TRPC3 Mediates T-cell Receptor-dependent Calcium Entry in Human T-lymphocytes*

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Stimulation of the T-cell receptor (TCR) activates Ca2+ entry across the plasma membrane, which is a key triggering event for the T-cell-associated immune response. We show that TRPC3 channels are important for the TCR-dependent Ca2+ entry pathway. The TRPC3 gene was found to be damaged in human T-cell mutants defective in Ca2+ influx. Mutations of the TRPC3 gene were accompanied by changes of TRPC3 gene expression. Introduction of the complete human TRPC3 cDNA into those mutants rescued Ca2+ currents as well as TCR-dependent Ca2+ signals. Our data provide the initial step toward understanding the molecular nature of endogenous Ca2+ channels participating in T-cell activation and put forward TRPC3 as a new target for modulating the immune response.

Following stimulation of the TCR through antigen-presenting cells, several signaling pathways are initiated that result in activation and proliferation of T-cells (for review see Ref. 1). One of those pathways involves receptor tyrosine kinase-dependent activation of PLCγ, which cleaves phosphorylatedinositol 4,5-bisphosphate and generates the second messengers InsP3 and DAG. InsP3 binds to the InsP3R in the membrane of the endoplasmic reticulum and initiates release of its stored Ca2+. The depletion of Ca2+ from the endoplasmic reticulum activates SOC channels in the plasma membrane. These channels contribute to long lasting elevations of the intracellular calcium concentration [Ca2+]i, necessary for antigen-dependent T-cell activation and central for the T-cell-dependent immune response. SOC channels are defined as cation or Ca2+ entry channels considered to open as a direct result of store depletion. They can be activated by passive store depletion independently of PLC-coupled receptor activation using endoplasmic reticulum Ca2+ pump blockers like TG. The mechanisms linking store depletion to Ca2+ entry are not fully understood, and it has been proposed that multiple mechanisms for signaling store operated Ca2+ entry may exist, including a conformational coupling of SOC channels to molecules located in the endoplasmic reticulum as well as a diffusible factor released from the stores (2).

In T-cells, store-operated Ca2+ channels resemble Ca2+-release-activated Ca2+ channels (CRAC) found in mast cells, (3, 4), rat basophilic leukemia cells (5), and DT40 B lymphocytes (6, 7). CRAC channels are the prototype of highly Ca2+-selective SOC channels, and their importance in T-cells is underscored by the devastating consequences of their functional impairment in some patients with severe-combined immunodeficiencies (8–10).

SOC channels could be viewed as a subfamily of receptor-operated Ca2+ channels, which are activated in a PLC-dependent manner following receptor stimulation and which depend on store depletion. Different PLC-dependent Ca2+ entry pathways, including activation by second messengers like InsP3 or arachidonic acid (reviewed in Ref. 11) coexist in one cell and might influence each other (12, 13). In DT40 B lymphocytes, it was shown that cation entry involves not only store-operated channels but also a population of Ca2+ channels requiring functional InsP3Rα (14).

Because receptor-operated Ca2+ channels are localized in the plasma membrane and can be easily accessed, they would constitute an excellent pharmacological target for drugs modulating immune function. The problem, however, is that in many cell types, including T-cells, the molecular composition and identity of those channels is still unknown. One approach to identify the molecular nature of TCR-dependent Ca2+ channels in T-lymphocytes was to generate random Jurkat T-cell mutants and select those with specific defects in Ca2+ entry and Ca2+-dependent gene expression (15). Five of those independent mutants, CJ-1 to CJ-5, have been characterized in detail. To varying degrees, they all show a reduction of Ca2+ channel activity and, correspondingly, a reduction of Ca2+ influx as well as Ca2+-dependent gene expression, strongly suggesting that mutations of the TCR-dependent Ca2+ channels underlie the mutant phenotypes. The molecular defects, however, were never identified.

Members of the family of mammalian TRP-related proteins (for nomenclature see Ref. 16) have been implicated in both receptor- and store-operated Ca2+ entry, but data linking a specific TRP protein with an endogenous Ca2+ influx channel is sparse (reviewed in Ref. 17). TRPC3 is one of the best studied members of the mammalian TRP family, but the analysis of its functional properties relies mostly upon the characterization of heterologously expressed proteins. Most reports agree that...
TRPC can be activated in a PLC-dependent manner and forms non-selective cation channels when expressed in heterologous expression systems (18). However, there is also evidence that, depending on the cell type used for overexpression, TRPC3 channels can be activated spontaneously, by InsP₃, DAG, Ca²⁺, and G-proteins, and following store depletion. Transient expression of TRPC3 in DT40 B lymphocytes for example revealed both InaP₃-R-dependent and InaP₃-R-independent channel activity (19, 20).

In other cell types, there is evidence that TRPC3 channel activity depends upon interaction with InaP₃ and/or rydogione receptors (21–26). This activity seems to be modulated by the binding of Ca²⁺/calmodulin (24, 27) and by the binding of phospholipase Cγ (28). The different findings regarding TRPC3 might at least in part be reconciled by the fact that TRPC3 is able to build heterooligomers with other members of the TRPC subfamily (29) that are to various degrees expressed endogenously in many cell types.

In this report, we describe the molecular and functional analysis of mutant T-cell lines with specific defects in Ca²⁺ entry and Ca²⁺-dependent gene expression (15) focusing on potential defects of TRPC proteins. We found the TRPC3 gene to be damaged in five independent mutant cell lines when compared with wild type cells, whereas TRPC1, TRPC4, TRPC5, and TRPC6 were unaffected. The alteration of the TRPC3 genotype was accompanied by a highly increased expression of truncated TRPC3 transcripts. These findings led to the hypothesis that reduced Ca²⁺ channel activity could be explained by defects in the TRPC3 gene. This was tested by transient transfection of the human wild type TRPC3 in two of the mutant cell lines. TRPC3 was able to restore TRC-activated Ca²⁺ channel activity and the resulting Ca²⁺ signals. We conclude that TRPC3 is part of endogenous Ca²⁺ channels in human T-lymphocytes and contributes to TCR-dependent Ca²⁺ entry in human T-lymphocytes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**Jurkat T-cell lines were grown in culture medium consisting of RPMI 1640 supplemented with 10% fetal calf serum and penicillin-streptomycin as described previously (15). Reagents include thapsigargin (TG, 1 μM stock in MeSO₂, Molecular Probes), isositol 1,4,5-trisphosphate (InsP₃, 20 μM stock in H₂O, Calbiochem), and phytylephedrinlhitin (PHA, 10 μg/ml stock in H₂O, Sigma). OX2 was a generous gift from Andreas Stahl (Hamburg). All other chemicals were from Sigma (highest grade).

**Southern Blot Analysis—**Genomic DNA was prepared according to standard protocols (30). 20 μg of DNA from Jurkat parental cells and the mutants CJ-1, CJ-2, CJ-3, CJ-4, and CJ-5 were each digested with EcoRI, BamHI, HindIII, BglII, or XbaI. Half of the material was loaded onto one lane of each of two 0.7% agarose gels, separated by electrophoresis, and transferred to nylon membranes by capillary transfer to obtain blots 1 and 2 (Fig. 1). The probes were labeled by random priming with [α-³²P]dCTP (6000 Ci/mmol). Hybridizations were performed at 68 °C in 0.9 M NaCl, 90 mM sodium citrate, 0.1% bovine serum albumin, 0.1% Ficoll Type 400, 0.1% polyvinylpyrrolidone, and 0.5% SDS (29). The membranes were washed under stringent conditions (29 °C, 15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS). In a first round of hybridization we used the coding cDNA of human TRPC3 (accession number U47050) as a probe for blot 1 and the complete cDNA of human TRPC4 (accession number AF175406) for blot 2. The filters were stripped, and blot 1 was sequentially hybridized to nucleotides 2069–4773 of human INADL (accession number AF294477) and to the coding cDNA of human TRPC6 (accession number AJ271066). Blot 2 was hybridized to the coding cDNAs of human TRPC1 (accession number NM003304) and mouse TRPC5 (accession number AJ006204). Results were confirmed in an independent second experiment. Filters were exposed for 3–10 days to x-ray films with intensifying screens (–80 °C). Northern blots were performed as described previously (31) using 10 μg of poly(A)⁺ RNA from Jurkat T-cells, rabbit brain and lung, human salivary gland adenocarcinoma cells HSG (kindly provided by Dr. Mitsubou Sato, Tokushima University School of Dentistry, Japan), human choriocarcinoma JEG-3 cells (ATCC HTB-36), and human placenta. For detection of TRPC transcripts the same procedures as for Southern blot analysis were used. TRP3 and TRP1 mRNAs were detected as described (32). The filters were exposed to x-ray films with intensifying screens at –80 °C. One filter was used for the analysis of TRPC3 and TRPC1 expression, and another filter was used for the analysis of TRPC5, TRPC4, and TRPC6 (in that order). For that purpose, filters were stripped, exposed to x-ray films to control residual signals, and hybridized with the next probe and, finally, exposed to x-ray film. Combined analysis of TRPC3 transcripts in the T-cell mutants was performed in two independent experiments with identical results.

**Generation and Purification of TRPC3 Antibodies—**To generate polyclonal anti-TRPC3 antibodies, we expressed a fusion protein consisting of the N-terminal 213 amino acids of TRPC3 (accession number U47050) fused to the maltose-binding protein (MBP) using the pMal system (New England BioLabs) similar as described (33). The MBP-TRPC3 fusion proteins were bound on amyllose resin columns and eluted as described by the manufacturer before they were fractionated by SDS-PAGE, electrophoresed (Bio-Rad), lyophilized, and used to immunize rabbits. Serum obtained before and after immunization was tested for its reactivity against TRPC3 fusion proteins. To purify the anti-TRPC3 antibodies, MBP and MBP-TRPC3 fusion proteins were linked to N-hydroxysuccinimidyl-activated-Sepharose resin columns (Amerham Biosciences). Antibodies directed against MBP alone were removed from the serum by chromatography on MBP-Sepharose. TRPC3 antibodies (AT3) were then enriched on MBP-TRPC3-Sepharose and checked for their reactivity against MBP-TRPC3 and MBP on Western blots. Specificity of the antibodies was determined as described under “Results.”

**Immunoprecipitation and Immunoblotting—**For immunoprecipitations, 10⁷ Jurkat cells were washed twice with ice-cold phosphate-buffered saline (136 mM NaCl, 2.7 mM KCl, 1.47 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.4), suspended in 1.5 ml of radiolabeled immune precipitation assay buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 0.5% sodium deoxycholate, 1% (octylphenoxypolyethoxyethanol (Igepal CA-630), 0.1% SDS, 5 mM EDTA, 0.1% phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μg/ml aprotinin, 10 μg leupeptin, 1 μg soybean protease inhibitor). The suspension was homogenized at 4 °C by a Polytron homogenizer and by subsequent pressing through a 26-gauge cannula. After centrifugation for 15 min at 12,000 g the supernatant was transferred to a fresh tube. Nonspecific bindings were removed by a preincubation with 100 μl of protein-A-Sepharose (Amerham Biosciences) for 1 h followed by centrifugation for 20 s at 12,000 g. 100 μl of the supernatant was kept to quantify the protein concentration using the BCA determination method. 1 ml of the supernatant was then incubated with 10 μl of AT3 or the polyclonal anti-TRPC4 antibody AT4 overnight at 4 °C (AK236 (34)). Immunocomplexes were precipitated by incubation with 100 μl of protein-A-Sepharose for 1 h, centrifuged, and washed three times with radiolabeled immune precipitation assay buffer. Immunocomplexes bound to protein-A-Sepharose were solubilized in 100 μl of 2 × Laemmli buffer at 95 °C for 5 min. The samples were centrifuged briefly at 12,000 g. 5–15 μl of each sample, corresponding to the same amount of protein before precipitation, were subjected to SDS-PAGE.

**In vitro translation of bovine TRPC4 was performed using the Tnt™ system (Promega). Immunoblots and preparations of lysates from HEK 293 cells and HEK 293 cells stably expressing human TRPC3 (accession number U47050, clone T3-9 (35)) or the short variant of human TRPC4 (accession number AF063823) were performed as described previously (34). Labeled proteins were detected using a horseradish peroxidase-labeled secondary antibody and the Renaissance Western blot chemiluminescence reagent (PerkinElmer Life Sciences). As negative control in Fig. 2B (upper panel) was reprobed with antibody AT4 (4 μg/ml, Fig. 2B, lower panel). For that purpose, the filter was stripped by 30-min incubation in 62.5 mM Tris-Cl, pH 6.8, 2% SDS, 100 μl β-mercaptoethanol at 50 °C and checked for remaining signals before application of AT4 and the secondary antibody.**
of green cells after transfection of IRES constructs was below 5% we obtained between 30 and 50% green cells in co-transfection experiments. The efficiency of co-transfection was tested by electroporation of 2 μg of EGFP-pCAGGS and 6 μg of EBFP-pCAGGS encoding the green and the blue fluorescent proteins, respectively. Analyzing more than a hundred green cells we found only one without blue fluorescence, indicating that under the chosen experimental conditions the rate of co-transfection is near 100%. For most of the patch-clamp experiments, we used the IRES constructs, because only a few transfected cells were needed, whereas for Ca2+ imaging we routinely used the co-transfection protocol to get a sufficient number of transfected cells. Imaging experiments were also repeated with the IRES constructs giving similar results.

**Electrophysiology**—The standard pipette solution for whole-cell patch-clamp recordings contained (in mM): 0.65 InsP3, 140 cesium aspartate, 10 NaCl, 3 MgCl2, 10 EGTA, and 10 Hepes (pH 7.2 with CsOH). The bath solution contained (in mM): 155 NaCl, 4.5 KCl, 22 CaCl2, 1 MgCl2, 10 glucose, and 5 Hepes (pH 7.4 with NaOH). Patch-clamp experiments were performed at 22 °C in the tight-seal whole-cell configuration using fire-polished patch pipettes (2–3 MΩ uncompensated series resistance). Pipette and cell capacitance were electronically cancelled before each voltage ramp with an EPC-9 patch-clamp amplifier controlled by Pulse 8.3 software (Heka Elektronik). Membrane currents were filtered at 1.5–2.3 kHz and digitized at a sampling rate of 5–10 kHz. Whole-cell currents were elicited by 100-ms voltage-clamp ramps from 0 to +60 mV from a holding potential of 0 mV and measure leak currents before activation of store-operated currents. 20 voltage ramps were applied within the first 5 s after establishment of the whole-cell configuration, followed by voltage ramps applied every second. All voltages were corrected for a liquid junction potential of –12 mV between internal solutions and the bath solution. Transfected green cells were identified by their EGFP fluorescence.

**Fluorescence Measurements**—Cells were loaded at room temperature (22–23 °C) for 20 min with 1 μM fura-2/AM in culture medium with 10 mM HepES added (to buffer pH), washed with fresh medium, and stored in the dark at room temperature until use. Loaded cells were allowed to adhere to poly-l-ornithine-coated glass coverslip chambers on the stage of an Olympus IX 70 microscope equipped with a ×20 UPlanApo 340 (numerical aperture (N.A.) 0.75) or a ×40 UPlanApo (N.A. 1.0) objective. Cells were alternately illuminated at 340 and 380 nm with a polychrome IV Monochromator (TILL Photonics). The fluorescence emissions at λ > 440 nm were captured with a charge-coupled device camera (TILL Imago), digitized, and analyzed using TILL Vision software. Ratio images were recorded at intervals of 5 s. [Ca2+]i was estimated from the relation [Ca2+]i = K3′Rmax + E3′Rmin where the values of K3′, Rmax, and Rmin were determined from an in situ calibration of fura-2 in Jurkat T-cells as described previously (37). Ca2+ Ringer’s solution contained (in mM): 155 NaCl, 4.5 KCl, 0–2 CaCl2, 1 MgCl2, 10 glucose, and 5 Hepes (pH 7.4 with NaOH). CaCl2 was replaced by MgCl2 in the 0-Ca2+ Ringer’s solution with 1 mM EGTA added. TRPC3 and TRPC6 endogenous and transfected proteins were identified using 480-nm excitation (excitation filter: HQ 470/40, dichroic mirror Q 495 LP, emission filter HQ 525/50; all from AHF Analysetechnik, Germany). To determine an objective criteria to identify a green (= EGFP-expressing) cell, we used the following approach: Cells only transfected with vector or a construct not coding for EGFP were analyzed first resulting in the mean ± S.D. arbitrary fluorescence of non-green cells following 480-nm excitation. Cells were considered to be EGFP-positive if the arbitrary fluorescence was higher than the mean of non-transfected cells plus three times the standard deviation. Cells of the same experiment were considered EGFP-negative (= control cells) if the arbitrary fluorescence was within the standard deviation of the mean of non-EGFP-transfected cells. Cells with an intermediate fluorescence (between one and three times the standard deviation) were disregarded for the analysis.

**RESULTS**

**T-cells with Defects in Store-operated Calcium Entry Are Mutated in the TRPC3 Gene**—To facilitate the molecular identification of Ca2+ channels in T-lymphocytes, Fanger et al. (15) generated random T-cell mutants with defects in TCR-dependent Ca2+ signaling pathways. The mutant lines were characterized in detail and were found to have reduced Ca2+ channel activity and reduced Ca2+−dependent gene expression. The molecular defects generating this phenotype, however, have not been elucidated. Proteins of the mammalian TRPC channel family are presently the best candidates to form receptor- and store-operated Ca2+ channels. We therefore systematically compared the genotypes of TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6 in five mutant T-cell lines C5-1 through C5-5 and wild type parental cells by Southern blot. Hydrolysis of genomic DNA with several restriction endonucleases and hybridization with the cDNA of TRPC4 and hINADL (Fig. 1A) and with TRPC1, TRPC5, and TRPC6 (data not shown) showed an identical pattern of genomic fragments indicating that these genes are not affected by the mutagenesis procedure. Using the TRPC3 cDNA as probe for hybridization, we found the same band pattern for genomic DNA in Jurkat parental cells and in peripheral blood lymphocytes from two control persons (Fig. 1B, left panel) apart from fragments at ~5.5 kb specific for Jurkat parental cells. These fragments might arise from genomic imbalances in the lymphoblastic cell line (38). In contrast, we found divergent signals in every mutant line when compared with parental cells with each of the restriction enzymes used for hydrolysis of the DNA (Fig. 1B, right panel) indicating defects of the TRPC3 gene. Every mutant was affected and no mutant was found to have the same TRPC3 genotype as one would expect after generation of mutants by random mutagenesis and selection for a specific phenotype. These results link the phenotype of reduced Ca2+ entry in the mutant T-cells to the TRPC3 gene and suggest that the TRPC3 protein is part of TCR-dependent Ca2+ channels in T-lymphocytes.

**Expression Pattern of TRPC Channels in Jurkat T-lymphocytes**—To investigate which TRPC genes are expressed in Jurkat T-lymphocytes, we performed Northern blot analysis (Fig. 2A) and found TRPC3 transcripts of ~4.0 kb corresponding in size to transcripts present in human brain (35) likely to encode the functional TRPC3 channel protein. Shorter transcripts of ~3.6 and ~1.2 kb were also detected, which might arise from alternative splicing. In addition, we found weak expression of TRPC1, and possibly TRPC4 transcripts, whereas TRPC5 and TRPC6 mRNA could not be detected. Recently, TRPV6 (CaT1, CaT-L) has been described to contribute to store-operated calcium current in Jurkat T-cells (39). We could not detect the TRPV6 transcripts of the expected size of 3.0 kb in Jurkat T-cells, whereas 3.0-kb TRPV6 transcripts are readily detected in human placenta and the human JEG-3 cells (Fig. 2A, right panel) as well as in pancreas and prostate carcinoma (32).

We also looked for the presence of TRPC channel proteins by Western blot (Fig. 2B). The polyclonal antibodies AT3 and AT4 raised against the N terminus of TRPC3 and the C-terminal peptide of TRPC4 (34), respectively, detected TRPC3 as well as TRPC4 in crude protein extracts of HEK293 cells stably over-expressing recombinant TRPC3 or TRPC4 channels but failed to detect endogenous TRPC proteins in HEK 293 cells (Fig. 2B) or in microsomal membranes of Jurkat parental cells (data not shown). To allow detection of intermediate or low protein expression, we used the antibodies for immunoprecipitation. With AT4 we could not detect any protein in Jurkat cells indicating that TRPC4 is not expressed or its abundance is very low. In contrast, we found endogenous TRPC3 proteins to be present in Jurkat parental cells. These proteins migrate in protein gels as a sharp band different to recombinant TRPC3 proteins overexpressed in HEK293 cells, which could be explained by the different levels of TRPC3 glycosylation occurring in these cells (40).

**Changes of TRPC3 Gene Expression in Mutants Defective in Store-operated Calcium**—We investigated if the differences of
the mutant TRPC3 genes are linked to changes of TRPC3 gene expression. Therefore, we analyzed TRPC3 transcripts in mutant and parental cells (Fig. 2C). Similar to the parental cells, the CJ-5 mutants expressed transcripts of ∼3.6 and ∼1.2 kb in size. In contrast, ∼4.0-kb transcripts were missing and additional shorter transcripts of ∼3.1 and ∼2.1 kb were present. In CJ-1 cells we also found additional mRNA species of ∼3.1 and ∼1.6 kb in length. These additional transcripts likely reflect the expression of an altered TRPC3 allele. Moreover, we found a strong enhancement of TRPC3 gene expression in the mutant cells. Similar changes of TRPC3 gene expression were also found in the mutants CJ-2, CJ-3, and CJ-4 (data not shown). Thus, the mutant phenotype of reduced TCR-dependent Ca2⁺ entry is apparently linked to changes of TRPC3 gene expression.

Analyzing TRPC3 on the protein level, we found expression in the parental cells and at a slightly higher density in CJ-1 and CJ-5 mutants (Fig. 2D). Potentially truncated TRPC3 proteins, corresponding to the additional transcripts observed in the mutated cell lines, could not be detected by this approach. Apparently, the epitopes recognized by the AT3 antibody were absent in truncated versions of TRPC3 or these variants are similar in size to the heavy chains of the AT3 antibody (∼55 kDa) and thereby escaped detection after immunoprecipitation.

Jurkat is a pseudodiploid cell line with polyploidy occurring at various degrees. In an initial attempt to clone human TRPC3 by RT-PCR, 13 independent clones were isolated and sequenced.² Among these 13 cDNAs, 7 different TRPC3 splice variants were identified, 5 of which yielded truncated TRPC3 proteins. In the parental cell line, the most abundant transcripts were ∼1.2 kb in length. Apparently, the parental Jurkat

² U. Wissenbach, S. Philipp, and V. Flockerzi, unpublished data.
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**Fig. 2. Expression pattern of TRP genes in Jurkat T-lymphocytes and differences of TRPC3 expression in T-cells mutants.** In A: upper panels, Northern analysis of TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, and TRPV6 in Jurkat cells, rabbit brain and lung, human HSG and JEG-3 cells, and human placenta. Lower panels, analysis of the same filters with the human glyceraldehyde-3-phosphate dehydrogenase cDNA. The letters correspond to the lanes in the upper panels. In B: upper panel, immunoblot using anti-TRPC3 antibodies (AT3) for detection. HEK, crude lysate of HEK 293 cells; HEK-TRPC3, crude lysate of HEK 293 cells overexpressing human TRPC3; HEK-TRPC4, crude lysate of HEK 293 cells overexpressing human TRPC4, i.e., TRPC4, in vitro translated bovine TRPC4, Jurkat IP-AT3, immunoprecipitated Jurkat proteins using anti-TRPC3 antibodies; Jurkat IP-AT4, immunoprecipitated Jurkat proteins using anti-TRPC4 antibodies. Lower panel, the same blot is shown after stripping and incubation with anti-TRPC4 antibodies (AT4). C, Northern blot analysis of TRPC3 transcripts of parental, CJ-5, and CJ-1 cells after exposure of the blot to x-ray films for 3 days (left panel) or 3 weeks (right panel). The same filter is shown after hybridization to the human glyceraldehyde-3-phosphate dehydrogenase cDNA (lower panel, 3-h exposure). D, Western blot using anti-TRPC3 antibodies (AT3). AT3 recognizes TRPC3 in HEK 293 cells stably expressing TRPC3 (HEK-TRPC3) and in AT3 immunoprecipitates from parental cells (Parental IP-AT3). CJ-1 mutants (CJ-1 IP-AT3), and CJ-5 mutants (CJ-5 IP-AT3). The band at ~55 kDa probably corresponds to the heavy chain of immunoglobulin G used for precipitation.

**TCR Stimulation Enhances Ca^{2+} Signals in TRPC3-transfected Mutant T-cells—**In the absence of external Ca^{2+}, stimulation of TCR with phytohemagglutinin (PHA) resulted in typical Ca^{2+} spikes due to Ca^{2+} release, which were similar in CJ-5 and parental cells (Fig. 3A). The Ca^{2+} influx across the plasma membrane following re-addition of external Ca^{2+}, however, was significantly decreased in the CJ-5 mutants as shown for representative single cells as well as for the average of all analyzed cells (Fig. 3, B and C).

The same protocol was used to analyze PHA-induced Ca^{2+} signals in CJ-5 mutants following transient overexpression of wild type TRPC3 (accession number U47050, Fig. 3, D–F). There was no difference between green cells transfected with EGFP alone and non-green control cells pooled from co-transfections of TRPC3 and EGFP. Analysis of the green cells from the TRPC3-EGFP co-transfection experiments, however, revealed that their Ca^{2+} influx is different from both control groups. The peak of the [Ca^{2+}], rise as well as the [Ca^{2+}], plateau were significantly increased (Fig. 3, E and F) indicating that TRPC3 channels enhance Ca^{2+} influx following T-cell stimulation with PHA.

To test our hypothesis with a more specific stimulus of the TCR, we used the anti-CD3 antibody OKT3, previously used to characterize the defects of Ca^{2+} influx in the T-cell mutants (15). Following stimulation with OKT3, Ca^{2+} influx was significantly increased in TRPC3-transfected CJ-5 cells (Fig. 4, A–C). Both, the OKT3 and the PHA experiments indicate that TRPC3 is able to restore TCR-dependent Ca^{2+} influx in the mutants.

Restoration of Ca^{2+} influx was also analyzed after transfection of TRPV6, recently suggested to participate in store-operated Ca^{2+} entry in Jurkat cells (39) and after transfection of TRPC4 previously shown to participate in receptor-dependent Ca^{2+} entry in other cell systems (34, 41, 42). Introduction of human TRPC4 did not enhance TCR-dependent Ca^{2+} signals in CJ-5 T-cells (Fig. 4D). Overexpression of TRPV6 was analyzed in CJ-1 mutants, because the endogenous Ca^{2+} entry is much smaller than in CJ-5, allowing a better comparison of
Ca\textsuperscript{2+} signals before and after TCR stimulation (Fig. 4E). TRPV6 increased the levels of resting [Ca\textsuperscript{2+}]\textsubscript{i} as has been previously reported (32). Comparing Ca\textsuperscript{2+} entry before and after TCR stimulation, however, no difference could be detected (compare arrows in Fig. 4E). These results indicate that TRPC3 but, under our experimental conditions, not TRPC4 or TRPV6 enhance TCR-dependent Ca\textsuperscript{2+} signals in human T-lymphocytes.

**Thapsigargin Increases Ba\textsuperscript{2+} but Not Ca\textsuperscript{2+} Influx in TRPC3-transfected Cells**—In the current picture of T-cell activation, Ca\textsuperscript{2+} entry is solely activated in a Ca\textsuperscript{2+} store-dependent manner (43). Thus, one would predict that TRPC3 should also enhance Ca\textsuperscript{2+} signals following stimulation with thapsigargin (TG), which depletes Ca\textsuperscript{2+} stores through inhibition of the SERCA Ca\textsuperscript{2+}-ATPase. To our surprise, TG-dependent Ca\textsuperscript{2+} influx was not significantly enhanced in TRPC3-transfected CJ-5 and CJ-1 cells (data not shown). In DT40 lymphocytes, it has been shown that TG-dependent TRPC3 activity can be resolved when using Ba\textsuperscript{2+} as a surrogate for Ca\textsuperscript{2+} (44). Endogenous Ca\textsuperscript{2+} channels in lymphocytes are also permeable to Ba\textsuperscript{2+} (45), the corresponding fura-2 ratio signal changes (340/380), however, are quite small when compared with the changes in fura-2 ratios with Ca\textsuperscript{2+} as the charge carrier (46).

We analyzed Ba\textsuperscript{2+} entry in T-cells and confirmed the small changes in fura-2 ratios following store depletion with TG. In TRPC3-transfected CJ-5 cells, we found a significant enhancement of Ba\textsuperscript{2+} entry compared with non-transfected cells (Fig. 5A). The subsequent Ca\textsuperscript{2+} rise as well as the plateau were not increased confirming that TG did not increase Ca\textsuperscript{2+} signals in TRPC3-transfected cells. To account for cell-to-cell variation of
the endogenous Ca\(^{2+}\) channel number, the initial rate of the Ba\(^{2+}\) rise was normalized against the initial rate of the Ca\(^{2+}\) rise for each individual cell (Fig. 5B), revealing a significant difference of Ba\(^{2+}\) entry between TRPC3-transfected CJ-5 cells and controls (non-transfected or EGFP-transfected). We conclude that TRPC3 can participate in store-operated Ba\(^{2+}\) entry in T-cells.

It is surprising that TG enhances Ba\(^{2+}\) but not Ca\(^{2+}\) entry in TRPC3-transfected cells. Because it has been shown that Ca\(^{2+}\) channels in T-cells are regulated by mitochondrial Ca\(^{2+}\) uptake (47, 48) and that TRPC3 activity depends on functional mitochondria (49), we analyzed the role of mitochondria on TRPC3-dependent Ca\(^{2+}\) entry. Fig. 5C shows that, in the presence of 1 \(\mu M\) CCCP, which completely inhibits mitochondrial Ca\(^{2+}\) uptake in T-cells (47), Ca\(^{2+}\) entry was enhanced in TRPC3-transfected CJ-5 cells compared with controls. This result is consistent with an interaction between TRPC3 and mitochondria in T-cells in a way that mitochondria take up much of the Ca\(^{2+}\), which enters through TRPC3 channels activated by TG. This model could explain the differences found between TCR- and TG-activated TRPC3-dependent Ca\(^{2+}\) entry in T-cells.

TRPC3 Mediates TCR-dependent Calcium Entry

TRPC3 Rescues InsP\(_3\)-induced Currents in the Mutant Cell Lines—TCR stimulation is coupled to the activation of PLC\(_{\gamma}\) resulting in the formation of the second messengers InsP\(_3\) and DAG, both of which have been implicated to activate TRPC3 channels (18). Application of the DAG analogue 1-oleoyl-2-acetyl-sn-glycerol (100 \(\mu M\)) induced a small Ca\(^{2+}\) influx in parental cells and non-transfected CJ-5 control cells (46). However, no Ca\(^{2+}\) increase compared with control CJ-5 cells was observed in TRPC3-transfected CJ-5 cells (three experiments, \(n = 194\) green cells, data not shown) indicating that TRPC3 channels in Jurkat T-cells are not activated by DAGs, which is in contrast to other studies overexpressing TRPC3 in Chinese hamster ovary cells (50) and HEK 293 cells (23).

The involvement of InsP\(_3\) in TRPC3 activation was analyzed in whole-cell patch-clamp experiments following transient transfection of CJ-1 and CJ-5 mutant cells. InsP\(_3\) activated a small inwardly rectifying current (Fig. 6A) in mock transfected CJ-1 control cells (only EGFP) resembling reduced CRAC currents previously described (15). The amplitude of the currents in the TRPC3-transfected CJ-5 cells was significantly enhanced as shown in single cells (Fig. 6B) and summarized in Fig. 6C. In both CJ-1 and CJ-5, inward currents were increased to a value very close to the amplitude found in mock transfected parental cells. In contrast to other studies, TRPC3 currents were found to be inwardly rectifying with no significant outward component resembling endogenous CRAC currents. Identical results were obtained using the co-transfection protocol in

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**Fig. 4.** OKT3-induced Ca\(^{2+}\) entry in Jurkat T-cells is mediated by TRPC3 but not by TRPC4 or TRPV6. A, raw data of representative CJ-5 single cells stimulated with 4 \(\mu g/ml\) OKT3. B, average of \([Ca^{2+}]_i\) of all CJ-5 cells (six independent transfections), which showed Ca\(^{2+}\) release transients following the application of OKT3 in 0-Ca\(^{2+}\) Ringer’s solution. C, statistical analysis of all cells averaged in B. Data are given as mean ± S.E. (number of cells as shown). [Ca\(^{2+}\)]\(_i\) peak and plateau were found to be significantly different (Student’s t test, \(p < 0.01\) in TRPC3-transfected cells compared with non-transfected control cells from the same dish. D, same conditions as for TRPC3, except that CJ-5 cells were transfected with the cDNA of TRPC4. An average of one experiment is shown (\(n = 14\) and 25 cells). Identical results were obtained in six independent transfections. E, Ca\(^{2+}\) signals in CJ-1 lymphocytes transfected with the cDNA of TRPV6 were compared with control CJ-1 cells. An average of one out of four identical experiments is shown (\(n = 10\) and 15 cells).
CJ-5 mutants (current density, $-2.73 \pm 0.44$ pA/picofarad, $n = 8$). We conclude that InsP$_3$ activates TRPC3-dependent inward currents in T-lymphocytes.

**Selectivity and Properties of the TRPC3-dependent Current**—To test whether the TRPC3 currents were carried by Ca$^{2+}$ or Na$^+$, ion replacement experiments were performed in CJ-5 cells transfected with TRPC3. A typical experiment is shown in Fig. 7A in which the inward current is depicted as a function of time. Current responses to voltage ramps of the same cell are represented in Fig. 7B, and the statistical analysis of all experiments is summarized in Fig. 7C. On average, replacement of Na$^+$ by N-methyl-d-glucamine only led to a minor reduction of the current amplitude, whereas removal of extracellular Ca$^{2+}$ drastically reduced the current amplitude typical for CRAC channels. We conclude that TRPC3-dependent channels in T-lymphocytes have a high selectivity for Ca$^{2+}$ very similar to endogenous CRAC channels (1:1000 for Ca$^{2+}$:Na$^+$ (45)).

**DISCUSSION**

Ca$^{2+}$ influx has long been known to play an essential role during T-cell activation. Without Ca$^{2+}$ influx across the plasma membrane, no functional T-cell response can be elicited (43). The importance of Ca$^{2+}$ channels for the immune response is also obvious from patients with severe combined immunodeficiencies whose T-lymphocytes show drastically reduced Ca$^{2+}$ channel activity (8–10). Although many proteins have been implicated to function as receptor-operated cation/Ca$^{2+}$ channels, the molecular nature of T-cell receptor (TCR)-dependent Ca$^{2+}$ channels in T-lymphocytes is unknown.

The molecular analysis of mutant T-cell lines unmasked TRPC3 as a very good candidate to participate in TCR-dependent Ca$^{2+}$ entry. The altered TRPC3 genotype in the mutant cell lines results, however, in increased expression of transcripts and, consecutively, slightly higher levels of the TRPC3 protein compared with the parental cell line. The additional smaller transcripts present in the mutant cell lines might give rise to additional smaller TRPC3 variants, which, apparently, may lack the epitopes recognized by the available antibodies or which are hidden behind the antibody’s heavy chains after immunoprecipitation. The phenotype of defective TCR-dependent Ca$^{2+}$ entry therefore cannot be explained by the absence of TRPC3 but may well arise from the expression of dominant negative proteins that disturb the correct channel function. A similar dominant negative effect of truncated TRPC3 proteins upon endogenous Ca$^{2+}$ entry has already been shown after heterologous overexpression of an N-terminal fragment of TRPC3 in human umbilical vein endothelial cells (51).

We tried to overcome the potential dominant negative effect of truncated TRPC3 proteins by overexpressing full-length TRPC3 cDNA, and we indeed found increased Ca$^{2+}$ channel activity following stimulation of the cells. High Ca$^{2+}$ selectivity is a hallmark of Ca$^{2+}$ channels in T-lymphocytes, and we found TRPC3 to form highly Ca$^{2+}$-selective channels similar to endogenous Ca$^{2+}$ channels. This is in contrast to recent findings.
describing TRPC3 to form non-selective cation channels in primary pontine neurons (33) and, following overexpression, in HEK293 cells (22, 52, 53), Chinese hamster ovary cells (54), and bovine pulmonary artery endothelial cells (CPAE) (55).

Because there is evidence that TRPC3 is able to form heteromultimers with other proteins of the TRPC family (29), the differences of selectivity between TRPC3-dependent channels in T-cells and other cells could be explained by their different subunit compositions. A good candidate is TRPC1, which regulates store operated Ca2+ entry in DT40 B-lymphocytes (56) and is also expressed in T-cells.

Our molecular and physiological experiments identify TRPC3 as a protein important for TCR activated Ca2+ entry. This activation is InsP3-dependent, which is consistent with previous studies reporting an interaction of the C terminus of TRPC3 with the InsP3 receptor (21, 22, 24, 25) and the activation of a TRPC3-dependent cation conductance by InsP3 in HEK293 cells (22, 25) and in bovine pulmonary artery endothelial cells (55).

TRPC3-dependent activation of PLCγ and the subsequent formation of InsP3 is the physiological pathway to activate Ca2+ channels in T-cells but Ca2+ entry can also be activated by store depletion without the generation of InsP3 following application of thapsigargin (TG). In DT40 B-lymphocytes it has even been shown that the endogenous Ca2+ channels, which have very similar properties to the ones in T-cells, can be activated independently of InsP3 receptors (6, 7, 57). We could not detect a significant increase of Ca2+ entry following store depletion with TG in TRPC3-transfected T-cells, which compares well with some TRPC3 studies (54, 55, 58) but not with others (19, 22, 35, 44). Ba2+ has been used as a surrogate for Ca2+ in recent TRPC3 studies and therefore we tried Ba2+ in T-cells and observed that TG is able to activate TRPC3-dependent Ba2+ entry. Because Ba2+ is a poor substrate for Ca2+-dependent regulatory mechanisms, these findings might reflect a strong Ca2+-dependent regulation of TRPC3 channels. This hypothesis is supported by the finding that TRPC3 channel activity is tightly regulated by the interaction of TRPC3 with both the InsP3 receptor and Ca2+/calmodulin (24). Furthermore, mitochondrial Ca2+ uptake is likely to regulate Ca2+ entry through TRPC3 channels (49, this study), a mechanism very important for Ca2+ entry through CRAC channels in T-cells (47, 48).

Recently, Putney and colleagues (19, 44) have made an important step toward understanding the activation of TRPC3 by showing that receptor-dependent TRPC3 channels differ in their properties from store-operated TRPC3 channels in DT40 B lymphocytes. From their data, it appears likely that TRPC3 can be part of independent ion channels important for Ca2+ entry, which are activated and regulated in different ways. How this would fit in with the present picture of T-cell activation, can, at the moment, not be resolved. However, stimulation of the TCR is the physiological way to activate T-cells, and because TRPC3, which is endogenously expressed in T-cells, is activated through TCR-induced signaling, we have to conclude that TRPC3 mediates Ca2+ entry in T-cells and is important for the subsequent Ca2+-dependent activation of the cells.

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