Ca²⁺ entry through store-operated Ca²⁺ release-activated Ca²⁺ (CRAC) channels is essential for T-cell activation and proliferation. Recently, it has been shown that 3,5-bistrifluoromethyl pyrazole (BTP) derivatives are specific inhibitors of Ca²⁺-dependent transcriptional activity in T-cells (Trevillyan, J. M., Chiou, X. G., Chen, Y. W., Ballaron, S. J., Sheets, M. P., Smith, M. L., Wiedeman, P. E., Warrior, U., Wilkins, J., Gubbins, E. J., Gagne, G. D., Fagerland, J., Carter, G. W., Luly, J. R., Mollison, K. W., and Djuric, S. W. (2001) J. Biol. Chem. 276, 48118–48126). Whereas inhibition of Ca²⁺ signals was reported for BTP2 (Ishikawa, J., Ogha, K., Yoshino, T., Takezawa, R., Ichikawa, A., Kubota, H., and Yamada, T. (2003) J. Immunol. 170, 4441–4449), it was not found for BTP3 (Chen, Y. W., Smith, M. L., Chiou, X. G., Ballaron, S., Sheets, M. P., Gubbins, E., Warrior, U., Wilkins, J., Surowy, C., Nakane, M., Carter, G. W., Trevillyan, J. M., Mollison, K., and Djuric, S. W. (2002) Cell. Immunol. 220, 134–142). We show that BTP2 specifically inhibits CRAC channels in T-cells with an IC₅₀ of ~10 nM. It does not interfere with other mechanisms important for Ca²⁺ signals in T-cells, including Ca²⁺ pumps, mitochondrial Ca²⁺ signaling, endoplasmic reticulum Ca²⁺ release, and K⁺ channels. BTP2 inhibits Ca²⁺ signals in peripheral blood T-lymphocytes (in particular in CD4⁺ T-cells) and in human Jurkat T-cells. Inhibition of Ca²⁺ signals is independent of the stimulation method as Ca²⁺ entry was blocked following stimulation with anti-CD3, which activates the T-cell receptor, and also following stimulation with thapsigargin or inositol 1,4,5-trisphosphate. BTP2 also inhibited Ca²⁺-dependent gene expression (interleukins 2 and 5 and interferon γ) and proliferation of T-lymphocytes with similar IC₅₀ values. BTP2 is the first potent and specific inhibitor of CRAC channels in primary T-lymphocytes. The inhibition of CRAC channels as well as Ca²⁺-dependent signal transduction with similar IC₅₀ values in T-lymphocytes emphasizes the importance of CRAC channel activity during T-cell activation. Furthermore, BTP2 could be proved to finally unmask the molecular identity of CRAC channels.

Ca²⁺ entry through store-operated Ca²⁺ channels, also referred to as CRAC channels in T-cells, is an essential step during T-lymphocyte activation (1). Following antigen presentation, the TCR initiates several signaling cascades, including activation of phospholipase Cγ, which cleaves phosphatidylinositol 4,5-bisphosphate and generates the second messengers InsP₃ and diacylglycerol. InsP₃ releases Ca²⁺ from the endoplasmic reticulum and, following Ca²⁺ depletion, subsequently opens CRAC channels in the plasma membrane (2, 3). Ca²⁺ influx through those channels activates several transcription factors, which regulate the expression of cytokine genes critical for the immune response (4). The importance of CRAC channels for T-cell activity is evident by the close correlation of CRAC channel activity and gene expression (5) and by the devastating consequences of CRAC channel impairment in some patients with severe combined immunodeficiencies (6–8).

CRAC channels have been characterized in great detail in many different cell types, including T-lymphocytes (1, 9, 10). Their molecular identity, however, is still largely unknown. Specific inhibitors of CRAC channels could facilitate their molecular identification and would be excellent tools to study CRAC channel function. Unfortunately, all CRAC channel blockers described so far, including the most potent ones, SK&F 96365 and econazol (11, 12) or 2-aminoethylidiphenyl borate (13–18), have IC₅₀ values in the micromolar range and are nonspecific. Recently, Djuric and colleagues (19, 20) have described pyrazole derivatives that interfere specifically with the expression of Ca²⁺-dependent cytokine production following TCR stimulation, but they could not detect inhibition of TCR-dependent Ca²⁺ signals in T-cells (21). On the contrary, Ishikawa et al. (22) could demonstrate that one of the pyrazole derivatives named BTP2 (or YM-58483) is a potent inhibitor of store-operated influx in Jurkat T-cells. They did, however, not discriminate which of the mechanisms contributing to Ca²⁺ signals in T-cells were affected by BTP2.

We show that BTP2 is a very potent inhibitor of CRAC channels themselves in peripheral blood T-lymphocytes and Jurkat T-cells (IC₅₀ around 10 nM). It does not interfere with Ca²⁺ pumps, mitochondrial Ca²⁺ signaling, ER Ca²⁺ release, and K⁺ channels in T-cells, all of which greatly influence store-operated Ca²⁺ signals. BTP2 inhibits Ca²⁺-dependent gene expression and cell proliferation with similar potency as found for CRAC channels and the corresponding Ca²⁺ signals. BTP2 is the first specific and potent inhibitor of CRAC channels in primary human CD4⁺ T-cells from blood and in Jurkat T-cells.
**EXPERIMENTAL PROCEDURES**

**Cells—**PBls were purified from leukocyte reduction filters from the local blood bank. Cells were collected by back-flushing the filter with 125 ml of HBSS (PAA, 15–099). Peripheral blood mononuclear cells (PBMCs) were isolated by a density gradient centrifugation at 450 × g for 30 min at RT (Ficoll-Paque™ plus, Amersham Biosciences, #17144002) in 50 ml Leucosep tubes (Greiner, #227290). The PBMC layer was washed in HBSS. Remaining red blood cells were removed by the addition of 3 ml of lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1% Bovine serum albumin (BSA) for 3 min. After lysis, PBMCs were resuspended with HBSS (200 × g, 10 min, RT). Viability of the cells was checked by trypan blue. Cells were further purified by adhering to plastic for 24 h in RPMI 1640 complete medium at 37 °C (1.5 × 10⁶ cells/ml). Non-adherent cells, mostly PBls, were collected and used for proliferation assays.

**CD4⁺ T-lymphocytes were isolated from venous blood (250 ml) of healthy donors anti-coagulated with citrate (0.3% w/v) by successive purification steps. Centrifugation, percoll gradient, and two counter-current centrifugal elutriation steps were performed as described elsewhere (23). Afterward, lymphocytes were re-suspended in PBS containing 2% (v/v) FCS to a cell density of 1 × 10⁶ cells per 40 μl. CD4⁺ T-cells were obtained by negative selection using the CD4⁺ T-Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). CD8⁺ T-cells, γδT-cells, monocytes, dendritic cells, granulocytes, B-cells, erythroid cells, and NK-cells were labeled by biotin-modified antibodies directed against CD8, CD14, CD16, CD19, CD36, CD56, CD123, γδT-cells, and NK-cells. They were incubated via the CD3 chain of the T-cell receptor and the co-stimulatory molecules (25). All chemicals not specifically mentioned were from Sigma (St. Louis, MO). Reagents—

**Experimental Procedures**

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with commercially available enzyme-linked immunosorbent assay kits (Junk's IL-2, IFNγ, or IL-5, Immunotech, Marseille, France) following the instructions of the manufacturer.

For determination of proliferation, [³H]thymidine (0.2 μCi/well, added in 10 μl of culture medium) was present during the last 17 h of culture (72 h). Cells were harvested on a Tomtec Harvester 96 (Dunn Labortecnik, Asbach, Germany). Filter plates (Uni Filter-96, GF/C, Canberra-Packard, Dreieich, Germany) were washed three times with water at RT and then dried for about 1 h at 60 °C. Scintillator (MicroSint, Canberra-Packard) was added, and radioactivity was measured using the TopCount microplate scintillation counter from Canberra-Packard.

Proliferation experiments using the EZAU assay were carried out in 96-well cell culture plates (BD Biosciences, #350597, flat bottom), and duplicates were measured as triplicates. 7500 Jurkat cells were cultured in a total volume of 200 μl in each well. Plates were incubated for 48 h at 37 °C and 5% CO₂. After incubation time, the number of living cells was determined by reduction of the tetrazolium salt EZAU to formazan derivatives (Biozol, #B1-5000). 20 μl of EZAU reagent was added to each well, and plates were incubated for another 4 h. Optical density (OD) was measured in a EL 800 plus universal microplate reader (BIO-TEK Instruments) at wavelength settings of 465–630 nm.

**Single Cell Ca²⁺ Imaging—**Cells were loaded at 22–23 °C for 30 min with 1 μM fura-2/AM (Molecular Probes) in culture medium with 10 μM HEPES added, washed with fresh medium, stored at RT for 10 min, and immediately used. Cells were allowed to adhere to poly-L-ornithine-coated T-fluoroset chambers on an Olympus IX 70 microscope equipped with a 20× (UApO/340, numerical aperture (NA) 0.75), or a 40× (Uplan/Apo, NA 1.0) objective. Cells were alternately illuminated at 340 and 380 nm with the Polychrome IV Monochromator (TILL Photonics). The fluorescence emissions at λ > 440 nm were captured with a charge-coupled device camera (TILL Imago), digitalized, and analyzed using TILL Vision software. Ratio images were recorded at intervals of 5 s. [Ca²⁺], was estimated from the relation \[ [Ca^{2+}] = K_r (R - R_{\infty})/(R - R_{\infty}) \]

where the values of \( K_r \), \( R_{\infty} \), and \( R_{\infty} \) were determined from an in situ calibration of fura-2 in Jurkat T-cells as described previously (24). Ca²⁺ Ringer’s solution contained (in millimolar): 156 NaCl, 4.5 KCl, 2 MgCl₂, 10 glucose, 5 Hepes (pH 7.4 with NaOH). CaCl₂ was replaced by MgCl₂ in the 0-Ca²⁺ Ringer’s solution with 1 mM EGTA added. 145 NaCl was replaced by 145 mM KCl in the high K⁺ solution (TG, 1 μM stock, 1 mM in MeSO, Molecular Probes) and anti-C3d mAb (10 μg/ml, stock at 2 mg/ml, obtained from the hybridozy KOT3, ATCC CRL-8001) were used to stimulate the cells. A sandwich self-made chamber was used for all measurements, which allowed for a complete solution exchange <1 s.

**Ca²⁺ Imaging with the Flexstation—**Following isolation, CD4⁺ T-cells were kept in RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamate, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

Human Jurkat T-cell lines were grown in culture medium consisting of RPMI 1640 supplemented with 10% FCS and penicillin-streptomycin as described previously (5). Cells were continuously maintained in log-phase growth at 37 °C with 5% CO₂. For most Jurkat T-cell experiments, the diphtheria toxin-resistant version of the parental cell line (Jurkat (par) in the figures) generated by Fanger et al. (5) was used. For some of the experiments, the human Jurkat clone E6.1 (ATCC number TIB-152, Jurkat (E6.1) in the figures) was used.

**Reagents—**All chemicals not specifically mentioned were from Sigma (St. Louis, MO).

**Synthesis of BTP2—**BTP2 was re-synthesized following the procedure provided by Djuric et al. (19).

**Functional Assays of CD4⁺ Cells and PBls—**CD4⁺ cells were stimulated via the CD3 chain of the T-cell receptor and the co-stimulatory molecule CD28. After 72 h either the proliferation ([³H]thymidine incorporation) or the cytokine concentration in the culture medium (specific immune enzymeassays) were measured.

96-well microtiter plates (Microtect Tissue Culture Plate, Falcon, BD Biosciences, Le Pont de Claix, France) were pre-coated for 2.5 h at 37 °C with 0.5% CO₂ with anti-CD3 mAb (Orthoclone OKT-3, Janssen-Cilag, Neuss, Germany) by incubating with 50 μl per well of a solution containing 60 μg/ml anti-CD3 in PBS (corresponds to 3 μg/ml). Afterward, plates were washed at 4 °C for 30 min without removing the anti-CD3 solution. Before use plates were washed twice with PBS (150 μl/well). The stimulation of CD4⁺ cells was performed in a total volume of 200 μl/well in triplicates. 2 × 10⁶ cells/well in a volume of 180 μl/well were applied followed by the addition of 10 μl/well of the inhibitor (solved in MeSO and diluted in medium). In all samples, including control samples, MeSO was adjusted to a final concentration of 0.1% (v/v). After the addition of 10 μl of anti-CD28 mAb (final concentration), 3 μg/ml monoclonal antibody CD28, Immunotech, Marseille, France) cells were incubated for 72 h at 37 °C, 5% CO₂. Supernatants were removed, pooled, and stored at −20 °C until determination of cytokine concentration. Due to the high variability of cytokine levels from different blood donors, for each experiment and cytokine appropriate dilution factors had to be determined to guarantee that the cytokine levels were in the linear range of the enzyme-linked immunosorbent assay standard curve. Dilutions were performed in PBS containing 3% bovine serum albumin (w/v), and all cytokines were determined from the pool fraction in duplicate. Measurements of the cytokines were done.

**Ca²⁺ influx was induced by subsequent re-addition of 1 μM Ca²⁺ (final free concentration)/1 μM TG to the extracellular solution by adding 20 μl of Ringer’s solution/24 mM CaCl₂/16.5 mM EGTA/11 μM TG. Ca²⁺ influx was measured using a scanning fluorometer and integrated fluid transfer workstation (Flexstation, Molecular Devices) with an excitation wavelength of 485 nm and an emission wavelength of 525 nm. Data were collected as relative fluorescence units every 8 s. Ca²⁺ stores were depleted for 13 min in Ringer’s solution containing 1 mM EGTA (final free concentration 0.1 μM TG) (1 mM solution in MeSO, Molecular Probes) by adding 20 μl of Ringer’s solution/0.5 mM CaCl₂/16.5 mM EGTA/11 μM TG. Ca²⁺ influx was induced by subsequent re-addition of 1 μM Ca²⁺ (final free concentration)/1 μM TG to the extracellular solution by adding 20 μl of Ringer’s solution/24 mM CaCl₂/16.5 mM EGTA/11 μM TG. The inhibition was calculated as the percentage of the peak or plateau values (10 min after stimulation) of MeSO-to BTP2-treated cells. In control experiments, MeSO was used to stimulate the cells, the inhibition was calculated as percentage of either the peak values or plateau values (10 min after stimulation) of MeSO-to BTP2-treated cells. Basal fluorescence values were subtracted before calculation. To determine the kinetics of preincubation, cells were in-
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RESULTS

Store-operated Ca\(^{2+}\) entry through CRAC channels is the major Ca\(^{2+}\) entry route in lymphocytes, and it is necessary for cell activation and proliferation. To analyze the possibility that the 3,5-bistrifluoromethyl pyrazole derivative BTP2 (also called YM-58483), has the potential to inhibit store-operated Ca\(^{2+}\) entry in lymphocytes, Ca\(^{2+}\) signals in the presence and absence of BTP2 were compared.

Depletion of Ca\(^{2+}\) from the endoplasmic reticulum was induced by 1 \(\mu\)M TG in 0 mM Ca\(^{2+}\) Ringer’s solution. In the absence of extracellular Ca\(^{2+}\), TG causes a very small, transient Ca\(^{2+}\) rise in PBLs resulting from the unopposed leakage of Ca\(^{2+}\) from internal stores followed by Ca\(^{2+}\) extrusion across the plasma membrane. Exchanging the external solution to 1 mM Ca\(^{2+}\) Ringer’s solution allows Ca\(^{2+}\) influx through CRAC channels across the plasma membrane resulting in long lasting elevations of [Ca\(^{2+}\)]\(_i\), as illustrated in Fig. 1A. Preincubation of the cells with 100 nM BTP2 for 24 h leads to a marked decrease of store-operated Ca\(^{2+}\) entry (Fig. 1A). To quantify the reduction of store-operated Ca\(^{2+}\) signals, three parameters were analyzed in single cells: the initial influx rate as fitted by linear regression, the Ca\(^{2+}\) peak, and the Ca\(^{2+}\) plateau by BTP2. Between 206 and 486 cells were individually analyzed for each BTP2 concentration. Data are shown as mean ± S.E.

**Fig. 1.** Store-operated Ca\(^{2+}\) signals in PBLs are inhibited by BTP2 in the low nanomolar range. Single cell analysis was performed with the imaging set-up. A, Typical experiment in the absence and presence of 100 nM BTP2. Cells were preincubated with 1 \(\mu\)M TG in 0-Ca\(^{2+}\) Ringer’s solution to fully deplete Ca\(^{2+}\) stores, followed by re-addition of 1 mM Ca\(^{2+}\). Ringer’s solution to allow Ca\(^{2+}\) entry through CRAC channels. The initial Ca\(^{2+}\) influx rate, the Ca\(^{2+}\) peak, and the Ca\(^{2+}\) plateau were measured as depicted. Averages of 85 (control) and 78 (BTP2) PBL cells are shown. B–D, dose-dependent inhibition of the initial influx rate, the Ca\(^{2+}\) peak, and the Ca\(^{2+}\) plateau by BTP2. Between 206 and 486 cells were individually analyzed for each BTP2 concentration. Data are shown as mean ± S.E.

Electrophysiology—The standard pipette solution for whole cell patch-clamp recordings contained (in millimolar): 0.05 InsP \(_3\), (50 mM stock in H\(_2\)O, Calbiochem) 140 Cs aspartate, 10 NaCl, 1–5 MgCl\(_2\), 10 EGTA, and 10 Hepes (pH 7.2 with CsOH). The bath solution contained (in millimolar): 155 NaCl, 4.5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 Na\(_2\)glucose, and 5 Hepes (pH 7.4 with NaOH). Patch-clamp experiments were performed at 22–23 °C in the tight-seal whole cell configuration using fire-polished patch pipettes (2–3 M\(\Omega\) uncompensated series resistance). Pipette and cell capacitance were electronically cancelled before each voltage ramp with an EPC-9 patch-clamp amplifier controlled by Pulse 8.4 software (Heka Elektronik, Germany). 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 200-ms voltage-clamp ramps from –100 mV to +100 mV from a holding potential of 0 mV. To 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.

Data Analysis—Data were analyzed using commercially available programs, including TILL Vision (TILL Photonics), SoftMax Pro (Molecular Devices), Igor Pro (WaveMetrics), Microsoft Excel (Microsoft), and GraphPad Prism (GraphPad Software Inc.). Averages are presented as mean ± S.D. or S.E. as indicated. For statistical analysis a paired or unpaired two-tailed t test was used.
Fig. 2. Store-operated Ca$^{2+}$ signals but not Ca$^{2+}$ release in Jurkat cells are inhibited by BTP2 in the low nanomolar range. Single cell analysis was performed with the imaging set-up. A, a typical experiment as described in Fig. 1A is shown in the inset. The symbols refer to the average Ca$^{2+}$ concentrations depicted in the lower part of the figure. BTP2 had no effect on resting [Ca$^{2+}$], or Ca$^{2+}$ release by TG up to concentrations of 1 μM. Points reflect 113–288 individually analyzed cells. B–D, dose-dependent inhibition of the initial influx rate, the Ca$^{2+}$ peak, and the Ca$^{2+}$ plateau by BTP2. 113–288 cells were individually analyzed for each BTP2 concentration. Data are shown as mean ± S.E.

are a mix of different lymphocytes (about 50% CD4$^{+}$ and 25% CD8$^{+}$ T-cells). Because all cells showed a homogenous reduction in Ca$^{2+}$ signals (data not shown), it is concluded that store-operated Ca$^{2+}$ signals in CD4$^{+}$ and CD8$^{+}$ T-cells are reduced by BTP2. No difference of resting [Ca$^{2+}$], levels or the TG-dependent Ca$^{2+}$ release was observed in PBLs between control conditions and BTP2 concentrations up to 1 μM (data not shown). Because Ca$^{2+}$ release signals following TG stimulation in PBLs are very small, the same measurements were repeated in human Jurkat T-cells (Fig. 2A, inset). Analyzing resting [Ca$^{2+}$], levels, TG-dependent Ca$^{2+}$ release peaks, and Ca$^{2+}$ extrusion, no differences were observed between control conditions (no BTP2) and preincubation of up to 1 μM BTP2 for 24 h (Fig. 2A). Similar to the PBLs, there was, however, a marked inhibition of the initial Ca$^{2+}$ influx rate (Fig. 2B), the Ca$^{2+}$ peak (Fig. 2C), and the Ca$^{2+}$ plateau (Fig. 2D) in Jurkat T-cells by BTP2. The IC50 values of the sigmoidal regressions in Jurkat T-cells were 5, 10, and 7 nM, respectively. We conclude that BTP2 inhibits store-operated Ca$^{2+}$ entry in CD4$^{+}$, CD8$^{+}$, and Jurkat T-cells in the low nanomolar range, whereas resting [Ca$^{2+}$], levels and TG-dependent Ca$^{2+}$ release from the endoplasmic reticulum are not affected.

Immediate inhibition of BTP2 was also observed, however, the onset of the block was rather slow with an half-maximal inhibition between 300 nM and 1 μM (data not shown). Therefore, we analyzed the effect of the preincubation time dependence of BTP2 in more detail and found that inhibition of Ca$^{2+}$ signals by 200 nM BTP2 was almost complete within the first 2 h of incubation at 37 °C in CD4$^{+}$ and Jurkat T-cells (Fig. 3). Fig. 3A shows examples of Ca$^{2+}$ release transients in Jurkat cells following store depletion by TG and subsequent activation of Ca$^{2+}$ entry at various incubation times. Data are summarized in Fig. 3B revealing that about 75% of the inhibition in CD4$^{+}$ or Jurkat T-cells is complete within 2 h of preincubation. TG is an unphysiological stimulus of Ca$^{2+}$ release signals by 200 nM BTP2 was almost complete within the first 2 h of preincubation time at 37 °C. The onset of the block was rather slow with a half-maximal inhibition between 300 nM and 1 μM (data not shown). There was a slight but not significant decrease of the number of cells responding to anti-CD3 with a clear Ca$^{2+}$ release transient. 50 nM BTP2 did not interfere with the amplitude of Ca$^{2+}$ release transients (Fig. 4B). There was a slight but not significant decrease of the number of cells responding to anti-CD3 with a clear Ca$^{2+}$ release transient in the...
presence of 50 nM BTP2 (40% responders under control conditions and 38% responders when BTP2 was present). For both donors, a reduction of Ca\textsuperscript{2+} signals following application of 1 mM Ca\textsuperscript{2+} Ringer’s solution was observed (Fig. 4A), which manifested itself in a clear reduction of the initial Ca\textsuperscript{2+} influx rate, the Ca\textsuperscript{2+} peak, and the Ca\textsuperscript{2+} plateau in the statistical analysis (Fig. 4B). In population measurements with the Flexstation, the IC\textsubscript{50} values for BTP2 inhibition of anti-CD3-activated Ca\textsuperscript{2+} influx in E6.1 Jurkat cells were determined from experiments as depicted in Fig. 5A. Because single cells respond with different latencies to anti-CD3 stimulation (data not shown), the observed Ca\textsuperscript{2+} peak reflects a mixture of Ca\textsuperscript{2+} release by anti-CD3 and subsequently activated Ca\textsuperscript{2+} entry. It is thus not surprising that the Ca\textsuperscript{2+} peak was not inhibited fully by high BTP2 concentrations (Fig. 5B), because the Ca\textsuperscript{2+} release was not affected by BTP2 (compare Fig. 4, A and B, first panel). The IC\textsubscript{50} of the inhibition was 20 nM and very similar to the TG-induced influx peak. The same was true for the anti-CD3-induced Ca\textsuperscript{2+} influx plateau, which was inhibited by BTP2 with an IC\textsubscript{50} of 13 nM (Fig. 5B). The plateau was also not inhibited fully by BTP2, which was probably due to oscillating Ca\textsuperscript{2+} release sometimes observed following anti-CD3 stimulation (data not shown). We conclude that store-operated Ca\textsuperscript{2+} entry in T-cells is inhibited by BTP2 in the low nanomolar range regardless of the T-cell stimulation method.

Store-operated Ca\textsuperscript{2+} signals in T-cells depend on the activity of the following transport mechanisms: CRAC channels, which are responsible for the Ca\textsuperscript{2+} influx, Ca\textsuperscript{2+}- and potential-dependent K\textsuperscript{+} channels, which control the membrane potential and therefore the electrical driving force for Ca\textsuperscript{2+} entry, Ca\textsuperscript{2+}-

Fig. 4. T-cell receptor dependent Ca\textsuperscript{2+} signals in PBLs are inhibited by BTP2 in the low nanomolar range. Single cell analysis was performed with the imaging set-up. A, same stimulation protocol as shown in Fig. 1A, except that 4 μg/ml anti-CD3 was used (and not TG) to stimulate the T-cell receptor. B, P1 and P2 refer to PBLs from two different donors. Analysis of the Ca\textsuperscript{2+} release, initial influx rate, Ca\textsuperscript{2+} peak, and Ca\textsuperscript{2+} plateau for control (40 and 103 cells) and cells preincubated with 50 nM BTP2 (22 and 145 cells). Release peaks were not different between control and BTP2 cells (Student’s t test, p > 0.1), whereas initial influx rate, Ca\textsuperscript{2+} peak, and Ca\textsuperscript{2+} plateau were significantly different (Student’s t test, p < 0.01). Data are shown as mean ± S.E.
ATPases in the plasma membrane and the endoplasmic reticulum, which transport Ca\(^{2+}\) out of the cytosol, and mitochondrial Ca\(^{2+}\) homeostasis, which controls CRAC channel inactivation. A Na\(^{+}\)-Ca\(^{2+}\) exchanger would also contribute to the Ca\(^{2+}\) signals, however, there is good evidence that it does not play any role for T-cell Ca\(^{2+}\) signals, because BTP2 is effective in the low nanomolar range. E6.1 Jurkats were chosen for these experiments, because they have larger CRAC currents than parental Jurkat T-cells and thus allow relatively easy measurements at low external Ca\(^{2+}\) concentrations. CRAC channels were activated by 50 nM BTP2 (data not shown). Application of 0-Ca\(^{2+}\) Ringer's solution subsequently to the Ca\(^{2+}\) re-addition resulted in an exponential decay of [Ca\(^{2+}\)]. The average time constant of 143 control cells and 167 cells preincubated with 50 nM BTP2 for 24 h. A, same stimulation as in Fig. 1A, except that a high K\(^{+}\) external solution (149.5 mM KCl) was used. Averages of 181 (control) and 247 (BTP2) PBLs are shown. B, same stimulation as in Fig. 1A, except that 1 \(\mu\)M carbonyl cyanide m-chlorophenylhydrazone was present as indicated. Averages of 806 (control) and 1057 (BTP2) PBLs are shown.

Fig. 6. BTP2 inhibition of Ca\(^{2+}\) entry in PBLs is independent of membrane potential, mitochondrial Ca\(^{2+}\) homeostasis, and Ca\(^{2+}\) export. BTP2-treated cells were incubated with 50 nM BTP2 for 24 h. A, same stimulation as in Fig. 1A, except that a high K\(^{+}\) external solution (149.5 mM KCl) was used. Averages of 306 (control) and 142 (BTP2) PBLs are shown. B, same stimulation as in Fig. 1A, except that 1 \(\mu\)M carbonyl cyanide m-chlorophenylhydrazone was present as indicated. Averages of 181 (control) and 247 (BTP2) PBLs are shown. C, analysis of the time constant \(\tau\) of experiments as shown in Fig. 4A. Application of 0-Ca\(^{2+}\) Ringer's solution subsequently to the Ca\(^{2+}\) re-addition resulted in an exponential decay of [Ca\(^{2+}\)]. The average time constant of 143 control cells and 167 cells preincubated with 50 nM BTP2 is given. The values are not significantly different (Student's t test, \(p > 0.1\)). Data are shown as mean \(\pm\) S.E.

Fig. 7A shows maximal CRAC currents in two cells, one control cell and one cell incubated with 100 nM BTP2 for 24 h. The normalized currents were clearly reduced in the BTP2-treated cell, and this effect was also obvious when comparing all analyzed cells (Fig. 7B). Activity of K\(^{+}\) channels was also measured in each experiment and was not significantly affected by 100 nM BTP2 (data not shown). Similarly, we could not observe a reduction of Mg\(^{2+}\) -inhibited channel activity (27–30) with 100 nM BTP2 present having only 1 mM Mg\(^{2+}\) in the pipette (data not shown). We conclude that low nanomolar BTP2 concentrations specifically block CRAC channel activity in T-cells.

To better understand the mechanism of the BTP2 block, a detailed patch-clamp analysis with parental Jurkat T-cells was
performed using 10 mM external Ca\(^{2+}\), which results in CRAC current amplitudes comparable to the ones found in E6.1 cells measured in 2 mM external Ca\(^{2+}\). No instantaneous block of 200 nM BTP2 on a time scale of up to 3 min after BTP2 application could be observed following maximal activation of CRAC currents (n = 5 cells, data not shown). On the other hand, we could observe a slow instantaneous block by 1 \(\mu\)M BTP2 when applying it during the TG-induced Ca\(^{2+}\) plateau (Fig. 8A). The inhibition was, however, not as fast as reported by Ishikawa et al. (22). To analyze whether BTP2 might inhibit CRAC channel activity from the cytosolic site through accumulation of BTP2 in the cytosol, we performed patch-clamp experiments with 1 \(\mu\)M BTP2 in the patch pipette. Fig. 8B shows that, under these conditions, CRAC currents activated with a normal amplitude and, more importantly, no inhibition of currents was observed over a time course of 7 min in this example as was also the case in control experiments (data not shown). In two cells, we succeeded to measure CRAC currents in the presence of 1 \(\mu\)M BTP2 for almost 10 min and did not observe any inhibition. The lower part of Fig. 8B shows the current-voltage relationship of net CRAC currents 100 s after establishing the whole cell configuration. The data of all cells are summarized in Fig. 8C, indicating that the very high concentration of 1 \(\mu\)M BTP2 in the patch-pipette had no effect on the CRAC current amplitude compared with control conditions. From these data we conclude that it is very unlikely that BTP2 acts from the cytosolic site, for instance by inhibiting the activation mechanism of CRAC channels or the intracellular part of the channel pore. An intracellular action of BTP2 appears also unlikely, because whole cell perfusion of 1 \(\mu\)M BTP2 led to drastic morphological changes of the cells (data not shown), which was never observed when incubating cells for 24 h with the same concentration. We conclude that BTP2 inhibits CRAC channel activity from the extracellular space.

To further analyze the dose and time dependence of the BTP2 block, patch-clamp experiments following different incubation times with three different BTP2 concentrations (10 nM, 100 nM, and 1 \(\mu\)M) were performed. Single cell examples of such measurements are shown in Fig. 8D and summarized in Fig. 8E. Each point in Fig. 8E reflects 4–11 pooled cells. The data were fitted by exponential functions to illustrate the time dependence of the block at the different concentrations. The time constants of the exponential regressions were 64 min (1 \(\mu\)M), 98 min (100 nM), and 447 min (10 nM). These data very well correlate with the Ca\(^{2+}\) imaging data in which an almost maximal effect was observed after 120 min with 200 nM BTP2. Combining these data with our results of the experiments with 1 \(\mu\)M BTP2 in the patch pipette, we conclude that BTP2 interferes with CRAC channel activity in a dose-dependent way from the extracellular space. The on-rate of the block was slow, but it is specific for CRAC channels, because no other channels/transporters were affected by BTP2.

Ca\(^{2+}\) signals mediated through CRAC channel activity are very important for the activation of Ca\(^{2+}\)-dependent gene expression in T-cells. We analyzed whether Ca\(^{2+}\)-dependent gene expression was affected by BTP2. CD4\(^+\) T-cells were stimulated by anti-CD3/anti-CD28 co-stimulation and expression of various Ca\(^{2+}\)-dependent genes was assessed. BTP2 inhibited IL-2, IL-5, and interferon \(\gamma\) expression in a concentration-dependent way with IC\(_{50}\) values of 8 nM, 38 nM, and 22 nM, respectively (Fig. 9A). The IC\(_{50}\) of IL-2 inhibition depends strongly on the external Ca\(^{2+}\) concentration (Fig. 9B), because the IC\(_{50}\) was shifted from 15 ± 3 nM (measured in medium) to 35 ± 16 nM (1 mM Ca\(^{2+}\) added) or 84 ± 13 nM (2 mM Ca\(^{2+}\) added). These IC\(_{50}\) values are statistically different with p < 0.01 (paired two-tailed t test, the p value between “1 mM Ca\(^{2+}\) added” and “2 mM Ca\(^{2+}\) added” was 0.055). These experiments illustrate that the BTP2 inhibition of IL-2 expression very likely depends on the amount of Ca\(^{2+}\) entry and not on any Ca\(^{2+}\)-independent potential side effects of the drug. Ca\(^{2+}\)-dependent gene expression following T-cell stimulation is a prerequisite for T-cell proliferation and clonal expansion (1, 4). To verify the dependence of proliferation on Ca\(^{2+}\) influx, cell proliferation of Jurkat T-cells was measured while varying the concentration of free extracellular Ca\(^{2+}\) (Fig. 9C). We have measured the total Ca\(^{2+}\) concentration in the medium (RPMI plus FCS) to be 750 \(\mu\)M by mass spectroscopy (InfraServ KNAP-SACK, Germany). In addition, we have measured the free Ca\(^{2+}\) concentration in the medium with MagFura to be ~533 \(\mu\)M. Different EGTA concentrations were used to buffer the free Ca\(^{2+}\) of complete medium. The addition of 0.8 mM EGTA (which results in an approximately free external Ca\(^{2+}\) concentration of 2.5 \(\mu\)M) almost completely inhibited Jurkat T-cell proliferation indicating that a critical extracellular Ca\(^{2+}\) concentration in the low micromolar range is necessary for proliferation.

BTP2 was found to inhibit the proliferation of stimulated CD4\(^+\) in a concentration-dependent manner with an IC\(_{50}\) value of 75 ± 23 nM (Fig. 9D), which is slightly higher than the IC\(_{50}\) values for the inhibition of Ca\(^{2+}\) currents, Ca\(^{2+}\) signals, and Ca\(^{2+}\)-dependent gene expression. Similar to the expression of IL-2, BTP2 inhibition was dependent on the extracellular Ca\(^{2+}\) concentration (Fig. 9D). The IC\(_{50}\) was shifted from 75 ± 23 nM to 272 ± 156 nM when 1 mM Ca\(^{2+}\) was added to the medium. These IC\(_{50}\) values are statistically different with p < 0.05 (paired two-tailed t test). We conclude that BTP2 inhibits gene expression of IL-2 and T-cell proliferation in a Ca\(^{2+}\)-dependent way in the nanomolar range, very similar to its effect on store-operated Ca\(^{2+}\) signals and CRAC channel activity.
The Ca$^{2+}$ dependence of the BTP2 inhibition of IL-2 production and cell proliferation could imply that the BTP2 effect can be counterbalanced by an increased Ca$^{2+}$ entry, which would then be another argument for the specificity of the BTP2 effect. To test this, we analyzed Ca$^{2+}$ influx after the addition of different extracellular Ca$^{2+}$ concentrations in the absence or presence of a supra-maximal concentration of BTP2 (50 nM). The traces of all cells are averaged in Fig. 10A using 1 mM extracellular Ca$^{2+}$ and using 20 mM extracellular Ca$^{2+}$ in Fig. 10B. It is obvious that 50 mM BTP2 blocks the Ca$^{2+}$ signals better when 1 mM Ca$^{2+}$ was used. The same is reflected in Fig. 10 (C and D) in which the statistical analysis of the initial influx rate and the Ca$^{2+}$ plateau of the cells are shown for 1, 5, and 20 mM Ca$^{2+}$. Although CRAC currents are more than three times larger when measured with 20 mM instead of 1 mM external Ca$^{2+}$ (31), the Ca$^{2+}$ plateau was not increased to the same extent (Fig. 10, compare A and B). This can be explained by the fact that the Ca$^{2+}$ plateau in imaging experiments depended not only on CRAC channel activity but on many other parameters, including plasma membrane Ca$^{2+}$ ATPases (PM-CA). The PM-CA are heavily up-regulated when 20 mM external Ca$^{2+}$ is applied and do limit the Ca$^{2+}$ plateau (32). The cytosolic Ca$^{2+}$ concentration does usually not reach more than 1.5 μM. Therefore, increasing the extracellular Ca$^{2+}$ concentration resulted in an increase in the intracellular Ca$^{2+}$ concentration as long as the maximal [Ca$^{2+}$], of about 1.5 μM was not reached. A further increase in the extracellular Ca$^{2+}$ concentration may well lead to a further increase of Ca$^{2+}$ current through CRAC channels, but up-regulation of PM-CA guarantees that the [Ca$^{2+}$] stays at the maximal level of about 1.5 μM. Supra-maximal concentrations of BTP2 (e.g. 50 nM) do not inhibit 100% of the CRAC channels (see Figs. 7 and 8). The remaining open channels will conduct larger Ca$^{2+}$ currents as the driving force for Ca$^{2+}$ influx increases. Therefore, if BTP2 blocks the same number of channels in 20 mM and 1 mM external Ca$^{2+}$, inhibition of [Ca$^{2+}$], by 50 nM BTP2 will decrease in 20 mM Ca$^{2+}$ compared with 1 mM Ca$^{2+}$. The same effect is most likely responsible for the shift of block by increasing the external Ca$^{2+}$ concentration in the IL-2 and proliferation experiments. In the presence of higher external Ca$^{2+}$, the BTP2 block allows higher [Ca$^{2+}$], (although the same number of channels are blocked) and thus higher IL-2 expression or cell proliferation. From these data we conclude that BTP2 very likely has no unspecific effects in T-lymphocytes that are in any

![Fig. 8](image-url)  
**Intracellular, dose, and time dependence of the BTP2 CRAC channel block.** A, instantaneous effect of 1 μM BTP2 on Ca$^{2+}$ signals in parental Jurkat T-cells. An average of 114 cells is shown. B, effect of 1 μM BTP2 in the pipette solution, which was other than that identical to the one used in Fig. 7. CRAC currents in parental Jurkat T-cells were analyzed in 10 mM Ca$^{2+}$ Ringer's solution under the same conditions as in Fig. 7. In the example shown, 1 μM BTP2 was added to the pipette solution. Current activation is shown at -80 mV taken from the 200 ms voltage ramps from -100 to +100 mV. No inactivation of the current is seen over the time of the experiment. The lower part shows the current-voltage relationship of the net maximal CRAC current. C, average current amplitudes measured at -80 mV are not significantly different in BTP2-treated and control cells (Student's t test, p > 0.1). Data are shown as mean ± S.E. D, examples of the effect of different preincubation times and different concentrations of BTP2 on CRAC channel amplitudes. The experimental protocols are the same as in B. E, statistical analysis of all cells measured under the same conditions as in D. 4-11 cells were pooled for each point. Error bars reflect ± S.E. The dotted lines are exponential functions fitted to the data.

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**FIG. 7.** In the example shown, 1 μM BTP2 blocks the Ca$^{2+}$ influx rate and the Ca$^{2+}$ plateau in imaging experiments. In the presence of higher external Ca$^{2+}$, the BTP2 block allows higher [Ca$^{2+}$], (although the same number of channels are blocked) and thus higher IL-2 expression or cell proliferation. From these data we conclude that BTP2 very likely has no unspecific effects in T-lymphocytes that are in any...
way coupled to activation or proliferation of the cells, because
unspecific effects should not be modified by changing the extracellular Ca\(^{2+}\) concentration.

**DISCUSSION**

The pyrazole derivative BTP2 (19–22) was found to inhibit CRAC channels, the corresponding Ca\(^{2+}\) signals, Ca\(^{2+}\)-dependent gene expression, and cell proliferation in CD4\(^{+}\) and CD8\(^{+}\) human T-lymphocytes from peripheral blood and in the human T-cell line Jurkat. The IC\(_{50}\) of inhibition of all measured parameters was found to be \(\leq 75 \text{ nM}\) at physiological extracellular Ca\(^{2+}\) concentrations, which emphasizes the great importance of CRAC channel activity for T-cell function. Because other channels and transporters in T-cells were unaffected, BTP2 seems to be the first specific and potent CRAC channel inhibitor.

Pyrazole derivatives were only recently introduced as immunomodulators by Djuric and colleagues (19, 20) and were found to be potent inhibitors of nuclear factor of activated T-cells activation and cytokine production. They were later described to inhibit Ca\(^{2+}\)-dependent gene expression in mononuclear cells from peripheral blood (21). One of the pyrazole derivatives, BTP3, was tested on Ca\(^{2+}\) influx in Jurkat T-cells but was found to be ineffective (21). Ishikawa et al. (22) tested the potential inhibitory effect of another structurally very similar pyrazole derivative, BTP2 (also named YM-58483 by them), on Ca\(^{2+}\) signals in Jurkat T-cells. They found that store-operated Ca\(^{2+}\) signals are inhibited by BTP2 with an IC\(_{50}\) around 100 nM. We confirm that BTP2 inhibits store-operated Ca\(^{2+}\) signals in Jurkat T-cells; however, our IC\(_{50}\) values were one order of magnitude lower (around 10 nM). This difference could be due to the different preincubation times used, only minutes by Ishikawa et al. versus 24 h by us. In our hands, it takes more than 2 h for BTP2 to reach its full effect. Another possibility to explain the differences in the IC\(_{50}\) values is the different extracellular Ca\(^{2+}\) concentration used. In T-cells, Ca\(^{2+}\) imaging reveals very similar \([\text{Ca}^{2+}]_i\) following the activation of Ca\(^{2+}\) influx in the presence of 2 or 20 mM external Ca\(^{2+}\) (26). This phenomenon is explained by Ca\(^{2+}\) pump rate up-regulation, which efficiently counterbalances increased Ca\(^{2+}\) influx (32).

**FIG. 9.** Inhibition of gene expression and proliferation in human CD4\(^{+}\) T-lymphocytes by BTP2. **A**, cells (2 \(\times\) 10\(^5\) per well) were stimulated by pre-coated anti-CD3 mAb (3 \(\mu\)g/well) together with anti-CD28 mAb (3 \(\mu\)g/ml) for 72 h at 37 °C in the absence or presence of the inhibitor. Levels of cytokines were measured by specific immunoassays in the supernatants as described under “Experimental Procedures.” Data are shown as mean ± S.D. from four to five independent experiments. **B**, same conditions as in **A**. IL-2 levels were measured in the absence or presence of the inhibitor with different amounts of external Ca\(^{2+}\) added to the medium. Data are shown as mean ± S.D. from five independent experiments (paired experiments for each blood donor). IC\(_{50}\) values were separately calculated for each experiment. Significance was analyzed by a paired two-tailed \(t\) test. **C**, Jurkat T-cells (7.5 \(\times\) 10\(^3\) per well) were cultured in the presence of different EGTA concentrations for 48 h at 37 °C. Proliferation was determined by the EZ4U assay. Results are shown as mean ± S.E. of triplicates per data point. **D**, CD4\(^{+}\) T-lymphocytes (2 \(\times\) 10\(^5\) per well) were stimulated by pre-coated anti-CD3 mAb (3 \(\mu\)g/well) together with anti-CD28 mAb (3 \(\mu\)g/ml) for 72 h at 37 °C in the absence or presence of different BTP2 concentrations (filled squares). Proliferation was measured by cellular incorporation of [\(^{3}\text{H}\)]thymidine (0.2 \(\mu\)Ci/well), which was present during the last 17 h of incubation. Experiments performed either in medium (filled squares) or in medium with 1 mM Ca\(^{2+}\) added (filled triangles) were performed in parallel. Data are shown as mean ± S.D. from four independent experiments each. IC\(_{50}\) values were separately calculated for each experiment. Significance was analyzed by a paired two-tailed \(t\) test.
To analyze Ca^{2+} inhibitors, it is thus desirable to choose external Ca^{2+} concentrations below 2 mM to prevent “saturation” problems. Ishikawa et al. (22) used 2 mM free external Ca^{2+}, which could explain their higher IC_{50} values compared with the ones in the present study performed with 1 mM free external Ca^{2+}.

Ishikawa et al. reported that the instantaneous effect of BTP2 on Ca^{2+} signals was almost as strong as the effect of preincubating BTP2, a finding that we could not reproduce. In our hands, preincubation is clearly more effective than instantaneous application of BTP2. The reason for this discrepancy is at present unclear. The need for preincubation could also be the explanation why Chen et al. (21) did not find effects of BTP3 on Ca^{2+} signals in their system. It is disturbing that, on the one hand, our preincubation experiments reveal very low IC_{50} values for the BTP2 inhibition, whereas, on the other hand, the “instantaneous” effect of BTP2 was slower or less potent as was found by Ishikawa et al. (22). From our patch-clamp measurements we can exclude that BTP2 acts from the inside of the cell, making a cytosolic accumulation of the drug rather unlikely. To explain the differences between Ishikawa et al. and our study, one could speculate about potential differences regarding the cell lines used or differences of the synthesized BTP2. Because preincubation is very effective in both studies, these explanations appear, however, unlikely. We believe that we can rule out other measurement artifacts in our study, because experiments were carried out at two different locations with different stocks of BTP2 and different blood donors. In addition, our patch-clamp and imaging measurements correlate quite well. In the study by Ishikawa et al. the instantaneous block of BTP2 (140 nM) was slightly less effective than the one following preincubation (100 nM). Furthermore, the IC_{50} values for IL-2 production (17 nM) and IL-2 reporter gene assays (10 nM) reported by Ishikawa et al. are about one order of magnitude below their IC_{50} values for Ca^{2+} influx inhibition but do very well fit our results on IL-2 production (Fig. 9: A, 8 nM; B, 15 nM) raising the possibility that longer incubation times may well lead to a more potent effect of BTP2.

Ishikawa et al. (22) could only speculate about the exact mechanism of the BTP2 block. Our experiments reveal that CRAC channels themselves are the target of the BTP2 action and that BTP2 does not inhibit CRAC channel activity from the inside of the cell. It is therefore very unlikely that BTP2 interferes with the activation mechanism of CRAC channels or with intracellular structures of the channels. We postulate that BTP2 interferes with CRAC channels from the extracellular space, possibly with a rather slow on-rate. BTP2 is a specific CRAC channel blocker, because other transport mechanisms, which together with CRAC channels determine the amplitude of store-operated Ca^{2+} signals, remain unaffected. These include Ca^{2+} release by TG or T-cell receptor stimulation, transport by Ca^{2+} ATPases, mitochondrial Ca^{2+} homeostasis, and activity of K^{+} channels. In addition, Mg^{2+}-inhibited channels (27–30) were also unaffected. This makes BTP2 not only a potent but also a very specific CRAC channel inhibitor. It clearly sets apart BTP2 from other less specific CRAC channel inhibitors like 2-aminoethyldiphenyl borate (19–18), SK&F 96365 (11, 12), or econazole, the latter of which inhibits CRAC channels with a IC_{50} of about 600 nM in mast cells but, with similar IC_{50} values, inhibits all other channels tested in the same cells (11). The specificity of BTP2 is further underscored by the finding that it does not interfere with the activity of voltage-gated Ca^{2+} channels (22).

Most importantly, we found the inhibitory effect of BTP2 not only in Jurkat T-cells but in human peripheral blood T-lymphocytes following TCR stimulation. This makes BTP2 potentially a good immunosuppressive drug. Considering the finding that CRAC channels, the corresponding Ca^{2+} signals, expression of IL-2, IL-5, and interferon γ, and T-cell proliferation were all inhibited by BTP2 with similar potency, it is concluded that CRAC channel activity is translated linearly into T-cell activity. This finding not only stresses the importance of CRAC channels for T-cell activity, but it also makes CRAC channels excellent targets for fine-tuning the immune response.

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