Mechanisms of Signal Transduction:

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PHARMACOLOGY AND MOLECULAR CHARACTERIZATION OF THE MAJOR α PORE-FORMING AND AUXILIARY β-SUBUNITS*

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In T lymphocytes, engagement of the antigen receptor leads to a biphasic Ca^{2+} flux consisting of a mobilization of Ca^{2+} from intracellular stores followed by a lower but sustained elevation that is dependent on extracellular Ca^{2+}. The prolonged Ca^{2+} flux is required for activation of transcription factors and for subsequent activation of the T cell. Ca^{2+} influx requires as yet unidentified Ca^{2+} channels, which potentially play a role in T cell activation. Here we present evidence that human T cells express a non-voltage-gated Ca^{2+} channel related to L-type voltage-gated Ca^{2+} channels. Drugs that block classical L-type channels inhibited the initial phase of the antigen receptor-induced Ca^{2+} flux and could also inhibit the sustained phase of the Ca^{2+} signal suggesting a role for the L-type Ca^{2+} channel in antigen receptor signaling. T cells expressed transcripts for the $\alpha_1$1.2 and $\alpha_1$1.3 pore-forming subunits of L-type voltage-gated Ca^{2+} channels and transcripts for all four known β-subunits including several potential new splice variants. Jurkat T leukemia cells expressed a small amount of full-length $\alpha_1$1.2 protein but the dominant form was a truncated protein identical in size to a truncated $\alpha_1$1.2 protein known to be expressed in B lymphocytes. They further expressed a truncated form of the $\alpha_1$1.3 subunit and auxiliary β1- and β3-subunit proteins. Our data strongly suggest that functional but non-voltage-gated L-type Ca^{2+} channels are expressed at the plasma membrane in T cells and play a role in the antigen receptor-mediated Ca^{2+} flux in these cells.

In T lymphocytes the engagement of the T cell antigen receptor (TCR)[1] by antigen presented in the context of the major histocompatibility complex (MHC) induces a Ca^{2+} flux. An increase in cytosolic Ca^{2+} is a critical second messenger controlling T cell activation and proliferation through gene transcription. Engagement of the TCR induces the release of Ca^{2+} from intracellular stores followed by Ca^{2+} entry across the plasma membrane. The initial phase of calcium release is mediated through inositol (1,4,5)-P_3 and in non-excitable cells, store depletion activates Ca^{2+} influx across the plasma membrane. Prolonged Ca^{2+} elevation is required for a sustained translocation of the transcription factor nuclear factor of activated T cells (NFAT) into the nucleus. This activation of NFAT is crucial for production of interleukin-2 and T cell activation (1–3).

The sustained phase of the TCR-mediated Ca^{2+} signal requires the presence of extracellular Ca^{2+}, the production of cyclic ADP-ribose (4), and the expression of the type 3 ryanodine receptor (5). Ca^{2+} influx depends on the expression of a store-operated Ca^{2+} channel (SOC), the nature of which is the subject of much research. While SOCs have been defined electrophysiologically, their molecular identity remains unclear. The best characterized SOC current is the Ca^{2+} release-activated Ca^{2+} current, I_{CRAC}. Zweifach and Lewis (6) showed that TCR cross-linking activates a channel with properties identical to I_{CRAC}. In patients with a primary immunodeficiency there is an absence of I_{CRAC} and no TCR-activated Ca^{2+} influx (1). These studies demonstrate that the I_{CRAC} channel is activated following TCR cross-linking.

The molecular identity of the I_{CRAC} channel is unknown, but a small number of candidates have emerged. Mammalian homologues of the *Drosophila* Trp proteins have been reported to be expressed in Jurkat T lymphocytes (7, 8). The TrpV family member TrpV6 (CaT1) is the strongest contender for the I_{CRAC} channel (9, 10) although this has been disputed (11). It is possible that no single Trp family protein alone forms the I_{CRAC} channel, rather that there is a heteromultimeric channel formed from an assemblage of various Trp proteins. In support of this Cui *et al.* (10) found that Jurkat T lymphocytes express both TrpV1 and TrpV5 (ECaC1) in addition to TrpV6 and suggested that TrpV5 contributed to the I_{CRAC} current. Furthermore, Philipp *et al.* (12) isolated a series of random mutants of Jurkat T cells with defects in TCR-dependent Ca^{2+} entry. These all had mutations in TrpC3, and normal TCR-mediated Ca^{2+} entry could be restored by overexpression of normal TrpC3.

While there is growing evidence for the identity of the store-operated Ca^{2+} channel in T lymphocytes, it is also clear that SOCs are not the sole Ca^{2+} channel present in lymphocytes. There is some support for inositol trisphosphate receptors being expressed at the surface of T cells where they may function as Ca^{2+} channels (13, 14). A non-selective channel defined by the current it passes, the Ca^{2+} release-activated Ca^{2+} current (I_{CRANC}), has been described in human lymphocytes (15, 16), and there are data to indicate the expression of channels related to voltage-gated Ca^{2+} channels (reviewed in Ref. 17).

Voltage-gated Ca^{2+} channels are a family composed of 10

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[1] The abbreviations used are: TCR, T cell antigen receptor; NFAT, nuclear factor of activated T cells; SOC, store-operated Ca^{2+} channel; RT-PCR, reverse transcriptase-PCR; FACS, fluorescence-activated cell sorter; CRAC, Ca^{2+} release-activated Ca^{2+} current.

known types. They are classified according to the nature of the current they conduct following depolarization. The channels are composed of a pore-forming α_1-subunit and accessory β-, $\alpha_2\delta$-, and γ-subunits. The nature of the α_1-subunit defines the type of Ca^{2+} channel (18, 19). A growing body of evidence indicates that lymphocytes express a non-voltage-gated Ca^{2+} channel related to the L-type of voltage-gated Ca^{2+} channels. The L-type channels are defined by their expression of 1 of 4 types of α_1-subunit: $\alpha_1$1.1–$\alpha_1$1.4 (20). Initial evidence for the presence of L-type Ca^{2+} channels in lymphocytes relied on pharmacological studies using antagonists of these channels. A number of studies showed effects of these antagonists on B and T cell activation (21–24). However, the drugs were used at higher concentrations than those used to block authentic voltage-gated Ca^{2+} channels.

More substantial evidence for the expression of voltage-gated Ca^{2+} channel-related channels has been provided by the detection of Ca^{2+} channel transcripts and protein in various cell types. Akha *et al.* (25) showed that rat B lymphocytes expressed a channel related to the $Ca_V1.3$ (α_{1D}) L-type channel (25). We have demonstrated that human B lymphocytes express a truncated $\alpha_1$1.2 (α_{1C}) -subunit together with a regulatory β_1-subunit (26). Similar channels have been detected in T lymphocytes. Savignac *et al.* (27) showed surface staining of mouse T cells with an anti-$\alpha_1$1.3 antibody and demonstrated a transcript for $\alpha_1$1.3 in those cells. Brereton *et al.* (28) used RT-PCR to demonstrate the presence of transcripts for $\alpha_1$1.1 and $\alpha_1$1.2 in Jurkat T cells. Most recently, Kotturi *et al.* (29) showed the presence of an $\alpha_1$1.4 transcript in Jurkat and provided some pharmacological data to indicate that a voltage-gated Ca^{2+} channel-related channel may have a role in T lymphocyte activation (29). These studies have not directly addressed the expression of a voltage-gated Ca^{2+} channel-related protein in human T lymphocytes.

The body of studies showing the pharmacological effects of antagonists coupled with detection of multiple transcripts is confused by the absence of studies detecting protein expression in human T cells. Here we provide the first direct evidence for the expression of non-voltage-gated Ca^{2+} channel proteins in human T lymphocytes, together with accessory regulatory subunits.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—The T cell lines Jurkat, Molt-4, H9, Hut78, and CCRF-CEM were cultured as described in Ref. 30. The Kelly (human neuroblastoma) cell line was cultured as described in Ref. 26. Peripheral blood T cells were obtained from buffy coat preparations supplied by the National Blood Transfusion Service and were isolated as described in Ref. 31. Isolated peripheral blood T cells were used directly after isolation (quiescent cells) or were activated with 2 ng/ml phorbol 12-myristate 13-acetate and 1 μg/ml ionomycin for 72 h.

Intracellular Ca^{2+} Measurements—Intracellular Ca^{2+} concentration was measured using fura2-AM. Jurkat T cells (5×10^7) were loaded with 1 μM fura-2AM (Calbiochem) in culture medium plus 1 mM probenecid (Sigma) for 45 min at 25 °C. The loaded cells were washed twice in HBSS containing 1% (w/v) bovine serum albumin, 10 mM HEPES (pH 7.2), and 1 mM probenecid, resuspended at 4×10^6 cells/ml in this buffer, and stored on ice until use. Following a 5-min preincubation at 37 °C, intracellular Ca^{2+} measurements were performed using a Hitachi F2500 spectrophotometer, described in Ref. 26. For experiments where the effects of drugs on the sustained phase of a Ca^{2+} response were determined, the Ca^{2+} concentration was averaged over 100 s from 150 to 250 s after the initiation of the experiment.

RNA Isolation and RT-PCR Analysis—Total RNA was extracted using RNAwiz (Ambion) and was treated with DNase I before use. Primers 27U24 (5′-CTC GGA CTC TGG GGC ACA CTT CTT-3′) and 351L26 (5′-ACT CCC GCA TCT CCA TCA CCT TCT TC-3′) were used to amplify $\alpha_1$1.2 transcripts. Primers α1D4080 (5′-TCA GAG TTC CCA GGT GTA GCA G-3′) and α1D3858 (5′-ACG AGC AGT CCA AGA TGT TCA AT-3′) were used to amplify $\alpha_1$1.3 transcripts. The primers produce transcripts of 350 bp ($\alpha_1$1.2) and 243 bp ($\alpha_1$1.3). The PCR conditions for $\alpha_1$1.2 were 95 °C for 10 min followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, and final extension at 72 °C for 10 min using AmpliTaqGold polymerase (ABI) and 1 mM Mg^{2+}. PCR conditions for $\alpha_1$1.3 were 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min, and final extension at 68 °C for 15 min, using Expand DNA polymerase plus buffer 3 (Roche Applied Science).

Primers for human β-subunits were designed using GenBank™ accession NM_000723 (CACNB1), NM_000724 (CACNB2), XM_028766 (CACNB3), and NM_000726 (CACNB4). The β1-subunit primers were sense (5′-GGT GAA GGA GGG CTG TGA GG-3′) and antisense (5′-GAT GAT GTG TTT GCT GGG GTT GT-3′); β2-subunit primers were sense (5′-CCC AGT AAG CAC GCA ATA ATA GA-3′) and antisense (5′-GCT AAT GTA CGG CTA AGG AGA GG-3′); β3-subunit primers were sense (5′-GGG TTC AGC CGA CTC CTA CAC CA-3′) and antisense (5′-CGC CTG CTT TTG CTT CTG CTT G-3′); and β4-subunit primers were sense (5′-GGG CTC CAC GGC ACT CTC A-3′) and antisense (5′-ATC CTC GGT TCC TAT GGG GTT TG-3′). Cycling conditions were 95 °C for 10 min followed by 35 cycles of 95 °C for 1 min, 55 °C (β3) or 60 °C (β1, β2, β4) for 1 min and 72 °C for 2 min with final extension at 72 °C for 10 min using AmpliTaqGold polymerase (ABI) and 1.5 mM Mg^{2+}. Predicted PCR product sizes for β-subunits were 421 bp (β1), 414 bp (β2), 451 bp (β3), and 269 bp (β4). All PCR products were sequenced to verify their identity.

FACS—Jurkat T cells were fixed with 2% (w/v) paraformaldehyde at room temperature and permeabilized with 0.1% (w/v) saponin in phosphate-buffered saline containing 5% (v/v) fetal calf serum. Permeabilized cells were incubated with anti-$\alpha_1$1.2 or anti-$\alpha_1$1.3 (Alomone) at 3 μg/ml for 1 h followed by anti-rabbit Alexa 488 (Molecular Probes) used at 1:50 dilution. Negative controls were performed by staining with anti-$\alpha_1$1.2 or anti-$\alpha_1$1.3 (3 μg/ml) that had been preincubated for 1 h with an excess (3 μg/ml) of the peptide against which it had been raised. Secondary staining was performed using 1:50 anti-rabbit Alexa 488. Stained cells were analyzed using a FACS Calibur machine and CellQuest software.

Western Blotting—Whole cell lysates were prepared in radioimmune precipitation assay lysis buffer (50 mM Tris, pH 8.0, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 150 mM sodium chloride, 1 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 200 μg/ml benzamide, 8 μg/ml each of calpain inhibitors I and II, 200 μM phenylmethylsulfonyl fluoride, and 2 μg/ml pepstatin A. Positive controls were prepared from fresh rat tissue. Whole rat heart and brain were homogenized in 320 mM sucrose/4 mM HEPES (pH 7.0) buffer (rat heart) or 320 mM sucrose (rat brain) containing the protease and phosphatase inhibitors listed above. Homogenates were centrifuged at $2000 \times g$ for 10 min, and the supernatant centrifuged at $100{,}000 \times g$ for 60 min. The pellet was resuspended in 320 mM sucrose/4 mM HEPES (pH 7.0) buffer (rat heart) or 320 mM sucrose buffer (rat brain).

Protein (25 μg) was subjected to 7.5 or 10% SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane (Sigma). Nonspecific protein interactions were eliminated using the multiple antigen detection method described by Krajewski *et al.* (32). Briefly, the membrane was blocked with rabbit IgG (3 μg/ml) in 10% nonfat milk, probed with horseradish peroxidase-conjugated goat anti-rabbit IgG, and nonspecific binding was eliminated using an SG peroxidase substrate kit (Vector Laboratories) as per the manufacturer's instructions. The membrane was then blocked with 10% nonfat milk and probed with rabbit anti-$\alpha_1$1.2 (3 μg/ml), our in-house anti-$\alpha_1$1.2 antibody (3 μg/ml), anti-$\alpha_1$1.3 (3 μg/ml), or anti-β3 (3 μg/ml). For mouse anti-β1 (BD Transduction Laboratories) the SG substrate step was not performed. Anti-β1 was used at 2 μg/ml. Specific antibody binding was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG. Blots were developed with a chemiluminescence detection system (Pierce). Peptide blocking was performed by preincubating the antibody with an equal concentration of specific peptide for 1 h at room temperature before probing the membrane.

Statistical Analysis—Statistical analyses in all cases were performed by one-way analysis of variance. Significance was taken to be $p < 0.05$.

RESULTS

L-type Channel Antagonists Reduce TCR-induced Ca^{2+} Responses and Increase Intracellular Ca^{2+} in the Absence of TCR Stimulation—A number of recent reports show that drugs active against L-type channels can inhibit Ca^{2+} influx in non-excitable cells (26, 29). To eliminate the possibility of authentic voltage-gated Ca^{2+} channels being expressed in Jurkat T lym-

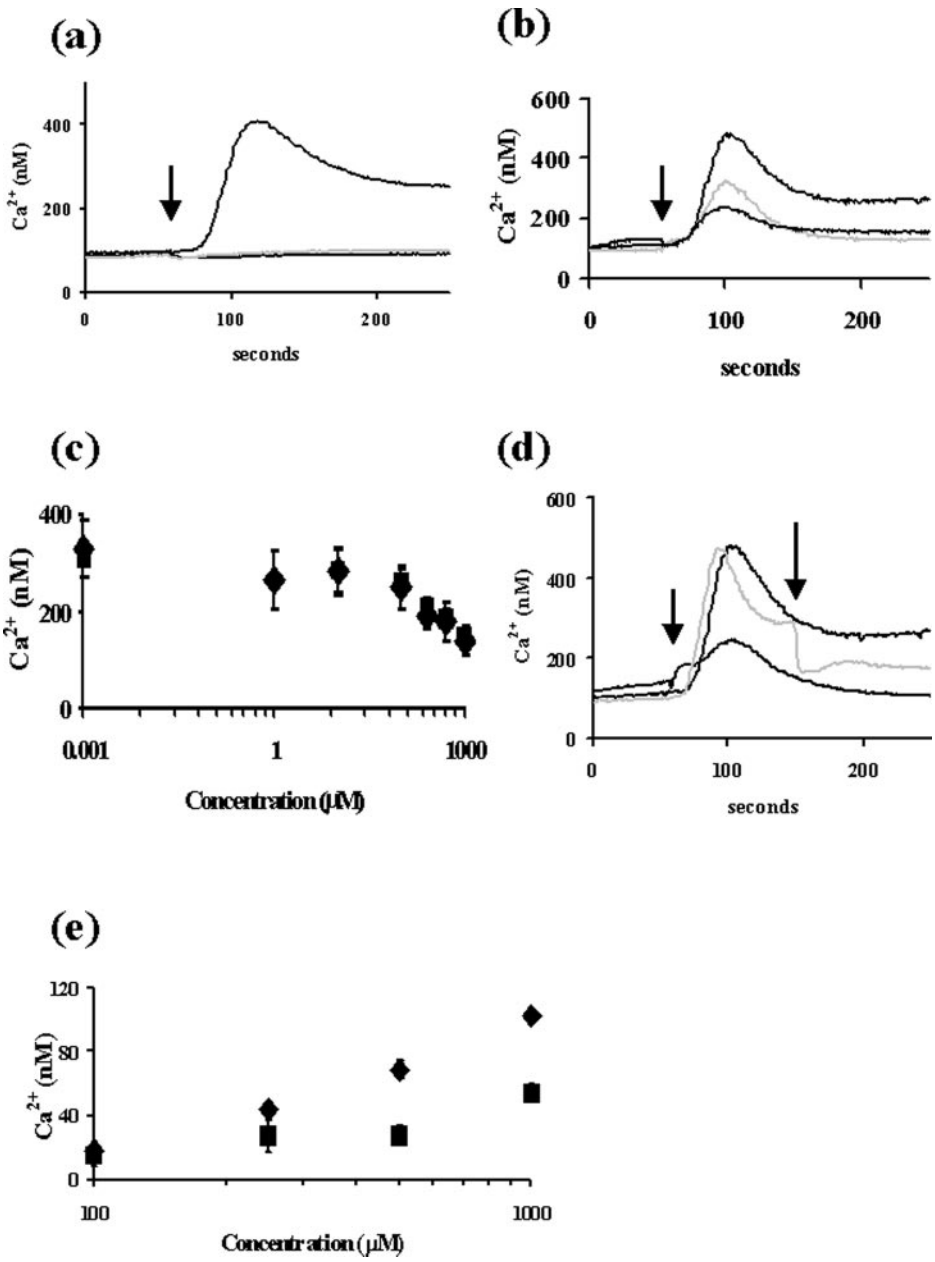

FIG. 1. **The effects of antagonists of L-type Ca^{2+} channels on TCR-induced Ca^{2+} responses.** *a*, cells were treated with 50 mM KCl (*lower black line*), 200 nM gramicidin (*light line*), or 1 μg/ml OKT3 (*upper black line*) added as indicated by the *arrow*. *b*, cells were treated with OKT3 alone (1 μg/ml) (*upper black line*) or OKT3 (1 μg/ml) in the presence of 500 μM verapamil (*light line*) or 1 mM diltiazem (*lower black line*). *c*, cells were treated with various concentrations of verapamil (*diamonds*) or diltiazem (*squares*) plus 1 μg/ml OKT3. Data are the elevation of Ca^{2+} above basal level and are the mean ± S.E. of four separate experiments. *d*, cells were treated with 1 μg/ml OKT3 alone (*upper black line*), 1 μg/ml OKT3 plus 1 mM verapamil added simultaneously (*lower black line*) or 1 μg/ml OKT3 followed by 1 mM verapamil added at the second *arrow* (*light line*). *e*, cells were treated with various concentrations of verapamil (*diamonds*) or diltiazem (*squares*) in the absence of OKT3 stimulation. Data are the elevation of Ca^{2+} above basal level and are the mean ± S.E. of three separate experiments. All data are representative of at least three separate experiments.

phocytes, cells were treated with 50 mM KCl to induce depolarization. Such treatment did not lead to an influx of extracellular Ca^{2+} (Fig. 1*a*). Treatment of the cells with 200 nM gramicidin (a K^+ ionophore) also failed to induce a Ca^{2+} influx (Fig. 1*a*). These data confirm that Jurkat cells do not possess a functional voltage-gated Ca^{2+} channel.

The major signal that activates a plasma membrane Ca^{2+} channel in T cells is cross-linking of the TCR. In our experiments, TCR cross-linking was initiated by the addition of OKT3, an anti-CD3ε antibody, which mimics the binding of foreign antigen to the TCR. Kotturi *et al.* (29) found that the dihydropyridine L-type Ca^{2+} channel antagonist nifedipine inhibited the anti-CD3-induced Ca^{2+} flux in Jurkat T lymphocytes in a dose-dependent manner. We extended these observations using representative drugs from two other classes of L-type Ca^{2+} channel antagonist. Verapamil (a phenylalkylamine) and diltiazem (a benzothiazepine) both inhibited the anti-CD3-induced Ca^{2+} flux in a dose-dependent manner (Fig. 1, *b* and *c*). The IC_{50} for the effect of verapamil was 182 ± 42 μM (n = 4) and the IC_{50} for the effect of diltiazem was 133 ± 60 μM (n = 4). These data are consistent with the expression of a non-voltage-gated L-type Ca^{2+} channel in T lymphocytes.

In B lymphocytes L-type Ca^{2+} channel antagonists are active on both the initial phase of the antigen-receptor-induced Ca^{2+} release from intracellular stores and on the sustained phase of the signal, which involves the influx of extracellular Ca^{2+} (26). In Jurkat T cells verapamil inhibited the TCR-induced initial Ca^{2+} flux (Fig. 1*d*) when added simultaneously with the anti-CD3 antibody. It also partially inhibited the sustained phase of the TCR-induced Ca^{2+} signal when added after the initial phase of the response (Fig. 1*d*). Neither verapamil nor diltiazem completely inhibited the TCR-induced Ca^{2+} flux.

To further explore the effects of these drugs on Ca^{2+} flux in T lymphocytes we treated Jurkat cells with either verapamil or diltiazem in the absence of any other stimulus. Both verapamil and diltiazem induced a dose-dependent rise in intracellular Ca^{2+} in the absence of TCR cross-linking (Fig. 1*e*). Consistent with our findings in B lymphocytes (26), these data suggest at least two different actions for the L-type antagonists, namely an inhibition of the TCR-mediated Ca^{2+} flux, plus a separate TCR-independent mobilization of Ca^{2+}.

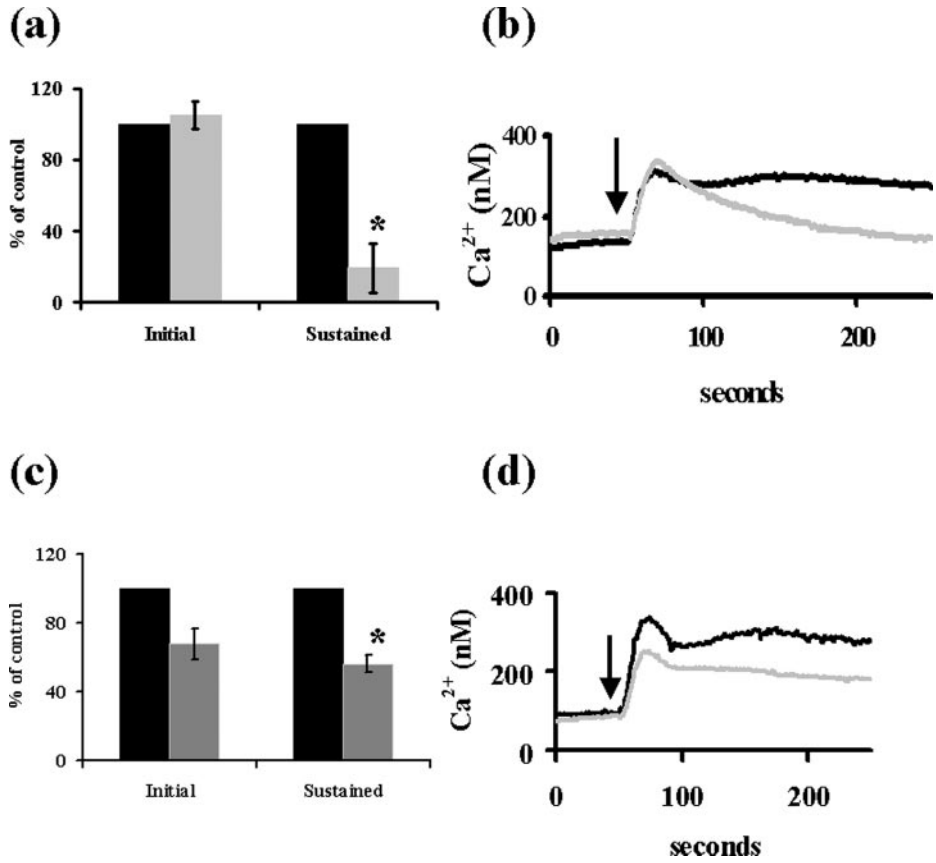

FIG. 2. **The effects of L-type Ca^{2+} channel antagonists on thapsigargin-induced Ca^{2+} flux.** *a*, Jurkat T cells were treated with 100 nM thapsigargin (*black bars*) or 100 nM thapsigargin plus 1 mM verapamil (*light bars*). The effects of verapamil were determined on the initial thapsigargin-induced release of Ca^{2+} from intracellular stores (*Initial*) and on the thapsigargin-induced sustained plateau of the Ca^{2+} flux (*Sustained*). The data are presented as percentage of control (Ca^{2+} flux in the presence of thapsigargin alone). The *asterisk* indicates significant difference from the control value at $p = 0.0009$, $n = 6$. *b*, a representative trace from the experiment described in Fig. 2*a*. The *black line* is treatment with 100 nM thapsigargin alone, and the *light line* is treatment with 100 nM thapsigargin plus 1 mM verapamil. *c*, Jurkat T cells were treated with 100 nM thapsigargin (*black bars*) or 100 nM thapsigargin plus 1 mM diltiazem (*light bars*). The effects of diltiazem were determined on the initial thapsigargin-induced release of Ca^{2+} from intracellular stores (*Initial*) and on the thapsigargin-induced sustained plateau of the Ca^{2+} flux (*Sustained*). The data are presented as percentage of control (Ca^{2+} in the presence of thapsigargin alone). The *asterisk* indicates significant difference from the control value at $p = 0.0329$, $n = 5$. *d*, a representative trace from the experiment described in the legend to Fig. 2*c*. The *black line* is treatment with 100 nM thapsigargin alone, and the *light line* is treatment with 100 nM thapsigargin plus 1 mM diltiazem.

L-type Ca^{2+} Channel Antagonists Partially Inhibit Thapsigargin-induced Ca^{2+} Influx—The second phase of the TCR-activated Ca^{2+} flux is dependent on the influx of extracellular Ca^{2+} through the I_{CRAC} channel. This Ca^{2+} channel is also activated following depletion of intracellular stores with thapsigargin (1). Furthermore, it has been suggested that dihydropyridines can inhibit SOCs in HL60 promyelomonocytic cells (33). Therefore we determined the effect of L-type Ca^{2+} channel antagonists on the thapsigargin-activated Ca^{2+} flux. Verapamil did not affect the initial release of Ca^{2+} from intracellular stores (p = N.S. compared with thapsigargin alone). However it did reduce the thapsigargin-induced plateau by 81 ± 13.9% compared with treatment with thapsigargin alone ($p = 0.0009$, $n = 6$, Fig. 2, *a* and *b*). Similarly, diltiazem did not inhibit the thapsigargin-induced release of Ca^{2+} from stores (p = N.S compared with thapsigargin alone). It did however inhibit the sustained plateau phase of the Ca^{2+} signal by 44 ± 4.9% compared with treatment with thapsigargin alone ($p = 0.0329$, $n = 5$, Fig. 2, *c* and *d*). These data suggest a possible role for L-type Ca^{2+} channels in the store-operated influx of Ca^{2+} in Jurkat T cells.

Human T Cells Express Full-length and Truncated $\alpha_1$1.2 Protein—The data presented suggest the possibility that Jurkat T lymphocytes express L-type Ca^{2+} channels. However the high drug concentrations used could lead to the possibility of nonspecific effects of these drugs. Therefore we sought to identify whether authentic L-type channels, or L-type-related channels were expressed in T lymphocytes. Previous studies have identified transcripts for the L-type $\alpha_1$1.1-, $\alpha_1$1.2-, and $\alpha_1$1.4-subunits in Jurkat T cells (28, 29). We have previously demonstrated that human B lymphocytes express $\alpha_1$1.2 transcripts and protein (26). Despite the pharmacological and molecular evidence indicating the possibility of L-type channels in human T cells, the expression of proteins from L-type α_1-subunit transcripts has not been demonstrated.

We designed PCR primers to a highly conserved region on the $\alpha_1$1.2- (α_{1C}) subunit of L-type voltage-gated Ca^{2+} channels. The amplified region spans exons 34–37 encoding domains IVS4 to IVS6. RT-PCR of cDNA from Jurkat T cells demonstrated that these cells express a transcript for the $\alpha_1$1.2-subunit (Fig. 3*a*). Sequencing confirmed 100% identity of the transcript with the L-type $\alpha_1$1.2-subunit. Transcripts for $\alpha_1$1.2 were also detected in another human T cell line, Molt-4, and in quiescent and activated peripheral blood T cells (Fig. 3*a*). To assess whether the mRNA produced functional protein we first investigated $\alpha_1$1.2 protein expression by flow cytometric analysis. FACS analysis of permeabilized Jurkat cells stained with a commercial anti-$\alpha_1$1.2 antibody revealed that these cells do indeed express $\alpha_1$1.2-subunit protein. Preincubation of the antibody with the peptide against which it was raised completely blocked the staining, confirming the specificity of the interaction (Fig. 3*b*). These data reveal that Jurkat T cells express

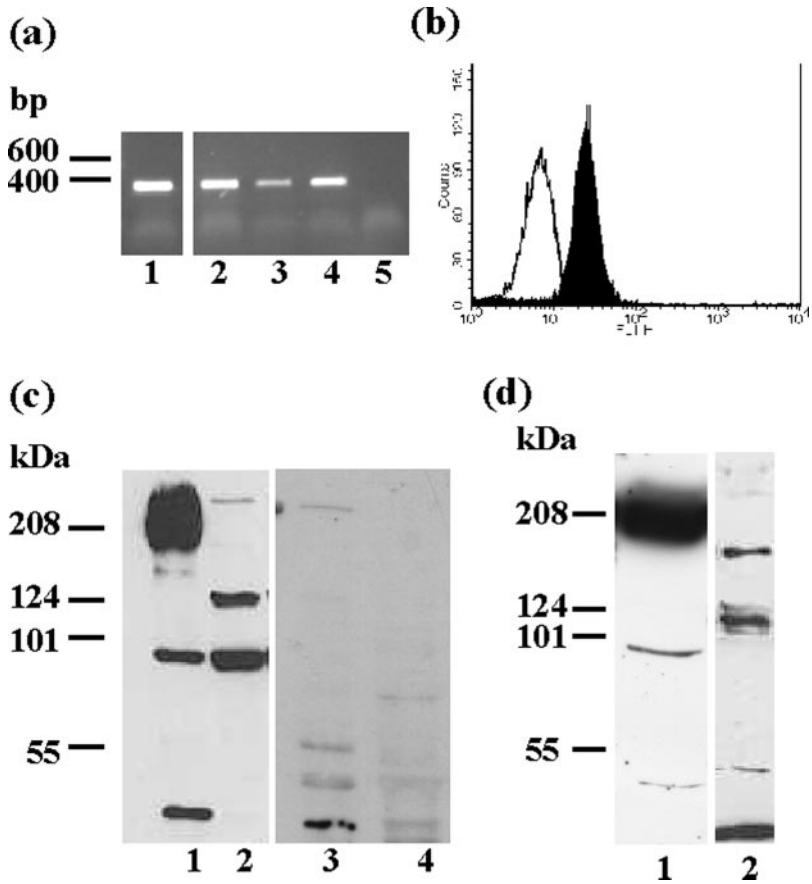

FIG. 3. **Human T cells express $\alpha_1$1.2 mRNA and protein.** *a*, RT-PCR for $\alpha_1$1.2 was carried out from total RNA from Jurkat T cells (*lane 1*), Molt4 T cells (*lane 2*), quiescent and activated $CD4^+$ peripheral blood T cells (*lanes 3* and *4*, respectively), and water as a negative control (*lane 5*). *b*, Jurkat T cells were fixed, permeabilized, and stained with anti-$\alpha_1$1.2 followed by anti-rabbit Alexa 488 secondary antibody. The filled histogram represents specific staining and the lined overlay represents the negative control (peptide-blocked anti-$\alpha_1$1.2). *c*, 25 μg of rat heart microsomes (*lanes 1* and *3*) and Jurkat T cell lysates (*lanes 2* and *4*) were subjected to 7.5% SDS-PAGE and immunoblotted with anti-$\alpha_1$1.2 (*lanes 1* and *2*) or anti-$\alpha_1$1.2 preincubated with its control peptide (*lanes 3* and *4*). *d*, 25 μg of rat heart microsomes (*lane 1*) and Jurkat T cell lysate (*lane 2*) were subjected to 7.5% SDS-PAGE and immunoblotted with our in-house anti-$\alpha_1$1.2 antibody. Data are representative of at least three separate experiments.

readily detectable protein related to the L-type $\alpha_1$1.2-subunit.

We recently reported that human B lymphocytes contain a truncated $\alpha_1$1.2-subunit protein (26). To investigate whether this was also the case in T cells, Western blots of Jurkat lysates and rat heart lysates were probed with a commercial anti-$\alpha_1$1.2 antibody (Fig. 3*c*, *lanes 1* and *2*). The positive control (rat heart lysates) showed the expected band for $\alpha_1$1.2 at 210 kDa. A small amount of the full-length protein was detected in the Jurkat lysates (Fig. 3*c*, *lane 2*, *faint band*), but the dominant band was a truncated protein of 119 kDa. A lower molecular mass protein of ~86 kDa was detected in both the rat heart and Jurkat lysates. When blots of rat heart and Jurkat lysates were probed with anti-$\alpha_1$1.2 that had been preincubated with its control peptide (Fig. 3*c*, *lanes 3* and *4*), no bands were detected. We also probed blots of control and Jurkat lysates with our own in-house anti-$\alpha_1$1.2 antibody raised against a different sequence to that used as the immunogen for the commercial antibody (26). This antibody similarly detected authentic full-length $\alpha_1$1.2 (Fig. 3*d*, *lane 1*) in the rat heart control and the truncated 119 kDa protein in the Jurkat lysates (Fig. 3*d*, *lane 2*). The multiple bands in the positive control have been reported previously (26, 34–36) and have been demonstrated to arise from post-translational proteolysis of the full-length protein (36). Our data show that while Jurkat T lymphocytes express a small amount of full-length $\alpha_1$1.2 protein, the predominant form expressed is a truncated 119 kDa protein.

Human T Cells Express a Truncated $\alpha_1$1.3-Subunit—To address the potential expression of the $\alpha_1$1.3 (α_{1D})-subunit in Jurkat T cells we designed PCR primers to a conserved region spanning domains IVS2 and IVS3. RT-PCR of Jurkat cDNA revealed the presence of a transcript for this subunit (Fig. 4*a*). Sequencing confirmed 100% homology of this transcript with the L-type $\alpha_1$1.3-subunit. To confirm whether this mRNA generated functional protein we investigated $\alpha_1$1.3 protein expression as for $\alpha_1$1.2. Thus, FACS analysis of permeabilized Jurkat cells using a commercial anti-$\alpha_1$1.3 antibody revealed the expression of $\alpha_1$1.3-subunit protein with its control peptide reversing the staining (Fig. 4*b*). To determine protein size, Western blots of rat brain microsomes and Jurkat cell lysates were probed with anti-$\alpha_1$1.3 (Fig. 4*c*, *lanes 1* and *2*). The positive control (rat brain microsomes) showed the expected bands for $\alpha_1$1.3 at 220 and 240 kDa (34). No full-length protein was detected in Jurkat T cells; however, truncated bands of 133 and 115 kDa were detected (Fig. 4*c*, *lane 2*). A lower molecular mass band of 57 kDa was expressed in both Jurkat and rat brain lysates. Several other lower molecular mass bands were expressed in the rat brain microsomes, but not in Jurkat cells. When blots of rat brain and Jurkat lysates were probed with anti-$\alpha_1$1.3 that had been preincubated with its control peptide, no bands were detected (Fig. 4*c*, *lanes 3* and *4*). Multiple bands in the positive control have previously been reported (34). Our data show that Jurkat T lymphocytes do not express a full-length $\alpha_1$1.3 protein. These cells do however express two truncated $\alpha_1$1.3 proteins of 133 and 115 kDa.

Multiple Transcripts for Auxiliary β-Subunits Are Co-expressed in Human T Cells—We have previously described the expression of a β1-subunit of voltage-gated Ca^{2+} channels in a human B cell line (26). Bichet *et al.* (37) demonstrated that β-subunits play an important role in chaperoning α_1 proteins to the cell surface by masking an endoplasmic reticulum retention signal. These regulatory proteins seem to be essential for functional expression of L-type α_1-subunits at the plasma membrane (38). Therefore we wanted to determine whether auxiliary β-subunits were also expressed in T lymphocytes. The primary screening was by RT-PCR. PCR primers were designed to all four known β-subunits and were designed to detect all currently known splice variants of these subunits.

There are three known β1 splice variants, β1a, β1b, and β1c,

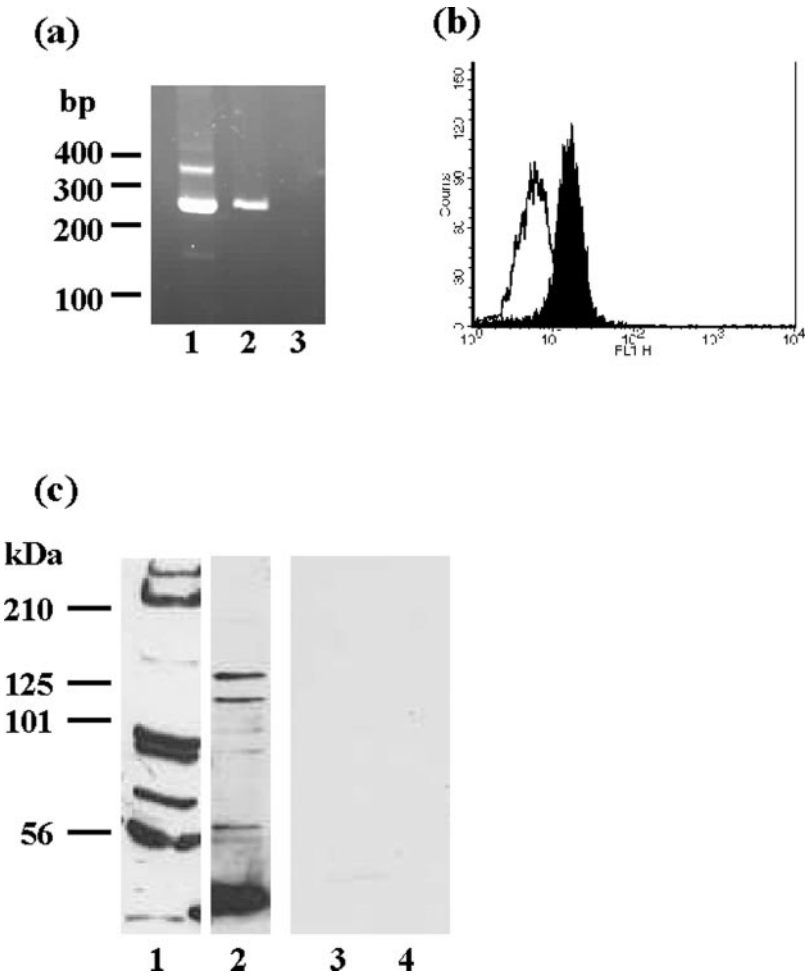

FIG. 4. **Human T cells express $\alpha_1$1.3 mRNA and protein.** *a*, RT-PCR for $\alpha_1$1.3 was carried out from total RNA from Kelly neuroblastoma cells (positive control, *lane 1*), Jurkat T cells (*lane 2*), and water as a negative control (*lane 3*). *b*, Jurkat T cells were fixed, permeabilized, and stained with anti-$\alpha_1$1.3 followed by anti-rabbit Alexa 488 secondary antibody. The *filled histogram* represents specific staining, and the *lined overlay* represents the negative control (peptide-blocked anti-$\alpha_1$1.3). *c*, 25 μg of rat brain microsomes (*lanes 1* and *3*) and Jurkat T cell lysates (*lanes 2* and *4*) were subjected to 7.5% SDS-PAGE and immunoblotted with anti-$\alpha_1$1.3 (*lanes 1* and *2*) or anti-$\alpha_1$1.3 preincubated with its control peptide (*lanes 3* and *4*). Data are representative of three separate experiments.

which differ in their central region and their C termini (39). Primers for β1 were designed to the central variable region. Splice variant β1a contains exon 7a, whereas β1b and β1c both contain exon 7b, therefore the PCR could distinguish between the presence of a β1a or β1b/β1c splice variant. β1 PCR was performed on cDNA from a range of T cell lines, including Jurkat. Each PCR product was cloned and sequenced to verify its identity. Three distinct bands were detected in all of the T cell lines. A faint band of 550 bp showed 100% identity with human β1 and contained exon 7a consistent with it being splice variant β1a (Fig. 5*a*, *top band*). A dominant central band of 400 bp was also detected in all cell lines examined (Fig. 5*b*, *central band*). This band showed 100% identity to the human β1 sequence but lacked either exon 7a or 7b. A smaller band of 350 bp was detected in all of the cell lines. This showed 100% sequence identity to human β1 and contained exon 7b; however it lacked exon 8. These latter two PCR products may represent new splice variants of β1.

There are five β2 splice variants presently known, β2a to β2e, which differ only in their N-terminal region (40). PCR primers for the β2 isoform were designed spanning exons 9–12. This region contains the guanylate kinase-like domain and is conserved between all presently known splice variants (40); therefore, PCR could not distinguish between the known splice variants. β2 transcripts were detected in a number of T cell lines including Jurkat (Fig. 5*b*), and sequencing confirmed their 100% identity with the reported human sequence.

There are currently two known human β3 splice variants, β3a and β3b (41). PCR primers for the β3 isoform were designed spanning exon 2 to the exon 6–7 boundary, corresponding to the SH3 domain of the protein and could not distinguish between the splice variants. A single β3 transcript was detected in all T cell lines examined (Fig. 5*c*). Sequencing confirmed its 100% identity with the human β3 sequence.

To examine the expression of β4, PCR primers were designed spanning exons 12 and 13 of the β4-subunit. These primers detect both known splice variants, namely β4a and β4b. These splice variants differ at their N termini (42). Two PCR products were detected in all of the T cell lines examined (Fig. 5*d*). Sequencing showed that the smaller (250 bp) band had 99% identity with isoforms β4a and β4b. The larger (300 bp) band also showed 99% identity with these two isoforms but contained an additional 69 nucleotides incorporated into the sequence at the end of exon 12. These additional nucleotides matched intronic sequence between exons 12 and 13 and were not continuous with either exon and are therefore unlikely to represent an alternative splice donor or acceptor site. It is possible that this additional sequence represents a new β4 exon.

Human T Cells Express Auxiliary β-Subunit Proteins—The PCR screening showed the expression of transcripts for all four known β-subunits in a variety of T cell lines, and several potential new splice variants were detected. To determine whether these subunits were expressed at the protein level, Western blotting of Jurkat lysates was carried out for β1 and β3: antibodies to the β2- and β4-subunits being unavailable.

To determine whether any of the β1 transcripts were translated into functional protein Western blots of rat skeletal muscle lysates and various T cell line lysates were probed with an anti-β1 antibody. The antibody was raised to the N-terminal region of the protein (amino acids 28–229) and detects all three known splice variants. All T cell lines examined expressed a

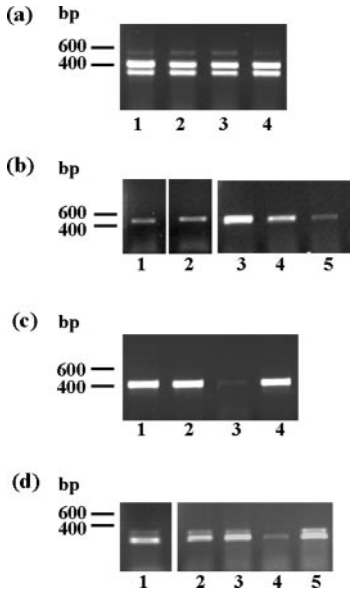

FIG. 5. **Human T cells express multiple transcripts for β-subunits.** RT-PCR was performed for the four known auxiliary β-subunits using primers designed to subunit-specific sequences and total RNA from a variety of human T cell lines. *a*, β1-subunit expression in Jurkat (*lane 1*), H9 (*lane 2*), CCRF-CEM (*lane 3*), and Hut78 (*lane 4*) T cell lines. *b*, β2-subunit expression in Jurkat (*lane 1*), Hut78 (*lane 2*), Molt-4 (*lane 3*) CCRF-CEM (*lane 4*), and H9 (*lane 5*) T cell lines. *c*, β3-subunit expression in Jurkat (*lane 1*), H9 (*lane 2*), CCRF-CEM (*lane 3*), and Molt-4 (*lane 4*) T cell lines. *d*, β4-subunit expression in Jurkat (*lane 1*), Molt-4 (*lane 2*), CCRF-CEM (*lane 3*), H9 (*lane 4*), and Hut78 (*lane 5*) T cell lines.

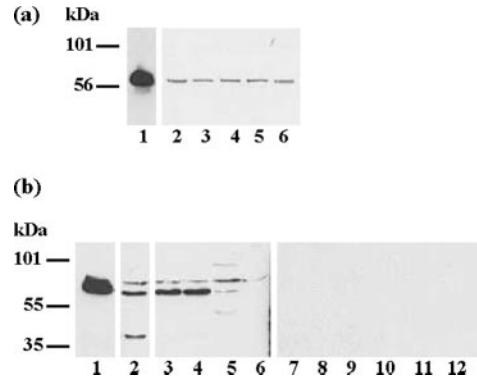

FIG. 6. **Human T cells express β1- and β3-subunit proteins.** Whole cell lysates from a range of T cell lines were subjected to 10% SDS-PAGE and blotted for β-subunit protein. *a*, β1 protein expression in rat skeletal muscle lysate (positive control, *lane 1*) Jurkat (*lane 2*), Molt-4 (*lane 3*), CCRF-CEM (*lane 4*), Hut78 (*lane 5*), and H9 (*lane 6*) T cell lines. *b*, β3 protein expression in rat brain microsomes (positive control, *lane 1*) Jurkat (*lane 2*), Molt-4 (*lane 3*), CCRF-CEM (*lane 4*), H9 (*lane 5*), and Hut78 (*lane 6*) T cell lines. *Lanes 7–12* are duplicate lanes immunoblotted with peptide-blocked anti-β3. Data are representative of three separate experiments.

single band of 57 ± 5 kDa (n = 3, Fig. 6*a*). This correlates well with the reported molecular mass for β1 of 55–58 kDa (43, 44). These data show that human T cells express a β1-subunit, but it is not clear which of the potential splice variants gives rise to this protein expression.

Similarly, to determine whether β3 protein was expressed, Western blots of rat brain microsome lysates and various T cell line lysates were probed with an anti-β3 antibody. This antibody was raised against the C terminus of β3 and is thought to detect all known human splice variants. The T cell lines expressed two distinct anti-β3-reactive proteins of 74 ± 2 kDa and 67 ± 4.2 kDa (n = 12), the latter correlating with the reported molecular mass for β3 of 67 kDa in neuronal cells (45). The exception was the Hut78 T cell line, which only expressed the 74 kDa protein. The RT-PCR only detected one β3 transcript. The protein data may therefore represent a splice variant of β3 outside of the region amplified by the set of primers used, or a post-translational modification of the full-length protein.

DISCUSSION

A growing number of reports support the suggestion that lymphocytes and other non-excitable hemopoietic cells express a Ca^{2+} channel related to the L-type of voltage-gated Ca^{2+} channels. Our observation that verapamil and diltiazem inhibit the TCR-mediated Ca^{2+} flux is an example of such a study. In common with other investigations, high concentrations of antagonists of L-type Ca^{2+} channels were required to inhibit the Ca^{2+} flux (26, 29). We observed this same phenomenon in B lymphocytes and attributed the high doses required to the lack of the high affinity state of the channel because of the absence of voltage gating and the consequent lack of use-dependence (26).

In the present study we showed that both the TCR and thapsigargin-induced Ca^{2+} influx could be inhibited by L-type Ca^{2+} channel antagonists. Since the Ca^{2+} influx activated by both of these stimuli has previously been characterized as the I_{CRAC} channel, the data may imply that this channel is composed wholly, or in part, by an L-type Ca^{2+} channel or that the L-type Ca^{2+} channel plays a regulatory role in I_{CRAC} activation. This conclusion is also supported by Kotturi *et al.* (29) who found that a dihydropyridine sensitive L-type Ca^{2+} channel had a role in the TCR-mediated Ca^{2+} influx in Jurkat T cells, and by Harper *et al.* (33) who reported that dihydropyridines could inhibit capacitative Ca^{2+} influx in HL60 cells. However, the high concentrations of the drugs give rise to the possibility of effects unrelated to their actions on voltage-gated Ca^{2+} channels. For example, high concentrations of dihydropyridines have been reported to have inhibitory effects on voltage-gated K^+ channels and Ca^{2+}-activated K^+ channels (46, 47), and antagonists of L-type channels, such as verapamil, can inhibit the multidrug transporter, P-glycoprotein (48). The effects of these drugs on other non-voltage-gated Ca^{2+} channels, *e.g.* Trp channels, are unknown, and the full range of Ca^{2+} channels expressed by lymphocytes is currently not ascertained. Our data also show that antagonists of L-type Ca^{2+} channels have at least two separate effects; namely the inhibition of the TCR-mediated Ca^{2+} flux and a separate mobilization of Ca^{2+} independent of the TCR (present study and Ref. 26). This makes a proper interpretation of the effects of these drugs problematic.

In an attempt to address the functional role of L-type Ca^{2+} channels in T lymphocytes, Kotturi *et al.* (29) used drugs active against this class of channel to show that they could affect Erk1/2 activation and influence the TCR-mediated up-regulation of NFAT and interleukin-2 production. They attributed these effects to actions on an L-type channel. Their conclusion was strengthened by the finding of an $\alpha_1$1.4 transcript in Jurkat cells. However, specific antagonists of Ca_V1.4 ($\alpha_1$1.4/α_{1F}) have not been demonstrated (19) although it has been assumed that drugs active against other L-type channels are also active against this channel (*e.g.* see Ref. 29). The possibility that the cells expressed other L-type Ca^{2+} channels was not addressed and therefore it is not certain whether the effects noted were because of $\alpha_1$1.4 expression, or unrelated effects as already noted.

Direct evidence supporting the expression of L-type Ca^{2+} channels in human T cells has been lacking. In mouse T cells, Savignac *et al.* (27) described a transcript for $\alpha_1$1.3 and found that an antibody against this protein stained permeabilized T cells in a FACS-based assay. In human T cells transcripts for

$\alpha_1$1.1, $\alpha_1$1.2, and $\alpha_1$1.4 have been described (28, 29), but the studies did not address whether the proteins were expressed. Our data reveal that human T lymphocytes express protein for at least two L-type α_1-subunits. Jurkat T cells expressed $\alpha_1$1.2 protein. A minor fraction was full-length but the predominant protein expressed was a truncated subunit of 119 kDa. This truncated $\alpha_1$1.2-subunit was also expressed in human B lymphocytes in conjunction with a second truncated $\alpha_1$1.2-subunit of 112 kDa (26). The 112 kDa subunit was not detected in Jurkat. Jurkat T cells also expressed $\alpha_1$1.3 transcript and protein. No full-length protein was detected, but two truncated $\alpha_1$1.3-subunits of 133 and 115 kDa were detected. We believe that ours is the first study to directly detect expressed proteins related to voltage-gated Ca^{2+} channels in human T lymphocytes.

Voltage-gated Ca^{2+} channels are heteromeric structures containing regulatory subunits in addition to the pore-forming α_1-subunit. β-Subunits are necessary for the expression of α_1-subunits at the plasma membrane (37) and also affect gating properties of the α_1-subunit (38). The β1-subunit has previously been detected in the spleen and in human B lymphocytes (26, 49). We detected multiple transcripts for all four known β-subunits in T cells, and revealed the potential for the expression of several new splice variants. One band corresponding to authentic β1 was present in all T cell lines examined. Two β3-related proteins were discovered; one probably corresponding to the previously described β3 protein and one larger protein, which was present in all of the T cell lines examined. The presence of two and possibly four β-subunits is fully consistent with these cells expressing Ca^{2+} channels with multiple features of authentic voltage-gated L-type Ca^{2+} channels.

We have presented data showing that T cells express two truncated non-voltage-gated Ca^{2+} channels highly related to L-type Ca^{2+} channels. These cells potentially express channels with characteristics of all four known L-type Ca^{2+} channels. The role played by these Ca^{2+} channels is unclear but the available evidence points to two separate possibilities. The first in the initiation of the TCR-mediated Ca^{2+} signal and the second in the influx phase of the Ca^{2+} signal. These data reveal an additional layer of complexity and flexibility to the TCR-mediated Ca^{2+} signal.

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