample, and the analysis was resumed. Red line, Me\_SO solvent (Control); yellow, 10 μM (+/-) Bay K 8644; green, 50 μM (+/-) Bay K 8644; gray, 100 μM (+/-) Bay K 8644; black, 100 μM (+/-) Bay K 8644 with no extracellular Ca\textsuperscript{2+} in medium. Indo-1 loading was assessed by stimulating Ca\textsuperscript{2+} influx with 2 μM ionomycin in Jurkat T cells (C) and human PBTs (D). The human PBTs contained 14% CD3\textsuperscript{+} CD4\textsuperscript{+} CD8\textsuperscript{+}, 79% CD3\textsuperscript{+} CD4\textsuperscript{+} CD8\textsuperscript{+}, 6.5% CD3\textsuperscript{+} CD4\textsuperscript{+} CD8\textsuperscript{-} and 0.5% CD3\textsuperscript{+} CD4\textsuperscript{-} CD8\textsuperscript{-} cells. The results are representative of three independent experiments.

In Jurkat T cells, 50 and 100 μM (+/-) Bay K 8644 stimulation resulted in a rapid and transient phosphorylation of both Erk1 and 2, which is similar to the level of Erk1/2 activated with 2 μM ionomycin (Fig. 4A). 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 with the ionomycin control (Fig. 4B). 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 Me\_SO alone (Control) did not activate Erk1/2. It should be noted that we also examined the effects of 100 μM (+/-) Bay K 8644 on naïve human PBTs that were immediately isolated from PBMCs and not previously cultured with 10 μg/ml plate-bound OKT3 and rIL-2. Unstimulated naïve human PBTs had the same level of Erk2 activation following (+/-) Bay K 8644 treatment as human PBTs grown for
absence of extracellular Ca$^{2+}$ (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. 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, Me$_2$SO solvent (Control); blue, 1 μM Nifedipine; yellow, 10 μM Nifedipine; green, 50 μM Nifedipine; gray, 100 μM Nifedipine; purple, 200 μM Nifedipine. The human PBTs contained 18% CD3$^+$ CD4$^+$, 45% CD3$^+$ CD8$^+$, 32% CD3$^+$ CD4$^+$ CD8$^+$, and 5% CD3$^+$ CD4$^+$ CD8$^+$ cells. The results depicted are representative of three independent experiments.

8 days in culture with OKT3 and rhIL-2 (data not shown).

Since there is very little information on what aspects of the T lymphocyte activation process are affected by inhibiting Ca$^{2+}$ influx with nifedipine treatment, we examined whether Erk1/2 activation induced by (+/-) Bay K 8644 could be specifically blocked by nifedipine. Pretreatment of Jurkat T cells with either 100 μM or 200 μM nifedipine, but not the Me$_2$SO solvent, significantly inhibited Erk1/2 activation induced by 100 μM (+/-) Bay K 8644 (Fig. 4C). As a positive control for Erk1/2 phosphorylation, we stimulated Jurkat T cells 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.

**Nifedipine Blocks NFAT-Transcriptional Activity in Anti-CD3-stimulated Jurkat T Cell Leukemia Line**—The activation of the transcription factor NFAT is dependent upon increased intracellular Ca$^{2+}$ (36). We investigated whether the block in Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels by nifedipine alters the transcriptional activity of NFAT 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. The maximum induction of 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 (Fig. 5). Low concentrations of nifedipine that weakly blocked Ca$^{2+}$ influx, such as 50 μM nifedipine, significantly reduced the transcriptional activity of NFAT. Higher doses of nifedipine, such as 200 μM, almost completely abolished NFAT activity. The Me$_2$SO 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.

**IL-2 Production and IL-2R Expression is Inhibited by Nifedipine in Jurkat T Cell Leukemia Line and Human PBTs**—Since IL-2 secretion is a definitive indicator of T cell activation, we assessed whether blocking L-type Ca$^{2+}$ channels with nifedipine can inhibit IL-2 production in both anti-CD3 stimulated Jurkat T cells (Fig. 6A) and human PBTs (Fig. 6B). In agreement with the results showing the effect of nifedipine on Ca$^{2+}$ influx (Fig. 3) and NFAT activation (Fig. 5), nifedipine significantly inhibited IL-2 secretion in both cell types and abolished IL-2 secretion completely at 200 μM nifedipine. The Me$_2$SO 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 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 with the viability of cells stimulated with OKT3/TPA and treated with DMSO (Fig. 6C and D). We 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 (Fig. 6E) and human PBTs (Fig. 6F) 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 with 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 (Fig. 7A), 200 μM nifedipine caused a 55% decrease in the log mean fluorescence intensity of IL-2R expression, whereas in human PBTs pre-treated with 200 μM nifedipine (Fig. 7B) a 70% decrease was observed compared with IL-2R expression of T cells stimulated with OKT3/TPA and treated with DMSO.

Nifedipine Suppresses Splenocyte Proliferation—To investigate whether nifedipine could block the proliferation of T lymphocytes, we assayed the effects of nifedipine on the proliferative response induced by a mixed lymphocyte reaction (MLR). Nifedipine significantly suppressed the proliferation of splenocytes in a dose-dependent fashion (Fig. 8A). Low doses of nifedipine, including 1 and 10 μM, weakly inhibited splenocyte proliferation, whereas 100 and 200 μM nifedipine completely abrogated proliferation. There was no significant inhibition of splenocyte proliferation by treatment with DMSO solvent alone.

Since nifedipine clearly inhibited MLR induced splenocyte proliferation in vitro, we evaluated whether nifedipine could block the proliferation of an antigen specific T cell response in vivo. To address this question we examined the proliferative response of female H-Y-specific TCR-Tg CD8^{+} T cells transferred into male C57Bl/6 mice treated with either one dose of 15 mg/kg nifedipine or vehicle control. In male recipients, H-Y-specific CD8^{+} T cells accumulating in the spleen proliferated in response to the male antigen. Treating male mice with one dose of 15 mg/kg nifedipine inhibited, but did not completely abro-
we have begun this investigation by demonstrating through nifedipine protein is expressed in T lymphocytes. As the presence of the same extent as the vehicle control, suggesting that nifedipine treatment resulted in an increased number of H-Y-specific gate, H-Y specific CD8+ T cell proliferation (Fig. 8B). Nifedipine treatment resulted in an increased number of H-Y-specific CD8+ T cells undergoing only 1–2 cell divisions and significantly fewer cells transiting to 3 divisions compared with the vehicle control. We addressed the specificity of nifedipine in this assay by determining whether administration of 15 mg/kg nifedipine 1 h (half-life of nifedipine 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 with vehicle control (37). In this experiment, H-Y-specific CD8+ T cells proliferated to the same extent as the vehicle control, suggesting that nifedipine is specifically blocking L-type Ca2+ channels and not inducing a nonspecific hormonal change in the mice, which could lead to decreased T cell proliferation (data not shown). We also assayed the proliferative response of H-Y-specific CD8+ T cells in female mice as a control for no proliferation. As expected, the H-Y-specific CD8+ T cells homed to the spleen but did not proliferate in the female mice due to the absence of the H-Y male antigen (Fig. 8B, Control, open bar).

**DISCUSSION**

The first step in the evaluation of the VDCC model was to provide molecular evidence that a functional L-type Ca2+ channel protein is expressed in T lymphocytes. As the presence of mRNA frequently correlates with the expression of a protein, we have begun this investigation by demonstrating through nested RT-PCR that the L-type α1d subunit gene, CACNA1F, is expressed in human T lymphocytes. Furthermore, the sequence of the PCR product unequivocally proves that the CACNA1F gene is expressed in T lymphocytes, which is a novel and exciting finding presently unreported in the literature.

In order to more directly assess the role of L-type Ca2+ channels in T lymphocytes, we noted that previous studies have used synthetic DHP derivatives to study the function of these channels in the plasma membranes of numerous cell types (32, 33, 38). In the present study, we used the DHP derivatives, (+/−) Bay K 8644 and nifedipine, to further investigate the presence of L-type Ca2+ channels in T lymphocytes and to assess the contribution of Ca2+ influx by L-type Ca2+ channels during the T lymphocyte activation process. Through an intracellular Ca2+ assay, we demonstrated that (+/−) Bay K 8644 exerts an agonistic action on the Ca2+ channels of Jurkat T cells and human PBTs. However, treatment with (+/−) Bay K 8644 did not induce maximal Ca2+ influx in either cell type since anti-CD3 stimulation of PBTs and Jurkat T cells resulted in a 2–4-fold larger increase in intracellular Ca2+, respectively. We also showed that nifedipine only partially blocks Ca2+ influx, and does not significantly effect Ca2+ release from intracellular Ca2+ stores in Jurkat T cells following stimulation through the TCR/CD3 complex. Therefore we conclude that nifedipine is blocking L-type Ca2+ channels found in the plasma membrane, with minor inhibitory effects at higher concentrations on Ca2+ release from intracellular stores.

Although the specificity of action of DHPs has been confirmed by determining the DHP binding sites on the channel-forming α1d-subunit of L-type VDCCs (39, 40), there are studies demonstrating the nonspecific inhibitory effects of high micromolar concentrations of DHPs on voltage-dependent potassium (Kv) channels and Ca2+-activated potassium (K+)-channels (41–43). Even though these observations could be of concern in the present study, Fagni et al. (42) reported that micromolar concentrations of both nifedipine and the stereospecific enantiomers of Bay K 8644 exhibited an inhibitory effect on K+ current through Kv and Ca2+-activated K+ channels. This is in contradiction to our results, since we show that (+/−) Bay K 8644 has an agonistic effect while nifedipine antagonizes Ca2+ influx in T lymphocytes. In addition (+/−) Bay K 8644 does not activate K+ channel currents (42). Therefore, we conclude that the overall observed effect of the DHPs in this study was due to modulation of Ca2+ influx through L-type Ca2+ channels. Minor inhibitory effects of DHPs on K+ 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 Ca2+ influx is still observed (41).

After confirming that (+/−) Bay K 8644 and nifedipine can mediate Ca2+ influx in T lymphocytes, we investigated whether Ca2+ influx through an L-type Ca2+ channel can modulate early Ca2+-dependent signaling events, such as MAP kinase activity. It has been postulated that Ca2+ 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 Ca2+-dependent Erk1/2 enzymatic activation (44). We report here that (+/−) Bay K 8644 could induce phosphorylation and activation of both Erk1 and Erk2 in Jurkat T cells, weak activation of Erk2 in human PBTs, and that the activation of Erk1/2 by (+/−) Bay K 8644 could be blocked by pretreatment with nifedipine. These results support the hypothesis that Ca2+ influx through an L-type Ca2+ 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 Ca2+ to induce Erk activity compared with transformed T lymphocytes. We observed that the Ca2+ ionophore, ionomycin, induces a 2.5-fold greater increase in intracellular Ca2+ in human PBTs compared with Jurkat T cells, which corresponds with a more robust phosphorylation of Erk1/2 by ionomycin in human PBTs. In addition, Ca2+ entry induced by (+/−) Bay K 8644 in human PBTs was only transient compared with the sustained Ca2+ influx in Jurkat T cells. Therefore the difference in the amount of Ca2+ 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\textsuperscript{2+}/H1\textsuperscript{1001} channel can mediate more downstream Ca\textsuperscript{2+}-dependent signaling events during T lymphocyte activation, we studied the effects of nifedipine on the transcriptional activity of NFAT, IL-2 secretion and IL-2R.

**Fig. 6. Nifedipine prevents IL-2 secretion in 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. IL-2 secreted in the supernatant was measured by standard sandwich ELISA techniques. The control is Jurkat T cells or human PBTs treated with the Me\textsubscript{2}SO (DMSO) solvent alone. The human PBTs contained 19% CD3\textsuperscript{1}CD4\textsuperscript{2}CD8\textsuperscript{2}, 72% CD3\textsuperscript{1}CD4\textsuperscript{2}CD8\textsuperscript{1}, 8.0% CD3\textsuperscript{1}CD4\textsuperscript{2}CD8\textsuperscript{1}, and 1.0% CD3\textsuperscript{1}CD4\textsuperscript{2}CD8\textsuperscript{1} cells. p < 0.01, relative to the Me\textsubscript{2}SO control. Viability of Jurkat T cells (C) and human PBTs (D) was assessed by staining with PI. p < 0.01, relative to the Me\textsubscript{2}SO control. To demonstrate whether additional Ca\textsuperscript{2+} 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\textsuperscript{1}CD4\textsuperscript{1}CD8\textsuperscript{2}, 86.5% CD3\textsuperscript{1}CD4\textsuperscript{1}CD8\textsuperscript{1}, 1.0% CD3\textsuperscript{1}CD4\textsuperscript{1}CD8\textsuperscript{1}, and 1.0% CD3\textsuperscript{1}CD4\textsuperscript{1}CD8\textsuperscript{1} 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 S.D. of assays from triplicate wells.

Jurkat T cells, and only weakly inducing activation of Erk activity in human PBTs.

To examine whether an L-type Ca\textsuperscript{2+} channel can mediate
expression. We demonstrated that inhibiting Ca\textsuperscript{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, we then examined the effects of nifedipine on IL-2 production and IL-2R expression (36). In both Jurkat T cells and human PBTs, IL-2 production was blocked in the presence of nifedipine. We confirmed that the overall inhibition in IL-2 secretion mediated by nifedipine was due to a block in Ca\textsuperscript{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 \mu M nifedipine was not due to drug cytotoxicity (23). We 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\textsuperscript{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 (45). Thus Ca\textsuperscript{2+} entry through L-type Ca\textsuperscript{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\textsuperscript{2+} channel mediates Ca\textsuperscript{2+} influx during T lymphocyte activation, we also investigated whether an L-type Ca\textsuperscript{2+} channel would regulate the proliferation of T lymphocytes. To this end, we assayed the proliferation of mouse splenocytes through an in vitro MLR and found that nifedipine markedly inhibited mouse splenocyte proliferation in a dose-dependent manner. Although the in vitro anti-proliferative effects of nifedipine have been documented, there are no previous studies describing nifedipine-mediated inhibition of T cell proliferation in vivo. We are the first to report that one dose of 15 mg/kg nifedipine treatment specifically slows down, but does not completely abolish the proliferation of H-Y-specific TCR-Tg CD8\textsuperscript{+} T lymphocytes in mice. These observations support our hypothesis that Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels is required for sustained Ca\textsuperscript{2+} influx during T lymphocyte proliferation in vitro and in vivo.
results depicted are representative of three independent experiments. The mean and S.D. of assays from four mice. *,

200 splenocytes from C57Bl/6 mice were incubated with 1–H9262, and 3 divisions (hatched bar). Each bar represents the proliferation of CFSE

4). Splenocytes were harvested 40 h after the initial i.v. injection and n

1/H11005 one intraperitoneal dose of vehicle (H11001). Each bar rep -

resents the mean and S.D. of assays from triplicate wells. /H9251/H9252 male mice with Tg TCR

B

FIG. 8. Nifedipine suppresses splenocyte proliferation. A, splenocytes from C57Bl/6 mice were incubated with 1–200 μM nifedipine in triplicate and then stimulated with irradiated Balb/c splenocytes. 5–6 days later, each sample was assayed for proliferation as reflected as lymphocyte number determined by flow cytometry. The control is splenocytes treated with Me2SO (DMSO) solvent alone. Each bar rep

resents the mean and S.D. of assays from triplicate wells. p < 0.01, relative to the Control. B, CFSE-loaded thymocytes from C57Bl/6 fe-

male 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 intraperitoneal 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 TCRαβ− splenocytes was quantified by flow cytometry. Bars represent percent of total cells exhibiting a discrete CFSE (FL1) intensity reflecting an equal number of cell divisions: no divisions (open bar), 1 division (solid bar), 2 divisions (hatched bar), and 3 divisions (dotted bar). Each bar represents the mean and S.D. of assays from four mice. *, p < 0.01, as comparing cell divisions between vehicle control and nifedipine treated male mice. The results depicted are representative of three independent experiments.

Taken together, the results in this study show that (+/−) Bay K 8644 and nifedipine partially modulate T lymphocyte activation and proliferation. Although we have provided evidence for the presence of a DHP sensitive L-type Ca2+ channel in the plasma membrane of T lymphocytes, additional Ca2+ channels may also contribute to the Ca2+ influx pathway. This conclusion is in agreement with recent studies, describing the involvement of TRP ion channels in regulating Ca2+ influx in T lymphocytes. Cui et al. (15) showed that the TRP-vanilloid receptor family member of ion channels, CaT1, is involved in generating LCRAC in Jurkat T cells, which is partially regulated through intracellular Ca2+ store depletion. Although CaT1 plays a significant role in mediating Ca2+ entry, overexpression of a dominant negative pore-region mutant of CaT1 did not completely abolish Ca2+ influx in Jurkat T cells, leading to the possibility that other channels, such as L-type Ca2+ channels are also involved in Ca2+ entry (15). TRP channels that are not modulated by intracellular Ca2+ store depletion have also been found in human T lymphocytes. For instance, Sano et al. (16) reported that the LTRPC2 protein is abundantly expressed in human peripheral blood and Jurkat T cells and mediates Ca2+ influx in response to elevated levels of pyrimidine nucleotides, adenosine 5′-diphosphoribose, and NAD. TRP6 mRNA and protein is also expressed in Jurkat T cells and P815s and Ca2+ influx through this channel is activated by diacylglycerol (14).

In conjunction with the results from this study and the recent discovery of TRP protein expression in T lymphocytes, it is highly probable Ca2+ entrance into T lymphocytes is mediated through multiple Ca2+ channels, including both TRP and L-type Ca2+ channels. Given that the amplitude and duration of Ca2+ signals in T lymphocytes are very diverse, a number of different channels may be necessary to coordinate the different Ca2+ responses required for T lymphocyte activation, proliferation and death.

Our study also raises the question of whether administration of DHPs, such as nifedipine, for treatment of cardiovascular disorders has deleterious side effects on circulating T lymphocytes (46). Currently nifedipine is widely prescribed by clinicians in the treatment of cardiovascular disorders, including hypertension and ischemic stroke (38, 47). In patients with cardiovascular disease, nifedipine blocks the action of L-type VDCCs, which are responsible for initiating the contraction of cardiac and smooth muscles (38). Interestingly, a single study has reported that lymphocytes in healthy humans treated with one 10 mg oral dose of nifedipine have decreased blastogenesis and IL-2 production (48). We have shown that one 15 mg/kg dose of nifedipine can suppress T lymphocyte proliferation in mice. Even though the doses of nifedipine in the present study are higher than the serum concentration of patients administered DHPs (49), long term treatment with nifedipine may act as an immunosuppressant, affecting the immune competence of patients with cardiovascular disorders. Our present findings should encourage further research into determining whether cardiac patients receiving Ca2+ channel blockers are immunosuppressed.

In summary, we have shown both molecular and extensive pharmacological evidence for the presence of an L-type Ca2+ channel in T lymphocytes. Elucidation of the role of the L-type Ca2+ channel α1p-subunit will provide the basis for a better understanding of the mechanisms controlling Ca2+ influx in T lymphocytes and may lead to the development of novel therapeu-

tic agents to control T lymphocyte activation and inactiva-

tion states. The results from the present study clearly support the hypothesis that an L-type Ca2+ channel is present in the plasma membrane of T lymphocytes and that this channel contributes to Ca2+ influx during T lymphocyte activation and proliferation.

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