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., Ohga, 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., Smith, M. L., Chiou, G. X., 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.

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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, #15-009). Peripherally blood mononuclear cells (PBMCs) were isolated by a density gradient centrifugation at 450 x 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% EDTA, pH 7.5). For 3 min. After washing, PBMCs were washed with HBSS (200 x 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 medium complete at 37 °C (1.5 x 10^6 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, perfcol and 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 x 10^6 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, γ/δT-cells, monocytes, dendritic cells, granulocytes, B-cells, erythrocyads, and NK-cells were labeled by biotin-modified antibodies directed against CD8, CD14, CD16, CD19, CD38, CD56, CD123, γ/δ TCR, and glycoporphin A. Labeling was performed by addition of 10 μl of antibody mixture per 1 x 10^6 cells to give a final volume of 50 μl and incubation at 4 °C for 10 min. After centrifugation the non-T-cells were magnetically labeled using MACS microbeads coupled to anti-human antibody (20 μl of antibody per 1 x 10^6 cells in 30 μl of PBS/0.2% FCS, 15 min, 4 °C). Cells were washed with PBS/2% FCS and re-suspended to a density of 1 x 10^6 cells per 500 μl of PBS/2% FCS. The negative selection of CD4+ cells was performed with the autoMACS separation unit using the separation program “deplete.” Finally cells were washed in PBS/2% FCS and re-suspended in RPMI 1640 medium supplemented with 10% FCS, 1% t-glutamine, 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 diphertheria 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 (highest grade).

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 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 Labortechnik, Asbach, Germany). Filter plates (Uni Filter-96, GFC, Cranberry-Cardack, Dreisch, Germany) were washed three times with water at RT and then dried for about 1 h at 60 °C. Scintillitor (Microsint O, Cranberry-Cardack) was added, and radioactivity was measured using the Topcount microplate scintillation counter from Cranberry-Cardack.

Proliferation experiments using the EZAU assay were carried out in 96-well cell culture plates (BD Biosciences, #356072, flat bottom), and data analysis was performed 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, #BI-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 EL800 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 mM 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 (100 μg/ml, Sigma) 96-well plates (Costar, #3590). Cells were pre-coated in an Olympus IX 70 microscope equipped with a 20× (U Apo/040, numerical aperture (N.A.) 0.75) or a 40× (Uplan/Apo, N.A. 1.0) objective. Cells were alternately illuminated at 340 nm 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 Imagex,数字化, and analyzed using TILL Vision software. Ratio images were recorded at intervals of 5 s. [Ca2+]i, was estimated from the relation \[ \text{[Ca}^{2+}]_i = K_i (R - R_{\text{min}})/(R_{\text{max}} - R) \]
where the values of K_i, R_{\text{min}}, and R_{\text{max}} were determined from an \textit{in situ} calibration of fura-2 in Jurkat T-cells as described previously (24). Ca2+ Ringer’s solution contained in (millimolar): 155 NaCl, 4.5 KCl, 2 MgCl2, 0.8 CaCl2, 10 glucose, 100 mM HEPES, and 5 Hepes pH 7.4. 0.7 M stock of MgCl2 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 hybridoma OKT3, ATCC CRL-8501) were used to stimulate the cells. A sandwiched self-made chamber was used for all measurements, which allowed for a complete solution exchange <1 s.

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

Cells were collected by centrifugation (170 x g, 10 min, RT) and re-suspended in Ringer’s solution (5 mM HEPES, 10 mM glucose, 4.5 mM KCl, 155 mM NaCl, 2.5 mM MgCl2, pH 7.4) with 0.5 mM CaCl2 to give a cell density of 5 x 10^6 cells/ml (CD4+) or 2.5 x 10^5 (Jurkat). 150 μl of cell suspension and 50 μl of Ca2+ dye (Ca2+ Assay Kit, Molecular Devices, Sunnyvale, CA; dissolved in 5 ml of Ringer’s solution/0.5 mM CaCl2) were added per well. Before use plates (Black Bottom Plates, Corning) were pre-coated with 40 μl/well poly-L-ornithine (0.1% solution, Sigma) for 2 h at RT. Cells were set to the bottom of the plate by centrifugation (430 x g, 4 min, RT). Cells were loaded with the Ca2+ dye for 45 min at 37 °C and 5% CO2. Plates were adjusted to room temperature within 2 min in the dark. Ca2+ imaging experiments were performed with 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: 1 mM EGTA) and 10 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 mM Ca2+ (final free concentration)1 μM TG to the extracellular solution by adding 20 μl of Ringer’s solution/24 mM CaCl2/1 mM EGTA/1 μM TG. The inhibition was calculated as the percentage of the peak dye fluorescence (max fluorescence) and the baseline dye fluorescence (max fluorescence) and the baseline dye fluorescence (max fluorescence) and the baseline dye fluorescence (max fluorescence). If maximum dye fluorescence was used to stimulate the cells, the inhibition was calculated as percentage of the peak value or plateau value (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-
cultivated for various periods of time at 37 °C in culture medium containing 200 nM BTP2. The time needed for dye loading and seeding of cells on the 96-well plate was included in the preincubation time (BTP2 was present during dye loading). To determine the concentration dependence of BTP2 inhibition of Ca2+ signals following anti-CD3 stimulation in Jurkat cells, cells were incubated with various concentrations of BTP2 for 24 h. Measurements were performed in quadruplicates.

**Electrophysiology**—The standard pipette solution for whole cell patch-clamp recordings contained (in millimolar): 0.05 InsP3, (50 mM stock in H2O, Calbiochem) 140 Cs aspartate, 10 NaCl, 1–5 MgCl2, 10 EGTA, and 10 Heps (pH 7.2 with CsOH). The bath solution contained (in millimolar): 155 NaCl, 4.5 KCl, 2 CaCl2, 1 MgCl2, 10 d-glucose, and 5 Heps (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 (2–5 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 8.4 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.

**RESULTS**

Store-operated Ca2+ entry through CRAC channels is the major Ca2+ 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 Ca2+ entry in lymphocytes, Ca2+ signals in the presence and absence of BTP2 were compared.

Depletion of Ca2+ from the endoplasmic reticulum was induced by 1 μM TG in 0 mM Ca2+ Ringer’s solution. In the absence of extracellular Ca2+, TG causes a very small, transient Ca2+ rise in PBLs resulting from the unopposed leakage of Ca2+ from internal stores followed by Ca2+ extrusion across the plasma membrane. Exchanging the external solution to 1 mM Ca2+ Ring-
er’s solution allows Ca2+ influx through CRAC channels across the plasma membrane resulting in long lasting elevations of [Ca2+]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 Ca2+ entry (Fig. 1A). To quantify the reduction of store-operated Ca2+ signals, three parameters were analyzed in single cells: the initial influx rate as fitted by linear regression, the Ca2+ peak fitted by polynomial regression, and the Ca2+ plateau after 300 s (averaging 5 points). Analyzing varying concentrations of 24 h preincubation with BTP2 resulted in a dose-dependent inhibition of the initial Ca2+ influx rate (Fig. 1B), the Ca2+ peak (Fig. 1C), and the Ca2+ plateau (Fig. 1D). The IC50 values of the sigmoidal regressions were 6, 14, and 12 nM, respectively. PBLs
before, we analyzed the effect of the preincubation time dependent Ca\textsuperscript{2+} release in Jurkat cells. Analyzing resting [Ca\textsuperscript{2+}] levels, TG-dependent Ca\textsuperscript{2+} release peaks, and Ca\textsuperscript{2+} extrusion, no differences were observed between control conditions (no BTP2) and preincubation of up to 1 \(\mu\)M BTP2 for 24 h (Fig. 2A). Similar to the PBLs, there was, however, a marked inhibition of the initial Ca\textsuperscript{2+} influx rate (Fig. 2B), the Ca\textsuperscript{2+} peak (Fig. 2C), and the Ca\textsuperscript{2+} plateau (Fig. 2D) in Jurkat T-cells by BTP2. The IC\textsubscript{50} values of the sigmoidal regressions in Jurkat T-cells were 5, 10, and 7 nM, respectively. We conclude that BTP2 inhibits store-operated Ca\textsuperscript{2+} entry in CD4\textsuperscript{+}, CD8\textsuperscript{+}, and Jurkat T-cells in the low nanomolar range, whereas resting [Ca\textsuperscript{2+}] levels and TG-dependent Ca\textsuperscript{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 \(\mu\)M (data not shown). Therefore, we analyzed the effect of the preincubation time dependence of BTP2 in more detail and found that inhibition of Ca\textsuperscript{2+} signals by 200 nM BTP2 was almost complete within the first 2 h of incubation time at 37 °C in CD4\textsuperscript{+} and Jurkat T-cells (Fig. 3). Fig. 3A shows examples of Ca\textsuperscript{2+} measurements in Jurkat cells following store depletion by TG and subsequent activation of Ca\textsuperscript{2+} entry at various incubation times. Data are summarized in Fig. 3B revealing that about 75% of the inhibition in CD4\textsuperscript{+} Jurkat T-cells is complete within 2 h of preincubation.

TG is an unphysiological stimulus of Ca\textsuperscript{2+} entry in T-cells. To test whether Ca\textsuperscript{2+} entry in PBLs following more physiological stimulation of the T-cell receptor was also inhibited by BTP2, anti-CD3 was used for T-cell activation. Fig. 4A shows Ca\textsuperscript{2+} imaging experiments of two individual cells that responded to anti-CD3 in 0-Ca\textsuperscript{2+} Ringer’s solution with a clear Ca\textsuperscript{2+} release transient. 50 nM BTP2 did not interfere with the amplitude of Ca\textsuperscript{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\textsuperscript{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 Ca²⁺/H₁₁₀₀₁ signals following application of 1 mM Ca²⁺/H₁₁₀₀₁ Ringer’s solution was observed (Fig. 4A), which manifested itself in a clear reduction of the initial Ca²⁺/H₁₁₀₀₁ influx rate, the Ca²⁺/H₁₁₀₀₁ peak, and the Ca²⁺/H₁₁₀₀₁ plateau in the statistical analysis (Fig. 4B). In population measurements with the Flexstation, the IC₅₀ values for BTP2 inhibition of anti-CD3-activated Ca²⁺/H₁₁₀₀₁ 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 Ca²⁺ peak reflects a mixture of Ca²⁺ release by anti-CD3 and subsequently activated Ca²⁺ entry. It is thus not surprising that the Ca²⁺ peak was not inhibited fully by high BTP2 concentrations (Fig. 5B), because the Ca²⁺ release was not affected by BTP2 (compare Fig. 4, A and B, first panel). The IC₅₀ of the inhibition was 20 nM and very similar to the TG-induced influx peak. The same was true for the anti-CD3-induced Ca²⁺/H₁₁₀₀₁ influx plateau, which was inhibited by BTP2 with an IC₅₀ of 13 nM (Fig. 5B). The plateau was also not inhibited fully by BTP2, which was probably due to oscillating Ca²⁺ release sometimes observed following anti-CD3 stimulation (data not shown). We conclude that store-operated Ca²⁺ entry in T-cells is inhibited by BTP2 in the low nanomolar range regardless of the T-cell stimulation method.

Store-operated Ca²⁺ signals in T-cells depend on the activity of the following transport mechanisms: CRAC channels, which are responsible for the Ca²⁺ influx, Ca²⁺- and potential-dependent K⁺ channels, which control the membrane potential and therefore the electrical driving force for Ca²⁺ entry, Ca²⁺-
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\(^{2+}\) homeostasis.店

To inhibit store-operated Ca\(^{2+}\) signals in T-cells, BTP2 could potentially block CRAC channels, K\(^{+}\) channels, or mitochondrial Ca\(^{2+}\) homeostasis or it could enhance Ca\(^{2+}\) transport by Ca\(^{2+}\)-ATPases. Because the inhibitory effect of 50 nM BTP2 was still present when high K\(^{+}\) Ringer's solution was used to clamp the membrane potential of PBLs to about 0 mV (Fig. 6A), it is concluded that K\(^{+}\) channels are not responsible for the BTP2 block. Similarly, BTP2 still inhibited store-operated Ca\(^{2+}\) signals when carbonyl cyanide m-chlorophenylhydrazone was present to fully suppress mitochondrial Ca\(^{2+}\) uptake, which is known to modulate CRAC channel activity (26). Mitochondria are thus also not responsible for the action of BTP2. Increased Ca\(^{2+}\)\(^{2+}\) \textit{in vivo} as well as the sarcoplasmic-endoplasmic reticulum calcium ATPase was also excluded to be responsible for the BTP2 inhibition of Ca\(^{2+}\) signals, because BTP2 was effective in the presence of TG, which fully inhibits the ATPase. In addition, the Ca\(^{2+}\) export rate was not enhanced following TG stimulation (compare last 0-Ca\(^{2+}\) addition in Fig. 6, A and B) or anti-CD3 stimulation (examples in Fig. 4A; statistics in Fig. 6C) excluding other Ca\(^{2+}\) clearance mechanisms as the BTP2 effector. These experiments leave the CRAC channel as the most likely target for BTP2.

A potential direct effect of BTP2 on CRAC channels was tested in E6.1 Jurkat T-cells using the patch-clamp technique. 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 \(\mu M\) InsP\(_3\)/10 mM EGTA in the patch pipette having only 2 mM Ca\(^{2+}\) in the external solution to mimic extracellular physiological Ca\(^{2+}\) concentrations. 5 mM Mg\(^{2+}\) in the pipette solution were used to completely inhibit Mg\(^{2+}\)-inhibited channels (27–30).

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 nM), 98 min (100 nM), and 447 min (1 \(\mu\)M). 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 only 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 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 nM 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 (PMCA). The PMCA 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 PMCA 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 extracellular 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
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 \(\approx 75\) 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 [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).

![Inhibition of gene expression and proliferation in human CD4\(^{+}\) T-lymphocytes by BTP2.](image-url)

**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 [\(^{3}\)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\textsuperscript{2+} inhibitors, it is thus desirable to choose external Ca\textsuperscript{2+} concentrations below 2 mM to prevent “saturation” problems. Ishikawa et al. (22) used 2 mM free external Ca\textsuperscript{2+}, which could explain their higher IC\textsubscript{50} values compared with the ones in the present study performed with 1 mM free external Ca\textsuperscript{2+}.

Ishikawa et al. reported that the instantaneous effect of BTP2 on Ca\textsuperscript{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\textsuperscript{2+} signals in their system. It is disturbing that, on the one hand, our preincubation experiments reveal very low IC\textsubscript{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\textsubscript{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\textsubscript{50} values for Ca\textsuperscript{2+} influx inhibition but do very well fit our results on IL-2 production (Fig. 9: A, 8 mM; B, 15 mM) 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\textsuperscript{2+} signals, remain unaffected. These include Ca\textsuperscript{2+} release by TG or T-cell receptor stimulation, transport by Ca\textsuperscript{2+} ATPases, mitochondrial Ca\textsuperscript{2+} homeostasis, and activity of K\textsuperscript{+} channels. In addition, Mg\textsuperscript{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-aminoethylidiphenyl borate (19–18), SK&F 96365 (11, 12), or econazole, the latter of which inhibits CRAC channels with an IC\textsubscript{50} of about 600 nM in mast cells but, with similar IC\textsubscript{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\textsuperscript{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\textsuperscript{2+} signals, expression of IL-2, IL-5, and interferon \(\gamma\), 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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Potent Inhibition of Ca$^{2+}$ Release-activated Ca$^{2+}$ Channels and T-lymphocyte Activation by the Pyrazole Derivative BTP2

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