T-lymphocyte activation requires sustained Ca$^{2+}$ signaling dependent upon capacitative Ca$^{2+}$ entry (CCE). The protein(s) that forms the stores-operated Ca$^{2+}$ channel (SOCC) responsible for CCE has long been sought but has not been definitively identified. Members of the TRPV family (transient receptor potential superfamily-vanilloid receptor subfamily) of channel genes have been proposed to encode SOCCs responsible for CCE in non-excitable cells. Here we present evidence that a member of the TRPV group, CaT1, is involved in generating I$_{CRAC}$, the CCE current that is necessary for T-cell activation. CaT1 is expressed in Jurkat T-lymphocytes. When overexpressed in Jurkat cells, CaT1 produces a Ca$^{2+}$ entry current that mimics the endogenous I$_{CRAC}$ in its dependence on external Ca$^{2+}$, inactivation by elevated concentration of internal Ca$^{2+}$, and pharmacological block by capsaicin. Overexpressed CaT1 is partially regulated by the release of internal Ca$^{2+}$ stores via thapsigargin or receptor-mediated generation of inositol 1,4,5-trisphosphate. A pore-region mutant of CaT1, TRIA-CaT1, fails to carry Ca$^{2+}$ currents and associates with co-expressed wild type CaT1 to functionally suppress permeation of Ca$^{2+}$ ions. Expression of the TRIA-CaT1 mutant in Jurkat cells results in suppression of the endogenous I$_{CRAC}$. Taken together these results indicate that CaT1 is the channel protein that contributes to T-lymphocyte SOCCs either alone or as a subunit in a heterogeneous channel complex.

Capacitative calcium entry (CCE)$^1$ via stores-operated calcium channels (SOCCs) is a basic mechanism for sustained Ca$^{2+}$ signaling in a variety of cells (1–3). CCE is activated by depletion of Ca$^{2+}$ from internal Ca$^{2+}$ storage sites (endoplasmic reticulum (ER)) after activation of G-protein-coupled receptors or tyrosine kinase-based receptors, that stimulate phospholipase C $\beta$ or $\gamma$. Phospholipase C subsequently hydrolyzes membrane phospholipids to release inositol 1,4,5-trisphosphate (IP$_3$) that binds to the IP$_3$ receptor located on the Ca$^{2+}$ storage sites stimulating rapid release of Ca$^{2+}$ to the cytoplasm. When the concentration of Ca$^{2+}$ declines in the lumen of the ER, a signal is transmitted to SOCCs on the cell surface persuading them to open and allow external Ca$^{2+}$ ions to enter the cell (4). The ionic current carried by Ca$^{2+}$ entering through SOCCs in several cell types has been termed the Ca$^{2+}$ release-activated current (I$_{CRAC}$) (5) or the depletion-activated current (6).

The electrophysiological properties of the I$_{CRAC}$ have been studied in a variety of cell lines (7). There are several defining features of the I$_{CRAC}$ as follows: high selectivity for Ca$^{2+}$; a very small single channel conductance in the presence of divalent cations; large Na$^+$ permeability in the absence of divalent cations; [Ca$^{2+}$]-dependent inactivation; and permeation or block by divalent cations, (5, 6, 8–14).

One cell type where I$_{CRAC}$ has been well studied and where the biological function is well established is the T-lymphocyte (2). The biophysical properties, pharmacology, and regulation of I$_{CRAC}$ in the T-lymphocyte model cell, Jurkat cell, have been investigated by several groups (6, 8–11, 13, 15–17). In Jurkat cells, I$_{CRAC}$ is induced by stores-depletion via stimulation of the T-cell antigen receptor. The role of I$_{CRAC}$ in T-lymphocytes is to sustain calcium signaling and oscillation after immune activation. The sustained calcium signal leads to proliferation and a program of altered gene expression by allowing translocation of the transcription factor NFAT to the nucleus at a critical point in T-lymphocyte activation (18–20).

The identity of I$_{CRAC}$ proteins, however, has lagged behind advances in physiology due to lack of high affinity ligands or blockers and because most commonly used systems for heterologous expression exhibit some endogenous CCE. Until recently the best candidates for I$_{CRAC}$ have been proteins of the TRP family (21, 22). Members in TRPC subfamily had been recently the best candidates for I$_{CRAC}$ (28–30). Clapham and co-workers (27) performed an in depth electrophysiological analysis of heterologously expressed CaT1 that gave further support for it as a credible molecular candidate for I$_{CRAC}$. A report from Nilius and co-workers (31) showed that despite the difference in permeation properties of CRAC and CaT1, CaT1 was capable of forming store-depletion activated conductances when heterologously expressed in RBL mast cells (33). Clapham and co-workers (31) reported that they detected CaT1 by RT-PCR in Jurkat cells (31). In this study we further de-
scribe the expression of CaT1 in Jurkat cells and provide evidence for its functional contribution to T-lymphocyte ICRAC.

MATERIALS AND METHODS

Cloning of CaT1 from Human Placenta—The human CaT1 was cloned by RT-PCR using total RNA from human adult placenta (Stratagene). The forward primer for PCR begins at the start codon of CaT1 cDNA (5′-GGGAATTCATGGGGTTTGTCACTGCCC, and the reverse primer starts with the stop codon of CaT1 (5′-CCGCTCGAGTCTGATTATCCACGC). PCR primers introduced unique EcoRI and XhoI sites that were used for subcloning products into the expression vector pCMV-Tag2A (Stratagene) that provides an N-terminal e-Myc epitope tag and into the vector p3XFLAG (Sigma) to provide an N-terminal FLAG epitope. TRIA-CaT1 was constructed by an overlap extension PCR strategy (34). The mutated Kpn1/XhoI fragment was subcloned into myc-CaT1 vector described as above. All PCR fragments were verified by DNA sequence analyses.

Northern Analysis—Northern blot was carried out using the NorthernMax system (Ambion). 10 μg of total RNA from Jurkat cells was electrophoresed in formaldehyde-agarose gel and transferred to nylon membrane. The membrane was probed with 32P-labeled CaT1 cDNA fragment (1392–1719), hybridized at 42 °C overnight, washed with low stringency buffer 2 times for 5 min at room temperature, and then washed with high stringency buffer 2 times for 15 min at 65 °C. Autoradiography was performed at ~80 °C for 1 week.

RT-PCR and Real Time PCR—The expression of CaT1 mRNA in Jurkat cells, HEK cells, CHO cells, spleen, and placenta was investigated by RT-PCR. cDNA was constructed from the total RNA of the cell line using CaT1-specific primers. Primers for CaT1 (1907–2178) are as follows: forward, 5′-GGCAAGATCTCAACCGGCC and reverse, 5′-CCGCTCGAGTCTGATTATCCACGC; primers for CaT1(861–1130) are as follows: forward, 5′-GGGAATTCATGGGGTTTGTCACTGCCC, and reverse, 5′-GGGAATTCATGGGGTTTGTCACTGCCC. GAPDH primers are as follows: forward, 5′-GGGTTGAGAACCACAATGATC, and reverse, 5′-GGGTTGAGAACCACAATGATC.

2178) are as follows: forward, 5′-GGCAAGATCTCAACCGGCC- and reverse, 5′-CCGCTCGAGTCTGATTATCCACGC. The amplified GAPDH fragment verifies the integrity and loading of the cDNA samples.

Quantitative detection of CaT1 in Jurkat cell was carried out by real time PCR using iCyycler Thermal Cycler (Bio-Rad). PCRs were performed using gene-specific primers in 15-μl volumes. 7.5 μl of SYBR Green PCR Master Mix (Applied Biosystems), 1.5 μl of 1:5 diluted CaT1 cDNA, and 1.5 μl of CaT1 primers (forward, 5′-GAGATGAGCAGTCCCTGCTG; reverse, 5′-GGGAATTCATGGGGTTTGTCACTGCCC) were mixed into the 96-well 200-μl volumes. 7.5 μl volumes. 7.5 μl of 1:10 diluted first-strand cDNA were well mixed into the 96-well 200-μl PCR Plates (Bio-Rad). Data were analyzed by iCycred optical system interface (version 2.3). All the samples were triplicates and were repeated twice. GAPDH primers were used as internal control to normalize the expression of genes. The gene expression was presented as fold change compared with the control sample.

Cell Culture and Transfection—CHO cells were maintained in Ham’s F-12 media supplemented with 10% fetal calf serum and penicillin/streptomycin at 37 °C under 5% CO2. Gene transfer was performed using 5 μg of Qingen Midiprep purified plasmid cDNA. Cells were electroporated in a 2-mm gap cuvette using BTX ECM 620 setting at 100–120 V, 500 μF, and 1800 microfarads. 4 h after transfection, 1 l of rabbit polyclonal anti-Myc antibody and 1 l of 1:10 diluted first-strand cDNA were well mixed into the 96-well 200-μl Thin Wall PCR Plates (Bio-Rad). Data were analyzed by iCycred optical system interface (version 2.3). All the samples were triplicates and were repeated twice. GAPDH primers were used as internal control to normalize the expression of genes. The gene expression was presented as fold change compared with the control sample.

Whole cell patch clamp membrane currents were recorded as described previously (8, 36). The external solution contained 2 mM CaCl2, 145 mM NaCl, 10 mM CsCl, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES (pH 7.4, osmolarity, 320 mOsm). When we needed external solutions that were nominally Ca2+-free, we substituted MgCl2 for CaCl2. Completely divalent-free (DVF) solution contained 115 mM NaCl, 10 mM HEPES, pH 7.4, 10 mM sodium EDTA, and 10 mM EGTA. 2 mM Mg-ATP, 1 mM MgCl2, 10 mM EDTA, and 10 mM Cs-HEPES (pH 7.2, osmolarity, 290 mOsm).

Immunoblot Analysis—CHO or HEK cells were transiently transfected with 1 μg of cDNA plasmids using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instruction. Cells were harvested 24 h after transfection for Western blot analysis. Proteins were separated by SDS-PAGE on a 10% gel. Anti-Myc A-14 polyclonal antibody was used for detection of myc-tagged CaT1 proteins.

For co-association of wild type and mutant CaT1 cells were detergent-lysed in NDET (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 0.4% deoxycholic acid, EDTA-free protease inhibitor mixture tablets (Roche Molecular Biochemicals)). Cell lysates were incubated with 30 μl of rabbit polyclonal anti-Myc antibody and protein G-agarose. Precipitated proteins were then eluted with SDS-PAGE sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose for immunoblotting with appropriate antibodies.

Immunofluorescence—HEK293s were transiently transfected with wild type CaT1 or TRIA-CaT1. 48 h after transfection, culture media were removed, and cells were fixed with 4% paraformaldehyde for 20 min, rinsed once with PBS, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. After washing with PBS, cells were blocked with 5% bovine serum albumin in PBS for 30 min. Cells were incubated with anti-Myc 9E10 monoclonal antibody in PBS containing 0.1% Nonidet P-40 for 1 h at room temperature. A goat anti-mouse Alexa Fluor...
488-conjugated secondary antibody was added at 1:500 dilution for 1 h in the dark. Images were captured using confocal microscopy (Bio-Rad, Radiance 2000) and analyzed by Adobe Photoshop.

**Measurement of \([\text{Ca}^{2+}]_i\)**—8100 Jurkat cells were co-transfected with 2 μg of cDNA plasmids and 0.5 μg of GFP using LipofectAMINE 2000 in 24-well plate. 24 h after transfection, cells were loaded at 37°C for 15 min with 2 μM fura-2/AM-ester in culture medium and subsequently washed with \([\text{Ca}^{2+}]_i\)-free external solution twice. Fura-loaded cells were allowed to adhere to a poly-L-lysine-coated glass coverslip chamber on the stage of an inverted microscope (Nikon, Tokyo, Japan) equipped with a 40x Fluor objective (NA 1.3). Cells were alternately illuminated at 340 and 380 nm (Lambda DG-4, Sutter Instrument Co.), and the fluorescence emissions at λ > 480 nm were captured with Quantix CCD camera (Photometrics, Ltd.) and digitized and analyzed using an Axon Imaging Workbench system (Axon Instruments). All experiments were conducted at room temperature.

**Reagents and Antibodies**—Thapsigargin and SKF 96365 were purchased from Calbiochem. Fura-2-AM and goat anti-mouse Alex Fluor 488 were from Molecular Probes (Eugene, OR). Anti-Myc A-14 was from Santa Cruz Biotechnology (Santa Cruz, CA). All reagents for Northern analysis were purchased from Ambion Inc. (Austin, TX). Other reagents were from Sigma.

**Statistical Analysis**—In all experiments, the data are expressed as the mean ± S.E. In the case of significance \((p < 0.05)\), Student’s t test was used to compare individual groups.

**RESULTS**

**Expression of CaT1 Transcripts in Jurkat T-lymphocytes**—Human CaT1 cDNA was first cloned from small intestine, and strong expression was found in placenta, kidney, pancreas, and prostate (37, 38). Although CaT1 mRNA was not initially detected in spleen or thymus by Northern analysis in original studies (29, 37), it was reported later that CaT1 was present in Jurkat T-lymphocytes by RT-PCR (31). To investigate CaT1 expression in Jurkat cells, we performed Northern analysis using total RNA from Jurkat cells and a 300-bp fragment of CaT1 cDNA as a probe. A clear 3-kb band was detected, corresponding to the size of CaT1 cDNA of 2902 bp (Fig. 1A).

**Figs. 2**. Heterologous expression CaT1 in CHO cells. Whole cell current in mock-transfected CHO cell (A) or CaT1-transfected CHO cells (B). Families of current responses to membrane voltage steps are shown on the left. The time course for development of inward \([\text{Ca}^{2+}]_i\) current after establishing whole cell configuration at time = 0 and responses to TG-induced store depletion and cycling between 0 and 2 mM external \([\text{Ca}^{2+}]_i\) are shown on the right. Inset shows voltage-ramp I-V curves at positions marked in time course plots. C, summary data for current densities at positions marked in A and B. Current densities derived from CaT1-transfected CHO cells are significantly larger \((p < 0.05)\) than that in control CHO cells and showed a partial enhancement due to \([\text{Ca}^{2+}]_i\) stores-depletion.
ECaC1 transcripts were also detected in placenta and Jurkat cells but with less robust amplification (Fig. 1B). RT-PCR fragments were verified by nucleotide sequence to confirm the specificity of the amplified CaT1 and ECaC1 signals. We were not able to detect CaT1 expression by RT-PCR from either HEK or CHO cells (Fig. 1C).

To estimate the level of CaT1 mRNA in Jurkat cells, we quantified the relative mRNA expression of CaT1 in Jurkat cell and placenta by real time PCR using CaT1-specific primers. mRNA abundance was calculated and normalized by the abundance of internal control GAPDH from Jurkat cell and placenta, respectively. Our results indicate that the mRNA abundance in Jurkat cell is about 40-fold lower than that in placenta (Fig. 1D).

Biophysical Characterization of CaT1 Overexpression in CHO Cell and Jurkat Cell—We heterologously expressed Myc-tagged CaT1 cDNA in CHO cells to determine the biophysical properties of our cloned channel. Although only a small background current was detected in mock-transfected cells (Fig. 2A), we observed a large inwardly rectifying current in CaT1-transfected CHO cells (Fig. 2B). As reported previously (31, 33, 39) for CaT1 in heterologous expression systems, upon establishing the whole cell configuration with 10 mM EGTA internally, a large inward Ca$^{2+}$ current was spontaneously activated and then subsequently inactivated (Fig. 2B). Direct Ca$^{2+}$-store depletion can be obtained with thapsigargin (TG), a specific inhibitor of smooth endoplasmic reticulum calcium ATPase pumps resulting in a relatively rapid depletion of intracellular Ca$^{2+}$ store independent from membrane receptor activation or generation of IP$_3$ (6, 9, 40). When we depleted intracellular Ca$^{2+}$ stores with 1 mM TG, we observed a modest enhancement in inward current density (from 4.0 ± 0.49 to 4.6 ± 0.55 pA/pF) (Fig. 2C). By switching to a nominally Ca$^{2+}$-free external medium the Ca$^{2+}$ current nearly disappeared. Re-introduction of 2 mM external Ca$^{2+}$, (* indicates p ≤ 0.05 by t test versus control Jurkat cells.)

To examine the possible role of CaT1 in I$_{CRAC}$, we overexpressed CaT1 cDNA within a cellular context containing...
known I_{CRAC} activity, the Jurkat T-cell. Overexpression of CaT1 in Jurkat cells resulted in a markedly enhanced I_{CRAC}-like inward Ca^{2+} current density compared with base line (Fig. 3, A and B). The biophysical characteristics of CaT1 in Jurkat cells were comparable with those in CHO cells and similar to the endogenous Jurkat current. The current was dependent on external Ca^{2+}; both fast and slow Ca^{2+}-dependent inactivation was seen, and the channels were selective for Ca^{2+} over Na+. In untransfected Jurkat cells thapsigargin is capable of inducing store depletion and activation of I_{CRAC}. In some untransfected cells, just establishing the whole cell patch clamp configuration spontaneously activates endogenous I_{CRAC}. In CaT1-overexpressing Jurkat cells Ca^{2+} entry currents were usually stimulated spontaneously upon break in to the whole cell mode and slowly relaxed to a partially inactivated state. Addition of thapsigargin resulted in a modest enhancement of the Ca^{2+} entry current density (Fig. 3D) suggesting that the CaT1 current is partially sensitive to the state of the Ca^{2+} stores. The [Ca^{2+}]_{i} dependence of the CaT1 current was demonstrated in its disappearance in a nominally Ca^{2+}-free external solution. When Ca^{2+} was reintroduced after a period in Ca^{2+}-free solution, there was a very large enhancement of the inward current beyond the initial values. This enhancement was transient as the current inactivated again to a lesser steady-state amplitude (Fig. 3D).

To examine a more physiological stimulation of CaT1, current experiments were performed in CaT1-transfected JHM1 cells, a Jurkat derivative that stably express type 1 muscarinic receptor (8, 41, 42). Upon stimulation of the muscarinic receptor in these cells, phospholipase C was rapidly activated producing IP_{3}, diacylglycerol, Ca^{2+} release, and subsequent signaling similar to stimulation through the T-cell receptor (41, 42). When we applied 250 μM carbachol to CaT1-transfected JHM1 cells, we observed an enhancement of the inward Ca^{2+} current consistent with a partial Ca^{2+}-stores regulation of the CaT1 current (Fig. 3E).

**Pharmacological Analysis of CaT1 Currents**—To evaluate whether CaT1 shares pharmacological features with endogenous I_{CRAC}, we tested the effect of a known SOC inhibitor SKF 96365 (43, 44) on CaT1. In Jurkat cells, the endogenous I_{CRAC}
was inhibited by 50 μM SKF 96365 (Fig. 4A). SKF 96365, however, had no effect on CaT1 expressed in CHO cells (Fig. 4B). Our result agreed with a previous report (45) that SKF 96365 was ineffective on ECaC1.

We next tested capsaicin on CaT1 activity. Capsaicin is a member of the vanilloid family and activates the vanilloid receptor, a member of the TRPV family (46, 47). At micromolar concentrations, however, capsaicin blocks CCE in PC12 cells (48) and the endogenous ICRAC in Jurkat cells (36). An analog of capsaicin, capsazepine, that inhibits the activity of capsaicin on the vanilloid receptor can partially block ECaC1 (45) and is effective in blocking ICRAC in Jurkat cells (36). Capsaicin (250 μM, a concentration that completely blocks endogenous ICRAC in Jurkat cells (36)) was effective in reversibly blocking Ca2+ entry currents in both Jurkat (Fig. 4C) and JHMI (Fig. 4D) cells that were transfected with CaT1. The similar capsaicin sensitivity of Jurkat ICRAC and CaT1 supports the hypothesis that CaT1 contributes to the endogenous current in Jurkat cells.

**Construction and Expression of Dominant Negative CaT1 Mutant**—To assess the contribution of CaT1 in Jurkat cell ICRAC, we constructed a mutant CaT1 that would suppress Ca2+ entry currents by assembling with wild type CaT1 subunits to form non-conductive channels. We substituted three alanines for residues 534–536 (FEL) that are conserved in the putative pore-lining region to create the mutant TRIA-CaT1 (Fig. 5A). Expression of TRIA-CaT1 alone in CHO cells produced a small, non-selective cationic current that failed to pass Ca2+ ions and showed no regulation by Ca2+-store depletion (Fig. 5B). Immunofluorescence analysis of Myc-tagged TRIA-CaT1 showed a cellular distribution identical to wild type CaT1 (Fig. 5C). When expressed in HEK cells CaT1 and TRIA-CaT1
exhibited comparable staining patterns with evidence of surface expression and staining in the Golgi and ER locations typical of transiently transfected membrane proteins. Western analysis of Myc-tagged CaT1 and TRIA-CaT1 showed similar patterns with a major band near 80 kDa (predicted mass by amino acid sequence is 83 kDa) and a higher molecular weight band suggesting varied post-translational processing such as glycosylation and/or phosphorylation (Fig. 5D). When differentially tagged wild type and mutant constructs were co-transfected, we could successfully detect co-immunoprecipitation indicating the capability of TRIA-CaT1 to associate with wild type CaT1 (Fig. 5D).

To examine the functional effect of TRIA-CaT1 on wild type CaT1, we co-transfected equal amounts of each cDNA in CHO cells. Cells co-transfected with TRIA-CaT1 and CaT1 yielded a Ca2+-entry current smaller in magnitude than cells transfected with wild type CaT1 alone (Fig. 5E). When we examined cells that were transfected with differing ratios of wild type and mutant CaT1, we observed a dose-dependent suppression that approached a nearly completely dominant negative effect for a tetrameric assembly (Fig. 5F). The reduction in Ca2+-dependent current was seen at steady state and upon cycling between 0 and 2 mM external Ca2+ (Fig. 5G). Thus, TRIA-CaT1 exerts dominant negative suppression on wild type CaT1 function.

We then examined the ability of TRIA-CaT1 to alter the endogenous I_{CRAC} in Jurkat cells. When TRIA-CaT1 was overexpressed in Jurkat cells the base-line current was similar to that of mock-transfected cells (Fig. 6A compared with Fig. 3A). Compared with GFP-transfected Jurkat cells or untransfected cells, those expressing TRIA-CaT1 showed markedly reduced inward Ca2+-current in response to thapsigargin (Fig. 6, B and C). Moreover, the Ca2+-current augmentation that normally occurs after cycling from Ca2+-free to Ca2+-containing external solutions (see Fig. 3C) was greatly blunted in Jurkat cells transfected with TRIA-CaT1 (Fig. 6, B and C). I_{CRAC} in Jurkat cells can be converted to a large conductance nonspecific cation current when external divalent species are reduced sufficiently (8, 13). When we examined cells in divalent-free external media (DVF), we observed a near-complete suppression in Jurkat cells transfected with TRIA-CaT1 (Fig. 6D). To confirm that overexpression of TRIA-CaT1 in Jurkat cells reduced endogenous CCE, we examined Fura-2-loaded Jurkat cells that were transfected with GFP, CaT1, TRIA-CaT1, or untransfected cells (Fig. 6E). Thapsigargin was applied to cells in Ca2+-free containing solution (2 mM) was superfused to observe the rate and extent of CCE. The extent of [Ca2+]i elevation in untransfected cells and GFP-transfected cells was comparable, whereas cells transfected with CaT1 accumulated internal Ca2+ faster and to a greater extent (Fig. 6E). Cells transfected with TRIA-CaT1 have significantly slower Ca2+-entry with less total accumulation of Ca2+ i. Thus, TRIA-CaT1 expression was capable of suppressing endogenous I_{CRAC} and CCE in Jurkat cells.
discuss ion

In summary, the data presented here confirm and quantify the expression of CaT1 mRNA in Jurkat T-lymphocytes and provide pharmacological and functional evidence for the contribution of CaT1 to the endogenous I_{CrAC}. Despite the critical role that CCE plays in T-lymphocyte activation, experimental evidence suggests that resting T-cells express relatively few active channels on the surface with the biophysical estimate at 100–400 SOCCs per Jurkat cell (13). Our demonstration that CaT1 expression is lower in Jurkat cells than in placenta corresponds with this estimate. The biological function of SOCC in the two tissues is also compatible with the relative abundance of the channels; in lymphocytes finely tuned Ca^{2+} signaling only occurs briefly during immune responses and effects a signal transduction program. In the placenta, however, CaT1 channels are likely required for nutrition with constitutively active absorption of large amounts of Ca^{2+}.

When we overexpress CaT1 in Jurkat cells, the resulting current possessed characteristics of the endogenous I_{CrAC}—however with some differences. Of note, the current was usually spontaneously activated upon breaking into a whole cell patch configuration. When high concentrations of Ca^{2+} chelators are present in the pipette solution, activation of CCE can occur; however, we observed the same activation when pipette solutions contained less chelator (from 10 to 0.5 mM EGTA, data not shown). The CaT1-dependent current was only partially responsive to Ca^{2+}-stores depletion in transfected cells. As discussed by Yue et al. (31) this may be due to mismatched numbers of surface channels and the proteins involved in sensing and transmitting the information of the state of Ca^{2+} stores. It is possible that CCE is composed of a macromolecular complex with a precise stoichiometry that may be disrupted or uncoupled by forced overexpression of one element such as the CaT1 subunit. The [Ca^{2+}]_{i}-dependent fast and slow inactivation of I_{CrAC} (10, 11), however, appears intact in heterologously expressed CaT1.

In assaying for molecular candidates responsible for I_{CrAC} in Jurkat cells, we also detected the expression of EcaC1a, a member of the TRPV family, ~75% amino acid homology to CaT1. The electrophysiological properties reported for these two channels are also similar (39). The sequence similarity between CaT1 and EcaC1 is such that the probe we used for Northern analysis may cross-react with EcaC1a, which would be indistinguishable from a CaT1 signal; however, primers used for RT-PCR were CaT1-specific. Accordingly, we cannot exclude the possibility that EcaC1a also contributes to I_{CrAC} in Jurkat lymphocytes. The suppression of endogenous I_{CrAC} by mutant TRIA-CaT1 would argue against a functional contribution by EcaC1a unless CaT1 and EcaC1a are capable of forming heterotetrameric channels. We also detected and cloned another member in the TRPV family, VRL1, from Jurkat T-cell RNA (data not shown). Heterologous expression of VRL1 in CHO cells, however, could not produce detectable current at base line or upon thapsigargin treatment despite verified protein expression (at room temperature). Considering that the VRL1 channel is activated by extremes of temperature or pH (46) and that it normally supports a relatively non-selective cation current, it seems unlikely that it is responsible for I_{CrAC} in Jurkat. As in the case of EcaC1a, we cannot exclude the possibility that VRL1 could also participate in a heterogenous macromolecular complex that enables CCE.

The evidence supporting a role for CaT1 in lymphocyte CCE includes its expression in cells, its biophysical similarity to the endogenous current, its partial regulation by Ca^{2+} stores, and the pharmacological block by capsaicin. The most compelling argument, however, comes from experiments with TRIA-CaT1, the CaT1 mutant with an altered pore region that abolishes Ca^{2+} conductance. We interpret the ability of TRIA-CaT1 to suppress current from wild type CaT1 as a specific co-assembly of wild type and mutant subunits into tetramers that fail to effectively pass Ca^{2+} ions. That TRIA-CaT1 also suppresses endogenous I_{CrAC} in Jurkat cells is strong evidence that the mutant is interacting with and dominating CaT1 that is responsible (at least in part) for the native current. That capsaiacin blocks both CaT1 and I_{CrAC}, but SKF 96365 only blocks the native current suggests that CaT1 might be the direct target of capsaicin, whereas SKF 96365 might indirectly inhibit CCE via the pathway between store-depletion and channel opening (thus explaining the slower kinetics of SKF 96365 action (43)).

The reported discrepancy between pore conductance properties of I_{CrAC} and CaT1 in RBL mast cells (32) may be due to one of several possibilities. Different cell types and tissues may express unique profiles of proteins to produce CCE, and as such I_{CrAC} in different cells may vary. This may be due to differential expression of channel subunits such as CaT1 or ECAC or other members of the TRP family. Variation due to alternative splicing is another possibility. Another very likely explanation is that heterologous expression of channel subunits frequently fail to completely recapitulate the endogenous phenotype because expression systems often lack additional subunits or accessory proteins that are present in the native tissue. Nonetheless, our results strongly point to CaT1 as a necessary contributing protein in the production of T-lymphocyte CCE. This provides a clear starting point for further identification of all the proteins that are involved in generating the signal cascade that begins with release of Ca^{2+} stores and results in sustained and complex Ca^{2+} signaling.

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