

**A pyrazole derivative potently inhibits lymphocyte  $\text{Ca}^{2+}$  influx  
and cytokine production by facilitating TRPM4 channel activity**

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## Running Title Page

Running Title: BTP2 blocks  $\text{Ca}^{2+}$  influx by facilitating TRPM4 channels

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## Abstract

3,5-bis(trifluoromethyl)pyrazole derivative (BTP2 or YM-58483) is an immunosuppressive compound that potently inhibits both  $\text{Ca}^{2+}$  influx and IL-2 production in lymphocytes. We here report that BTP2 dose-dependently enhances TRPM4, a  $\text{Ca}^{2+}$ -Activated Nonselective (CAN) cation channel, which decreases  $\text{Ca}^{2+}$  influx by depolarizing lymphocytes. The effect of BTP2 on TRPM4 occurs at low nanomolar concentrations and is highly specific, since other ion channels in T lymphocytes are not significantly affected and the major  $\text{Ca}^{2+}$  influx pathway in lymphocytes,  $I_{\text{CRAC}}$ , is blocked only at 100-fold higher concentrations. The efficacy of BTP2 to block IL-2 production is reduced about 100-fold when preventing TRPM4-mediated membrane depolarization, suggesting that the BTP2-mediated facilitation of TRPM4 channels represents the major mechanism for its immunosuppressive effect. Our results demonstrate that TRPM4 channels represent a previously unrecognized key element in lymphocyte  $\text{Ca}^{2+}$  signaling and that their facilitation by BTP2 supports cell membrane depolarization, which reduces the driving force for  $\text{Ca}^{2+}$  entry and ultimately causes the potent suppression of cytokine release.

## Introduction

In many cells, receptor activation evokes an increase in cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) composed of release from intracellular stores followed by store-operated  $\text{Ca}^{2+}$  entry via calcium release-activated calcium (CRAC) channels (Penner et al., 1993; Parekh and Penner, 1997; Putney et al., 2001). These channels are functionally best characterized in mast cells (Hoth and Penner, 1992; Hoth and Penner, 1993) and T lymphocytes (Zweifach and Lewis, 1993; Premack et al., 1994), and considered to be key elements for the activation of immune cells. The study of CRAC has been hampered by the lack of potent and specific pharmacological tools. Currently, the most widely used organic blockers of store-operated calcium entry pathways are imidazole antimycotics, such as econazole and the closely related compound, SKF-96365 as well as 2-APB (Mason et al., 1993; Chung et al., 1994; Franzius et al., 1994; Christian et al., 1996; Hermosura et al., 2002). However, neither of these compounds is very potent nor selective.

A class of pyrazole derivatives – bis(trifluoromethyl)pyrazoles (BTPs) – have been reported to act as potent immunosuppressive compounds by inhibiting cytokine release (IL-2, IL-4, IL-5, IFN- $\gamma$ , and others) from human lymphocytes as well as suppressing T cell proliferation (Djuric et al., 2000; Trevillyan et al., 2001; Chen et al., 2002; Ishikawa et al., 2003). In addition, these compounds have proven efficacious in various immune disease-relevant rodent and non-human primate models, where they inhibit trinitrochlorobenzene-induced contact hypersensitivity in mice (a model of T lymphocyte-mediated delayed type hypersensitivity), as well as *Ascaris*-induced immediate bronchoconstriction of cynomolgous monkeys (an asthma model) (Djuric et al., 2000; Ishikawa et al., 2003). Despite the rather detailed characterization about the compounds' potent effects in many immune-based cellular and animal models, the mechanism by which BTPs inhibit cytokine production in lymphocytes remains unknown. The effects of

BTPs are presumably linked to intracellular  $\text{Ca}^{2+}$  signaling, since the pyrazole derivative BTP2 (Fig. 2D) potently inhibits thapsigargin-evoked  $\text{Ca}^{2+}$  influx in the low nanomolar range (Ishikawa et al., 2003) and two recent reports suggest that BTP2 may inhibit the store-operated  $\text{Ca}^{2+}$  current  $I_{\text{CRAC}}$  (Zitt et al., 2004; He et al., 2005) as well as TRPC3 and TRPC5 channels (He et al., 2005).

The ‘transient receptor potential’ (TRP) proteins represent an important family of mammalian ion channels that are thought to be involved in  $\text{Ca}^{2+}$  signaling. Based on sequence similarities, the mammalian TRP channel family is divided into three subfamilies: TRPC, TRPV, and TRPM (Harteneck et al., 2000; Clapham et al., 2001; Montell et al., 2002a; Montell et al., 2002b). TRPM4, specifically the longer splice variant TRPM4b (which we will refer to as TRPM4 throughout this manuscript), is a widely expressed  $\text{Ca}^{2+}$ -activated nonselective cation channel of the TRPM ion channel subfamily that does not conduct  $\text{Ca}^{2+}$ , but instead mediates cell membrane depolarization (Launay et al., 2002). In electrically nonexcitable cells, a depolarization would tend to decrease the driving force for  $\text{Ca}^{2+}$  influx through store-operated  $\text{Ca}^{2+}$  channels. Many other ion channels, such as voltage-dependent potassium ( $\text{Kv}1.3$ ) channels (Lin et al., 1993; Hanson et al., 1999), intermediate/small-conductance calcium-activated potassium ( $\text{IK/SK2}$ ) channels (Lin et al., 1993; Jensen et al., 1999; Desai et al., 2000; Wulff et al., 2000), and swelling-activated chloride ( $\text{Cl}_{\text{vol}}$ ) channels (Lewis et al., 1993; Ross et al., 1994), are also known to be involved in the regulation of the driving force for  $\text{Ca}^{2+}$  influx, but they do so by hyperpolarizing the membrane potential (Penner et al., 1988; Cahalan and Chandy, 1997; Cahalan et al., 2001). Thus, the net  $\text{Ca}^{2+}$  influx into a cell is not only determined by  $\text{Ca}^{2+}$  entry pathways themselves, but also by ion channels that modulate the membrane potential.

In the present study, we have investigated the mechanism by which BTP2 inhibits  $\text{Ca}^{2+}$  signaling in Jurkat T cells. We demonstrate that BTP2 potently and selectively facilitates the activity of TRPM4, resulting in reduced  $\text{Ca}^{2+}$  entry and cytokine release. This compound therefore represents a novel and promising pharmacological tool to inhibit  $\text{Ca}^{2+}$  signaling in lymphocytes and other cell types that regulate  $\text{Ca}^{2+}$  influx by the concerted actions of store-operated  $\text{Ca}^{2+}$  channels and  $\text{Ca}^{2+}$ -activated cation channels.

## Materials and Methods

*Cells:* HEK-293 cells transfected with the FLAG-humanTRPM4b/pCDNA4/TO construct were grown on glass coverslips with DMEM medium supplemented with 10% FBS, blasticidin (5  $\Omega$ /ml), and zeocin (0.4 mg/ml). TRPM4b expression was induced one day before use by adding 1  $\Omega$ /ml tetracycline to the culture medium and patch-clamp experiments were performed 16-24 hours post-induction (for details see (Launay et al., 2002)). Human leukemic T cells (Jurkat cells) were grown in RPMI medium (10% FBS) and rat basophilic leukemia cells (RBL-2H3 cells) in DMEM (10% FBS).

*Materials:* BTP2 (N-[4-3,5-bis(trifluoromethyl)pyrazol-1-yl]-4-methyl-1,2,3-thiadiazole-5-carboxamide or YM-58483) and SKF-96365 were synthesized by Yamanouchi Pharmaceutical Co., Ltd (now Astellas Pharma Inc., Tokyo, Japan). Econazole, margatoxin, apamin, charybdotoxin, and stilbenedisulfonate 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) were purchased from Sigma and dissolved in DMSO. Phytohemagglutinin (PHA-P) was obtained from Sigma and dissolved in PBS.

*Solutions:* For  $I_{CRAC}$  measurements, the standard bath solution had the following composition (in mM): NaCl 140, KCl 2.8,  $CaCl_2$  10,  $MgCl_2$  2, CsCl 10, glucose 10, Hepes·NaOH 10, pH 7.2, with osmolarity adjusted to around 320 mOsm. Intracellular pipette-filling solutions contained (in mM): Cs-glutamate 140, NaCl 8,  $MgCl_2$  1, Cs-BAPTA 10, HEPES·CsOH, pH 7.2 adjusted with CsOH. In experiments where  $[Ca^{2+}]_i$  was buffered to elevated levels,  $CaCl_2$  was added as necessary (calculated with WebMaxC <<http://www.stanford.edu/~cpatton/webmaxcS.htm>>, T = 24 °C, pH = 7.2, ionic equivalent = 0.16). Solution changes were performed by pressure ejection from a wide-tipped pipette.

*Electrophysiology:* Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 21-25 °C. High-resolution current recordings were acquired by a computer-based patch-clamp amplifier system (EPC-9, HEKA, Lambrecht, Germany). Patch pipettes had resistances between 2-4 M $\Omega$  after filling with the standard intracellular solution. Immediately

following establishment of the whole-cell configuration, voltage ramps of 50-200 ms duration spanning the voltage range of  $-100$  to  $+100$  mV were delivered at a rate of 0.5 Hz over a period of 300 to 400 seconds. All voltages were corrected for a liquid junction potential of 10 mV between external and internal solutions when using glutamate as intracellular anion. Currents were filtered at 2.9 kHz and digitized at 100  $\Omega$ s intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp using the automatic capacitance compensation of the EPC-9. The low-resolution temporal development of membrane currents was assessed by extracting the current amplitude at  $-80$  mV or  $+80$  mV from individual ramp current records. Where applicable, statistical errors of averaged data are given as means  $\pm$  S.E.M. with n determinations.

Voltage-dependent potassium ( $K_v1.3$ ) currents were measured in Jurkat cells. The bath solution contained (in mM): NaCl 160, KCl 4.5,  $CaCl_2$  2,  $MgCl_2$  1, and HEPES $\cdot$ NaOH 10 (pH 7.2). The internal solution contained (in mM): K-glutamate 140,  $MgCl_2$  2,  $CaCl_2$  1, K-EGTA 10, and HEPES $\cdot$ KOH 10 (pH 7.2). Ramps were given every 30 s ( $-100$  to  $+100$  mV in 200 ms), and cells were held at  $-80$  mV between ramps. Currents were not leak-subtracted.

Calcium-activated potassium ( $SK_{Ca2}$ ) currents were measured in Jurkat cells. The bath solution contained (in mM): KCl 164.5,  $CaCl_2$  2,  $MgCl_2$  1, and HEPES $\cdot$ KOH 5 (pH 7.2). The internal solution contained the following (in mM): K-aspartate 135,  $MgCl_2$  2, K $\cdot$ EGTA 1.1, and HEPES $\cdot$ KOH 10 (pH 7.2). Ramps were given every 2 s ( $-100$  to  $+40$  mV in 200 ms), and cells were held at  $-80$  mV between ramps. Free intracellular  $[Ca^{2+}]_i$  was adjusted to 1  $\mu$ M.

Swelling-activated chloride ( $Cl_{vol}$ ) currents were measured in Jurkat cells. The bath solution contained (in mM): NaCl 160, KCl 4.5,  $MgCl_2$  1,  $CaCl_2$  2, and HEPES $\cdot$ NaOH 5 (pH 7.2). The internal solution contained (in mM): Cs-glutamate 160,  $MgCl_2$  2,  $CaCl_2$  0.1, Cs $\cdot$ EGTA 1.1, Na $\cdot$ ATP 4, glucose 100, and HEPES $\cdot$ CsOH 10 (pH 7.2). Ramps were given every 2 s ( $-100$  to  $+50$  mV in 200 ms), and cells were held at  $-60$  mV between ramps.



TRPM4 currents were measured in Jurkat cells and HEK-293 cells over-expressing TRPM4b. The bath solution contained (in mM): NaCl 140, KCl 2.8, MgCl<sub>2</sub> 2, CaCl<sub>2</sub> 1, glucose 10, and HEPES·NaOH 10 (pH 7.2). The internal solution contained (in mM): K-glutamate 120, NaCl 8, MgCl<sub>2</sub> 1, K·BAPTA 10, and HEPES·KOH 10 (pH 7.2). Ramps were given every 2 s (−100 to +100 mV in 50 ms), and cells were held at −80 mV between ramps. Free intracellular [Ca<sup>2+</sup>]<sub>i</sub> was adjusted as indicated in figure legends.

*IL-2 production assay:* Jurkat cells (5 × 10<sup>6</sup> cells/ml) were placed in a 96-well microplate and incubated with either 10  $\Omega$ g/ml or 2  $\Omega$ g/ml PHA (Sigma) for 24 h and the supernatant was collected from these cells after centrifugation (200xg, 24 °C for 3 min). The concentration of IL-2 was measured by the human IL-2 ELISA system (human IL-2 ELISA Kit DuoSet, Genzyme Co., Cambridge, MA). OD values at 450 nm were measured by a microplate reader (Spectra Max 190, Molecular Devices Corporation, CA). Data for BTP2-treated cells were normalized to untreated control cells.

*Calcium measurements:* For Ca<sup>2+</sup> measurements, fura-2 AM loaded cells (5  $\Omega$ M / 30 min / 37 °C) were kept in standard extracellular saline containing (in mM): NaCl 140, KCl 2.8, CaCl<sub>2</sub> 1, MgCl<sub>2</sub> 2, glucose 10, Hepes·NaOH 10, pH 7.2 and excited by wavelengths of 340 and 380 nm. Fluorescence emission of several cells were simultaneously recorded at a frequency of 1 Hz using a dual excitation fluorometric imaging system (TILL-Photonics, Gräfelfingen, Germany) controlled by TILL-Vision software. Signals were computed into relative ratio units of the fluorescence intensity of the different wavelengths (340/380 nm).

## Results

### *Effect of BTP2 on $I_{CRAC}$*

Since BTP2 inhibits thapsigargin-induced  $Ca^{2+}$  influx (Ishikawa et al., 2003) and reportedly inhibits the store-operated calcium current  $I_{CRAC}$  (Zitt et al., 2004), we analyzed the effects of BTP2 on  $I_{CRAC}$  in RBL-2H3 and Jurkat T cells.  $I_{CRAC}$  was activated by a standard protocol following whole-cell break-in with 20  $\mu$ M  $InsP_3$  in the pipette solution. The time course of whole-cell current was monitored by 200 ms voltage ramps that spanned  $-100$  to  $+100$  mV and were applied every 2 s. Each voltage ramp yields a full current-voltage (I/V) relationship (Fig. 1C) and the  $I_{CRAC}$  time course is shown in Fig. 1A. It was constructed by measuring the whole-cell current amplitude on the ramp trace corresponding to  $-80$  mV, normalizing the current amplitude to the value obtained just prior to application of compound, and plotting it as a function of time. Extracellularly applied BTP2 blocked  $InsP_3$ -evoked  $I_{CRAC}$  in Jurkat cells in a concentration-dependent manner (Fig. 1A). Complete inhibition of the current was obtained within 200 s after addition of 10  $\mu$ M BTP2. Lower concentrations also blocked, but they did so more slowly. Figure 1E shows the dose-response relationship for BTP2-mediated block of  $I_{CRAC}$  at 300 s after application, along with the dose-response curve obtained for the reference compound econazole under identical experimental conditions (raw data not shown). In Jurkat cells, the apparent half-maximal inhibitory concentrations ( $IC_{50}$ ) for BTP2 and econazole were 2.2  $\mu$ M and 4.0  $\mu$ M, respectively (Hill coefficient = 1.7, each).

Next, we studied the effect of BTP2 on  $I_{CRAC}$  in RBL-2H3 cells and observed that BTP2 also blocks  $InsP_3$ -evoked  $I_{CRAC}$  in these cells in a concentration-dependent manner (Fig. 1B). Interestingly, with an  $IC_{50}$  of 0.5  $\mu$ M (Fig. 1F; Hill coefficient = 1.4), BTP2 appears slightly

more potent in RBL cells than in Jurkat cells, whereas the potency of econazole in RBL cells ( $IC_{50} = 4.2 \text{ } \mu\text{M}$ ; Hill coefficient = 1.4) was similar to that observed in Jurkat cells. To study the site of action, we included BTP2 in the pipette solution. Intracellularly applied BTP2 (10  $\mu\text{M}$ ) did not exhibit any inhibitory effect on  $I_{CRAC}$  in either RBL-2H3 cells or Jurkat cells, nor did it affect the block of  $I_{CRAC}$  induced by extracellularly applied BTP2 in either cell type (Fig. 1D). This suggests that the inhibitory action of BTP2 on  $I_{CRAC}$  may be located extracellularly, however, we cannot completely rule out the possibility that the lipophilicity of the compound might allow it to escape rapidly across the plasma membrane before impacting the channel.

While the above results demonstrate that BTP2 can inhibit  $I_{CRAC}$  in both RBL and Jurkat cells, the potency appears to be too low to account for the compound's ability to suppress cytokine production, which occurs in the low nanomolar range. Hoth and colleagues recently reported that  $I_{CRAC}$  is inhibited by BTP2 at lower concentrations, but this effect requires hours to develop and they arrived at an  $IC_{50}$  of about 10 nM after 24 hours of pre-incubation with the compound (Zitt et al., 2004). Gill and colleagues reported that store-operated  $Ca^{2+}$  entry was reduced much quicker, within about 10 min, but the concentrations required to do so in HEK-293 cells and DT40 B cells were found to be at least one order of magnitude higher (He et al., 2005), similar to the efficacy of BTP2 in blocking  $I_{CRAC}$  in RBL cells (see Fig. 1F). To further assess the involvement of  $I_{CRAC}$ , we analyzed the kinetics of BTP2 action on both  $Ca^{2+}$  signaling and cytokine production. Figure 1G illustrates the effect of 100 nM BTP2 on Jurkat  $Ca^{2+}$  signals evoked by 10  $\mu\text{g/ml}$  phytohemagglutinin (PHA). This relatively low concentration of BTP2 suppressed the PHA-induced  $Ca^{2+}$  signals almost completely within about 10 minutes and therefore cannot be easily reconciled with the reported slow  $I_{CRAC}$  inhibition (time constant = 98 min) (Zitt et al., 2004). We next analyzed the temporal development of the efficacy of BTP2 to

suppress IL-2 release over variable time spans ranging from 2-24 hours. Jurkat cells were stimulated with 10  $\mu$ g/ml PHA and IL-2 production was measured by ELISA at various times following the co-application of PHA and various concentrations of BTP2. Figure 1H illustrates that there was no significant change in the potency with which BTP2 suppressed IL-2 release, since the  $IC_{50}$  remained constant in the low nanomolar range even when measuring IL-2 release as early as 2 hours after stimulation, again suggesting that the main mechanism of action of BTP2 is engaged quickly and does not require several hours to develop. This would suggest that the  $I_{CRAC}$  inhibition observed by Hoth and colleagues at low nanomolar concentrations may be secondary to another event rather than a direct effect on CRAC channels. Since BTP2 has been demonstrated to suppress lymphocyte proliferation (Trevillyan et al., 2001; Zitt et al., 2004), one possible reason for the decrease in  $I_{CRAC}$  amplitude following long BTP2 pre-incubations could be that lymphocytes become arrested at a certain cell-cycle stage in which  $I_{CRAC}$  is suppressed. Indeed, serum-starved RBL cells become arrested in G0/G1 and have been reported to lack significant  $I_{CRAC}$  (Bodding, 2001). Gill and colleagues found a relatively fast inhibition of store-operated  $Ca^{2+}$  influx, but the concentrations required likely reflect the inhibition of  $I_{CRAC}$  we observe in e.g. RBL cells. Given that the effect of BTP2 even at low concentrations is relatively quick compared to the time course over which it compromises  $I_{CRAC}$ , we considered alternative or additional mechanisms that BTP2 may be targeting.

### ***Effect of BTP2 on other ionic currents in Jurkat cells***

Whole-cell recordings permit direct measurement of several types of channel activity that may affect  $Ca^{2+}$  signaling in Jurkat cells such as the voltage-dependent K channel Kv1.3, the small-conductance  $Ca^{2+}$ -activated K channel  $SK_{Ca2}$ , and volume regulatory  $Cl^-$  channel  $Cl_{vol}$ . We first analyzed the efficacy of known inhibitors of these channels on PHA-stimulated cytokine

production in Jurkat T lymphocytes. Of the presumed  $I_{CRAC}$  inhibitors, BTP2, econazole, and SKF-96365 (10  $\mu$ M each), only BTP2 completely inhibited PHA-induced IL-2 production (Fig. 2A), consistent with its complete inhibition of  $I_{CRAC}$  at that concentration. Econazole also suppressed cytokine production, albeit not completely, whereas SKF-96365 was not very effective. The other inhibitors exhibited varying degrees of inhibition. The  $K^+$  channel blockers, including the Kv1.3-specific inhibitor margatoxin (MgTx, 1 nM), the  $SK_{Ca}$  inhibitor apamin (10 nM), and the IK inhibitor charybdotoxin (ChTx, 100 nM) produced inhibitory effects in the range of 30-50%, whereas the  $Cl_{vol}$  channel inhibitor DIDS (100  $\mu$ M) was ineffective. The efficacy of  $K^+$  channel blockers to affect IL-2 production is consistent with the fact that they inhibit ion currents that promote  $Ca^{2+}$  influx via a membrane hyperpolarization. However, none of the compounds was able to suppress cytokine production completely. Moreover, none of the  $K^+$  and  $Cl^-$  channel inhibitors tested in this study exhibited significant additive inhibitory effects on IL-2 production when it was already partially inhibited by 10 nM BTP2 (Fig. 2B).

To assess the specificity of BTP2, we tested for its effects on the  $K^+$  and  $Cl^-$  channels expressed in Jurkat cells. BTP2 did not significantly affect the activity of any of these channels even at the highest concentration (10  $\mu$ M) tested (Fig. 2C), with the possible exception of  $Cl^-$  currents, which were inhibited by ~50%. Also, BTP2 showed no inhibitory effect on ADP-ribose-activated TRPM2 channels expressed in HEK-293 cells (Perraud et al., 2001; Sano et al., 2001) and inward rectifier  $K^+$  currents in RBL cells (Lindau and Fernandez, 1986). Given the significant discrepancy in the potency of BTP2 to inhibit IL-2 production at low nanomolar concentrations versus  $I_{CRAC}$  inhibition with an  $IC_{50}$  of 2.1  $\mu$ M and the lack of effect on  $K^+$  channels, we considered TRPM4, a  $Ca^{2+}$ -activated nonselective cation (CAN) channel expressed in Jurkat cells (Launay et al., 2002; Launay et al., 2004), as a possible target.

### ***Voltage- and $[Ca^{2+}]_i$ -dependence of TRPM4***

We previously have shown that TRPM4 can be detected at RNA and protein level in various T cells of murine and human origin and that these channels are functional in Jurkat T cells (Launay et al., 2004). Moreover, we have observed calcium-activated TRPM4-like currents in human primary T cells (unpublished observations). To assess TRPM4 function, we here used Jurkat T cells as a model system and carried out experiments very similar to those described in Fig. 1A, except that the free  $Ca^{2+}$  concentration of the intracellular pipette solution was buffered to levels of 0.3 - 1.8  $\mu M$ . Under these conditions, Jurkat cells indeed produce large cation currents with current-voltage signatures indistinguishable from those of TRPM4, and we will refer to these currents as  $I_{CAN}$ . In initial experiments, application of BTP2 to maximally activated  $I_{CAN}$  did not yield any significant modification of the currents, either inhibitory or facilitatory (data not shown). However, these experiments were carried out at a holding potential of 0 mV, which yields maximal activation of  $I_{CAN}$  in Jurkat cells (see Fig. 4A), whereas the resting potential of these cells is considerably more negative. The resting membrane potential is an important factor in determining TRPM4 behavior, since the channel exhibits a striking voltage dependence (Launay et al., 2002; Hofmann et al., 2003; Nilius et al., 2003). To assess the influence of membrane voltage on TRPM4 activity, we studied the voltage dependence of TRPM4 in HEK-293 cells.

Figure 3A illustrates the activation of  $Ca^{2+}$ -activated currents in TRPM4 over-expressing HEK-293 cells kept at various membrane holding potentials and perfused with a solution in which  $[Ca^{2+}]_i$  was buffered to 1.3  $\mu M$ . Exemplary current-voltage-relationships of TRPM4 obtained by voltage ramps spanning -100 to +100 mV and delivered from holding potentials of -60 and +60 mV are illustrated in Fig. 3B. They illustrate the typical characteristics of TRPM4 as previously

reported (Launay et al., 2002; Hofmann et al., 2003; Nilius et al., 2003). The data sets obtained at different holding potentials demonstrate that the magnitude of inward currents carried by TRPM4 is strongly dependent on the holding potential from which the voltage ramps are delivered. The maximum current amplitude obtained when holding the cell membrane potential at negative values is considerably smaller than that obtained at more depolarized holding potentials (Fig. 3A). The bar graph of Fig. 3C illustrates the voltage dependence of maximum inward currents obtained by perfusing cells with 1.8  $\mu\text{M}$  free  $\text{Ca}^{2+}$ .

A more comprehensive analysis of this voltage dependence is presented in Fig. 3D, where we plot the maximum current amplitudes of TRPM4 currents (extracted from ramp currents at  $-80$  mV) as a function of  $[\text{Ca}^{2+}]_i$  and measured in cells that were held at different holding potentials ( $-60$  mV,  $-30$  mV and  $+60$  mV). From this analysis it is clear that the degree of TRPM4 activation is both dependent on  $[\text{Ca}^{2+}]_i$  and the membrane potential, with increasing  $\text{Ca}^{2+}$  and positive voltages synergizing to recruit larger maximal currents. The dose-response curves fitted to the various data sets of Fig. 3D reveal that the holding potential does not affect the responsiveness of TRPM4 to  $[\text{Ca}^{2+}]_i$ , since the apparent  $\text{EC}_{50}$  for TRPM4 activation remains fairly constant at  $\sim 500$  nM, regardless of the holding potential (Fig. 3D). From this, we infer that the primary effect of membrane potential on TRPM4 activity resides at the level of the channel's open probability, which is consistent with our previous observations in single-channel recordings of TRPM4, where negative membrane voltages strongly reduced their open probability (Launay et al., 2002).

### ***Effect of BTP2 on TRPM4 in HEK-293 cells***

Based on the above observations, we reasoned that BTP2 could potentially increase the open probability of TRPM4 at negative membrane potentials. We therefore kept the holding potential of HEK-293 cells over-expressing TRPM4 at  $-80$  mV and then tested BTP2 for possible augmentation of inward currents. Fig. 3E illustrates average inward currents carried by TRPM4 at  $-80$  mV with  $[Ca]_i$  clamped at  $500$  nM. Control cells indeed showed a significantly reduced TRPM4 current at this negative holding potential and these were greatly enhanced when cells were pre-treated with  $10$   $\mu$ M BTP2 for several minutes. The current-voltage relationships illustrated in Fig. 3F were obtained from representative control and BTP2-treated cells at  $170$  s after whole-cell establishment and they demonstrate the massive upregulation of TRPM4 currents by BTP2. These results suggest that BTP2 can facilitate TRPM4 channels over-expressed in the heterologous expression system.

### ***Effect of BTP2 on $I_{CAN}$ in Jurkat T cells***

Figure 4A demonstrates that  $I_{CAN}$  in Jurkat cells also exhibits strong voltage dependence. Cells kept at a holding potential of  $0$  mV and perfused with pipette solutions in which  $[Ca^{2+}]_i$  was buffered to  $500$  nM yielded large cation currents (Fig. 4A) with the typical current-voltage relationship of TRPM4 (Fig. 4B). Under the same experimental conditions, cells kept at a holding potential of  $-80$  mV, exhibited strongly reduced  $I_{CAN}$  current amplitudes (Fig. 4A), quite analogous to the observations made with TRPM4 in the heterologous expression system (cf. Fig. 3A). Under these conditions, BTP2 did not enhance any currents when  $[Ca^{2+}]_i$  was buffered to resting levels of  $100$  nM or below (data not shown). However, when intracellular solutions were buffered to slightly elevated  $[Ca^{2+}]_i$  of  $500$  nM, the pre-incubation of Jurkat cells with various



concentrations of BTP2 resulted in a dose-dependent enhancement of  $\text{Ca}^{2+}$ -activated inward currents (Fig. 4C). The effect manifested itself in both an increase in maximal current amplitude and in the acceleration of the kinetics with which  $I_{\text{CAN}}$  developed. We analyzed the facilitation of  $I_{\text{CAN}}$  by constructing dose-response relationships for BTP2-mediated increases in inward currents as a function of BTP2 concentration at 300 s after whole-cell recording (Fig. 4D). The apparent half-maximal effective concentration ( $\text{EC}_{50}$ ) was 8 nM. Thus, the potency of BTP2 to enhance  $I_{\text{CAN}}$  is roughly 100-fold higher than that of inhibiting  $I_{\text{CRAC}}$  and close to its efficacy in inhibiting IL-2 production. We therefore propose that the primary action of BTP2 is based on its ability to shift the voltage dependence of TRPM4, so that open probability is increased at more negative membrane potentials. This effect enhances  $I_{\text{CAN}}$ -mediated cell membrane depolarization and thereby reduces the driving force for  $\text{Ca}^{2+}$  influx.

We sought to further corroborate the above hypothesis by assessing IL-2 production in Jurkat cells under experimental conditions that would negate the depolarizing action of  $I_{\text{CAN}}$ . This can be accomplished by extracellular solutions in which  $\text{Na}^+$ , the primary charge carrier of  $I_{\text{CAN}}$ , is replaced by an impermeant cation such as choline. This largely prevents TRPM4-mediated membrane depolarization and has been shown to enhance  $\text{Ca}^{2+}$  influx (Launay et al., 2002). We analyzed the effect of BTP2 on PHA-stimulated IL-2 production in Jurkat cells cultured in the presence or absence of  $\text{Na}^+$  in the extracellular medium. As illustrated in Fig. 4E, BTP2 potently inhibited IL-2 production in the presence of  $\text{Na}^+$  with an  $\text{IC}_{50}$  of 0.25 nM. This value is lower than the previously reported  $\text{IC}_{50}$  of ~10 nM and may be a consequence of the Ringer-like extracellular solution used in the present study. When performing the IL-2 assay in choline-based  $\text{Na}^+$ -free media under otherwise identical conditions, the  $\text{IC}_{50}$  for BTP2-mediated inhibition of IL-2 production was reduced by about 100-fold ( $\text{IC}_{50} = 14$  nM). This result is entirely consistent

with the notion that at low concentrations, BTP2 acts primarily via facilitation of  $I_{CAN}$ . The specificity of the  $Na^+$  removal experiment is demonstrated by the dose-response curves of econazole-mediated inhibition of IL-2 production under the same experimental conditions (Fig. 4F). Here, the removal of  $Na^+$  did not alter the efficacy of econazole to suppress cytokine production.

### ***Discussion***

Our results establish a novel principle in regulating cytokine release from lymphocytes, a process that is intimately linked to elevations in  $[Ca^{2+}]_i$ . We demonstrate that the pyrazole compound BTP2, a potent inhibitor of IL-2 release in lymphocytes, effectively facilitates the activity of the  $[Ca^{2+}]_i$ -activated nonselective cation channel TRPM4 both in a heterologous expression system as well as in Jurkat cells, which express the protein natively. The efficacy of facilitating  $I_{CAN}$  and the ability to suppress cytokine production occur in the low nanomolar concentration range within a few minutes of BTP2 exposure, suggesting that the primary mechanism of action of BTP2 is due to its effect on TRPM4. At higher concentrations, the compound can also inhibit  $I_{CRAC}$ . Even at these higher concentrations, the specificity of BTP2 for  $I_{CRAC}$  seems remarkable when compared to the effects on a range of other ion channels found in lymphocytes. Thus, of all compounds presently known to inhibit  $I_{CRAC}$ , BTP2 represents the most potent and most selective  $I_{CRAC}$  inhibitor, even though its primary mechanism of action appears linked to TRPM4.

BTP2 appears to be a very potent inhibitor of T cell activation in the low nanomolar range ((Djuric et al., 2000; Trevillyan et al., 2001; Chen et al., 2002; Ishikawa et al., 2003); see Figs. 1 and 4) and it inhibits  $[Ca^{2+}]_i$  signals within a few minutes (Fig. 1G). Any mechanism that accounts for this effect should match the potency and kinetic aspects of BTP2 action. So far,

three mechanisms of BTP2 have been proposed to account for its inhibitory effects on  $\text{Ca}^{2+}$  influx and cytokine production: the inhibition of the store-operated current  $I_{\text{CRAC}}$  (Zitt et al., 2004; He et al., 2005), the inhibition of the TRPC channels TRPC3 and TRPC5b (He et al., 2005), and the facilitation of TRPM4 (the present study).

All three studies agree that store-operated  $\text{Ca}^{2+}$  influx is compromised by BTP2, however, discrepancies exist in terms of potency and kinetics of block. Zitt et al. attribute BTP2's potent suppression of  $\text{Ca}^{2+}$  influx in lymphocytes in the low nanomolar range to a slow block of  $I_{\text{CRAC}}$  that develops over hours. He et al. did not measure  $I_{\text{CRAC}}$  directly, but found that thapsigargin and receptor-mediated  $\text{Ca}^{2+}$  signals were inhibited within about 10 min., but the potency was at least one order of magnitude higher ( $\text{IC}_{50} \sim 0.1\text{-}0.3 \text{ }\mu\text{M}$ ). It should be noted that the  $\text{IC}_{50}$  values reported by Gill and colleagues are not directly comparable to the values provided by Zitt et al. or our own study, as they represent only indirect measures of channel activity as determined from changes in  $[\text{Ca}^{2+}]_i$  and therefore likely overestimate the true potency at the channel level. Our study finds that  $I_{\text{CRAC}}$  in RBL and Jurkat T cells can indeed be inhibited relatively quickly within a few minutes and potencies range from 0.5 to 4  $\mu\text{M}$  as derived from direct current inhibition profiles. While the study of Hoth and colleagues is compatible with the potency range of BTP2 on inhibition of cytokine production, the slow kinetics of  $I_{\text{CRAC}}$  inhibition reported by Zitt et al. is at odds with the relatively fast inhibition of  $\text{Ca}^{2+}$  influx (see Fig. 1G). Conversely, the studies by Gill and colleagues and our own data are compatible with the kinetics of inhibition of store-operated currents, but neither study found a low nanomolar potency that would be required to explain the efficacy of BTP2 in terms of  $I_{\text{CRAC}}$  inhibition alone. Likewise, the efficacy of BTP2 in blocking other channels such as heterologously expressed TRPC3 and TRPC5, which Gill and colleagues estimate to be  $\sim 0.3 \text{ }\mu\text{M}$ , does not approach the low BTP2 concentrations that suffice

to mediate inhibition of cytokine release in native T cells. These channels also would not seem to be responsible for BTP-mediated inhibition of cytokine release from lymphocytes, since the only  $\text{Ca}^{2+}$  permeable current elicited by either antigen or thapsigargin stimulation appears to be  $I_{\text{CRAC}}$  and no ion channels that fit the profile of TRPC3 or TRPC5 have so far been reported in the literature. From these considerations, and based on the data present in this study, it would seem that the only mechanism that fits the criteria for BTP2 effects in T cells in terms of potency and kinetics is provided by the facilitation of TRPM4. Not only are the potency and kinetic properties adequate, but also the fact that these channels have been identified in T cells as important factors in determining the amount of  $\text{Ca}^{2+}$  influx (Launay et al., 2004). At higher concentrations,  $I_{\text{CRAC}}$  inhibition may contribute to this inhibition. Similarly, at these higher concentrations and in cell types that express TRPC3/TRPC5, the BTP2 effects may also involve inhibition of TRPC channels.

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## Footnotes

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## Figure Legends

**Fig. 1. Effects of BTP2 on  $I_{CRAC}$ .** Whole-cell currents were recorded in Jurkat cells and RBL-2H3 cells.  $I_{CRAC}$  was activated using intracellular solutions containing 20  $\mu$ M  $InsP_3$  and 10 mM BAPTA and monitored by applying voltage ramps of 50 ms duration that ranged from  $-100$  to  $+100$  mV from a holding potential of 0 mV. Data are  $\pm$  S.E.M. where applicable. **(A)** Average  $I_{CRAC}$  at  $-80$  mV ( $n = 3-10$ ) in Jurkat cells. BTP2 (closed circles = 1  $\Omega$ M, open squares = 3  $\Omega$ M, open circles = 10  $\Omega$ M) was applied extracellularly as indicated by the bar. Currents were normalized to the amplitude at 180 s. **(B)** Average  $I_{CRAC}$  at  $-80$  mV ( $n = 3-10$ ) in RBL-2H3 cells. BTP2 (closed circles = 10 nM, open squares = 1  $\Omega$ M, open circles = 10  $\Omega$ M) was applied extracellularly as indicated by the bar. Currents were normalized to the amplitude at 180 s. **(C)** Representative current-voltage ( $I/V$ ) relationships for fully activated  $I_{CRAC}$  at 180 s and fully inhibited  $I_{CRAC}$  (by 10  $\mu$ M BTP2) at 480 s in Jurkat cells. **(D)** Jurkat and RBL cells were perfused with standard internal solution containing 10  $\mu$ M BTP2 ( $n = 10$ ) which failed to block  $I_{CRAC}$ . At 180 s into the experiment, 10  $\Omega$ M BTP2 was applied extracellularly and caused complete block of  $I_{CRAC}$ . **(E)** Dose-response relationship for BTP2- and econazole-mediated block of  $I_{CRAC}$  in Jurkat cells ( $IC_{50}$  for BTP2 = 2.2  $\Omega$ M and  $IC_{50}$  for econazole = 4.0  $\Omega$ M with Hill coefficients of 1.7, each). **(F)** Dose-response relationship for BTP2- and econazole-mediated block of  $I_{CRAC}$  in RBL-2H3 cells ( $IC_{50}$  for BTP2 = 0.5  $\Omega$ M and  $IC_{50}$  for econazole = 4.2  $\Omega$ M, with Hill coefficients of 1.4, each). **(G)** Average  $Ca^{2+}$  signals in intact Jurkat T cells stimulated by PHA (20  $\Omega$ g/ml). Control cells (open circles;  $n = 32$ ) produced sustained changes in  $[Ca^{2+}]_i$ , whereas  $Ca^{2+}$  signals in cells that were exposed to 100 nM BTP2 (closed circles;  $n = 58$ ) returned to baseline within  $\sim 10$  min. **(H)** Dose-response relationships for BTP2-induced inhibition of IL-2 production over different stimulation times. Jurkat cells ( $5 \times 10^6$  cells/ml) were incubated with PHA (10  $\mu$ g/ml) and various concentrations of BTP2. At the indicated times (range 2-24 h), IL-2 content in the supernatant was measured by ELISA and normalized to control cells not treated with BTP2 (the absolute amounts of IL-2 in the absence of BTP2 were: 2hrs =  $9 \pm 1.9$  pg/ml, 4hrs =  $135 \pm 54$  pg/ml, 8hrs =  $702 \pm 157$  pg/ml, 24hrs =  $1500 \pm 329$  pg/ml. Mean  $\pm$  S.E.M.).

**Fig. 2. Effects of channel blockers on IL-2 production and membrane currents in Jurkat cells.** Jurkat cells ( $5 \times 10^6$  cells/ml) were incubated with PHA (2  $\mu$ g/ml). 24h after stimulation,

IL-2 content in the supernatant was measured by ELISA. Data are  $\pm$  S.E.M. where applicable. **(A)** Effects of different individual channel blockers on IL-2 production ( $n = 3$  each). 10  $\Omega$ M BTP2 is indicated in gray, the other compounds are shown as open bars. **(B)** Additive effects of different channel blockers (at the same concentration as in (A)) on IL-2 production in cells that were treated with 10 nM BTP2 plus the channel blockers (open bars) as compared to 10 nM BTP2 inhibition alone (gray bar; 10 nM BTP2 caused  $\sim$ 40% inhibition;  $n = 3$  each). For comparison purposes, the dashed line indicates the level of inhibition by 10 nM BTP2 alone. **(C)** Effects of BTP2 (gray bars) and reference compounds (open bars) on different ion channels in Jurkat T cells ( $n = 5-8$ ). **(D)** Chemical structure of BTP2.

**Fig. 3. Voltage-dependence of TRPM4 and facilitation by BTP2.** Whole-cell currents were recorded in HEK-293 cells over-expressing TRPM4. Data are  $\pm$  S.E.M. where applicable. **(A)** TRPM4 currents were activated using intracellular solutions with  $[Ca^{2+}]_i$  buffered to 1.3  $\Omega$ M and monitored by applying voltage ramps of 50 ms duration that ranged from  $-100$  to  $+100$  mV from a holding potential of either  $-60$  or  $+60$  mV ( $n = 6$  each). Note the strong suppression of inward currents of TRPM4 at holding potential of  $-60$  mV. **(B)** Representative current-voltage (I/V) relationships for TRPM4 at the two holding potentials collected 6 sec after whole-cell break-in. **(C)** Average inward peak current sizes measured with 1.8  $\Omega$ M internal calcium and at holding potentials indicated ( $n = 3-6$ ). **(D)** Dose-response curves for maximal TRPM4 inward currents at  $-80$  mV as a function of  $[Ca^{2+}]_i$  and at different holding potentials as indicated in the graph. The values at the right of each data set represent the half-maximal  $Ca^{2+}$  concentration for TRPM4 activation. Note that these values are very similar, demonstrating that the  $[Ca^{2+}]_i$ -dependence of TRPM4 remains unaffected by the holding potential ( $n = 3-6$ ). **(E)** Average inward currents carried by TRPM4 at  $-80$  mV with  $[Ca^{2+}]_i$  clamped at 500 nM ( $n = 5$ ). Control cells produce very small TRPM4 inward currents, whereas cells pretreated for 2-3 min. with 10  $\Omega$ M BTP2 produce large TRPM4 currents even at this negative holding potential ( $n = 5$ ). **(F)** Current-voltage relationship under experimental conditions as in (E), obtained from a representative cell 170 s after whole-cell establishment.

**Fig. 4. Effects of BTP2 on TRPM4-like current and IL-2 production in Jurkat cells.** Average whole-cell currents in Jurkat cells at  $-80$  and  $+80$  mV, respectively. Data are  $\pm$  S.E.M. where applicable. **(A)** TRPM4-like currents were activated using intracellular solutions with

[Ca<sup>2+</sup>]<sub>i</sub> buffered to 500 nM and voltage ramps were delivered from a holding potential of either -80 or 0 mV (n = 3 each). Note the strong suppression of currents at holding potential of -80 mV. **(B)** Representative current-voltage (I/V) relationships for TRPM4 at the two holding potentials collected 200 s after break-in. **(C)** Average inward currents carried by TRPM4-like channels at -80 mV with [Ca]<sub>i</sub> buffered at 500 nM in the presence of various concentrations of BTP2 (n = 10 for each set) and 0.1% DMSO as a control (n = 3). **(D)** Dose-response relationship for BTP2-mediated block of TRPM4-like currents at 300 s after whole-cell establishment (K<sub>D</sub> = 8 nM, Hill coefficient = 1). **(E)** Dose-response relationship for BTP2-mediated inhibition of IL-2 production in PHA-stimulated Jurkat cells (10  $\Omega$ M) assayed in either Na<sup>+</sup>- or choline-based extracellular solutions (n = 3 each). Note the ~100-fold shift in efficacy in choline-based solution. Absolute amounts of IL-2 in Na<sup>+</sup>-based ringer were: 12  $\pm$  2 pg/ml (without PHA), 40  $\pm$  85 pg/ml (with 10  $\Omega$ g/ml PHA). Absolute amounts of IL-2 in choline-based ringer were: 14  $\pm$  3 pg/ml (without PHA), 370  $\pm$  66 pg/ml (with 10  $\Omega$ g/ml PHA). Mean  $\pm$  S.E.M. **(F)** Dose-response relationship for econazole-mediated inhibition of IL-2 production in PHA-stimulated Jurkat cells (10  $\Omega$ M) assayed in either Na<sup>+</sup>- or choline-based extracellular solutions (n = 3 each). Note the lack of shift in efficacy.









