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Potent Inhibition of Store Operated Ca^{2+} Influx and Superoxide Production in HL60 Cells and Polymorphonuclear Neutrophils by the Pyrazole Derivative BTP2

Running title: BTP2 inhibits calcium influx in neutrophils

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ABSTRACT

Store operated calcium entry is a key regulator in the activation of leukocytes. 3,5-bistrifluoromethyl pyrazole (BTP) derivatives have recently been identified as inhibitors of T lymphocyte activation. The inhibitory effect of one of these compounds, BTP2, appears to be due to inhibition of store operated calcium influx. Polymorphonuclear neutrophils provide effective protection against bacterial infection but they are also involved in tissue damage during chronic inflammation. As for T lymphocytes, their activation relies on store operated calcium entry. We therefore investigated the effect of BTP2 on calcium homeostasis and functional responses of human neutrophils. BTP2 significantly inhibited the calcium influx after stimulation with thapsigargin or fMLF. This inhibition was seen after 5 min of incubation with 10 µM BTP2 and after 24h with lower concentrations. With 24h incubation, the effect appeared irreversible since the removal of BTP2 3 hours before the experiment did not reduce this inhibition in granulocyte differentiated HL60 cells. In human neutrophils BTP2 reduced superoxide anion production by 82% after 24h of incubation. On the contrary, phagocytosis, intraphagosomal radical production and bacterial killing by neutrophils were not significantly reduced even after 24h treatment with 10 µM BTP2. This work suggests that BTP2 could become an important tool to characterize calcium signaling in neutrophils. Furthermore, BTP2 or related compounds could constitute a new approach to the down regulation of neutrophils in chronic inflammatory disease without compromising antibacterial host defense.

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INTRODUCTION

Intracellular calcium is a key regulator in all cells of the immune system. Engagement of T-cell receptors causes long lasting calcium oscillations, which are essential for cytokine production and proliferation (1). Activation of polymorphonuclear neutrophils (PMN) involves rapid and transient elevations in the intracellular calcium concentration triggering bactericidal functions. Calcium is released from intracellular stores or enters the cell through ion channels in the plasma membrane. The exact nature and the mechanism of activation of these channels are not yet known. However, ion channels are excellent drug targets and the prospect of modulating the immune system by selective ion channel inhibitors is quite attractive.

In a search for safer immunosuppressive drugs, a new family of calcium influx inhibitors in lymphocytes has recently been described: the 3,5-bistrifluoromethyl pyrazole (BTP) derivatives inhibit cytokine gene transcription in Th1 and Th2 lymphocytes by suppressing the calcium dependent translocation of the transcription factor NFAT (2, 3). One of these compounds, BTP2 [N-(4-[3,5-bis(Trifluoromethyl)-1H-pyrazol-1-yl]phenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide] (also called YM-58483) turned out to inhibit store operated calcium entry (SOCE) in T lymphocytes (4). This calcium entry mechanism is based on the depletion of endoplasmic reticulum calcium stores and subsequent activation of plasma membrane ion channels (5). In mast cells and lymphocytes, SOCE generates a significant ionic current called I_{CRAC}. It has been suggested that BTP2 could either directly inhibit I_{CRAC} (4) or activate a non-selective cation channel, which causes membrane depolarization and thereby reduces the driving force for calcium entry (6). The responsible ion channel may belong to the TRP protein family (7). A direct inhibitory effect of BTP2 on two similar TRP channels, TRPC3 and TRPC5, was shown by expressing these proteins in several cell lines (8). A third member of the TRP family, TRPV6, remained unaffected by BTP2 (8, 9).

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Polymorphonuclear neutrophils are the most abundant circulating cells of the human immune system. They are essential for the phagocytosis and killing of pathogenic bacteria. However, they are also involved in tissue damage during chronic inflammation or ischemia-reperfusion injury. Neutrophil action against bacteria relies on the production of reactive oxygen intermediates by the phagocyte NADPH oxidase and on the release of proteases and pore forming bactericidal peptides. The same effector mechanisms cause tissue destruction in the case of inappropriate neutrophil activation. The control of the critical balance between antimicrobial defense and inflammatory tissue damage needs to be understood to identify new drug targets that reduce inflammation and preserve host defense. Since intracellular calcium and in particular SOCE play a central role in neutrophil activation, they are potential drug targets (10). Several mechanisms involving multiple ion channels appear to contribute to SOCE in neutrophils (11).

Electrophysiological and pharmacological data suggest that different immune cells have different calcium entry mechanisms (10). However, they all have store operated calcium influx, which is central for the control of their cellular functions. Therefore, we wanted to determine whether BTP2 acts on neutrophils and whether it affects neutrophil functions. We show here that BTP2 is a potent inhibitor of store operated calcium influx in human neutrophils and granulocyte differentiated HL60 cells and causes significant inhibition of certain cellular functions without impairing bacterial killing.

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MATERIALS AND METHODS

Cell culture

HL60 cells (ATCC, Manassas, Virginia) were cultured at 37°C with 5% CO_2 in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 2 mM L-glutamine and 100 U/ml penicillin-streptomycin. To differentiate HL60 cells into the neutrophil lineage, cells were grown in the presence of 1.3% DMSO for 3 days. Afterwards, the medium was replaced by fresh medium containing 0.65% DMSO and the culture continued for 3 more days.

Neutrophil preparation

Neutrophils (PMN) were purified from fresh, healthy human blood samples recovered on EDTA by the French blood bank (EFS, Vandoeuvre-les-Nancy, France). The protocol had been approved by the appropriate review committee. Whole blood was diluted in Hank's buffer, loaded onto Polymorphprep® (Axis-Shield PoC AS, Oslo, Norway) and centrifuged for 45 min at 450g. The neutrophil layer was collected, washed two times in PBS and resuspended in 2 ml of ES buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes pH 7.2, 9 mM glucose). PMN purity using this method was above 95% as determined by nuclear staining with Türk's solution. Some of our experiments used PMN after 24h of culture. In this case, purified neutrophils were cultured in the presence or absence of BTP2 at 3.5 10^5 cells/ml in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin-streptomycin (except for killing experiments) at 37°C with 5% CO_2. After 24h of incubation, the viability of the cells was controlled by flow cytometry (Beckman Coulter, EPICS® XL-MCL) with the Vybrant® Apoptosis assay kit (Molecular Probes™,

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Invitrogen). Necrotic cells were labeled by propidium iodide while annexin V (Alexa Fluor® 488 conjugate) binding indicated the beginning of apoptosis.

Intracellular calcium

Neutrophils and differentiated HL60 cells (5.10^6 cells/ml) were incubated in 500µl of loading buffer (ES buffer with 4 µM indo-1 AM, 250 µM sulfinpyrazone, 0.02% pluronic acid and 0.1% BSA) for 1 hour at room temperature. Then, cells were washed and resuspended in 500 µl of ES buffer with 250 µM sulfinpyrazone and maintained in this buffer until use. Just before the measurement, cells were washed and resuspended at 2.5 10^6 cells/ml in ES buffer with or without Ca^{2+}. Fluorescence of indo-1 was monitored in black 96 well plates (Costar® 3916, Corning Incorporated, NY, USA) at 37°C with a multilabel plate reader Wallac 1420 (Perkin Elmer, Courtaboeuf, France) at 405 and 485 nm with excitation at 355 nm every 9.5 or 12 s. Calibration was obtained by measuring the signal of cells incubated for 1h with 10µM ionomycin in ES buffer (maximal calcium signal) or with 10 µM ionomycin and 30 mM EGTA in Ca^{2+}-deprived ES buffer (minimal calcium signal). The intracellular calcium concentration was calculated with the following equation (12): $[Ca^{2+}]_i = Kd \times Q \times (R-R_{min})/(R_{max}-R)$, where Kd = 230 nM, $Q = F^{485}{}_{min}/F^{485}{}_{max}$ and $R = F^{405}/F^{485}$. Intracellular Ca^{2+} response was stimulated with 1 µM of formylmethionyl-leucyl-phenylalanine (fMLF) or with 200 nM of thapsigargin (TG). A control experiment was performed with DMSO, the solvent of fMLF and TG, to check that DMSO did not stimulate calcium influx.

Manganese influx

Neutrophils or HL60 cells were loaded with fura-2 as described above for indo-1. Quenching of fura-2 fluorescence in 10^6 cells in 1.7 ml was recorded 10 times per second at 360 nm excitation and 505 nm emission wavelengths at 37°C under constant stirring

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(spectrofluorimeter Varian Eclipse, Les Ulis, France or FL200 Safas, Monaco, Monaco). Where indicated, cells were incubated with 1 or 10 µM BTP2 or solvent (DMSO) for 8 min and 200 nM thapsigargin for 4 min prior to addition of 0.5 mM $MnCl_2$. At the end of each experiment, 5 mM EGTA were added to de-quench extracellular fura (ΔFe). Mn^{2+}-influx (Mi) was analyzed in the linear portion of the fluorescence decay between the point of Mn^{2+} addition (F0) and 40s (HL60) or 120s (PMN) later (F1) according to Demaurex et al. (13). In addition the Mi values were normalized to the fluorescence immediately before Mn^{2+} addition: $Mi = (F0 – F1 – \Delta Fe) / F0$

Secretion of β-glucosaminidase by HL60 cells

The protocol for granule release was adapted to microplate format (14, 15). Briefly, 2.10^5 cells in ES buffer were loaded per well of a 96 wells culture plate (353072 Microtest®, Falcon®) with 5 µg/ml of cytochalasin B and the compound to be tested (BTP2, Calbiochem/VWR, France). After an incubation of 10 min at 37°C, 1 µM of fMLF was added. After 15 min of incubation at 37°C, 100 µl of ice cooled ES buffer was added to stop the reaction. The plate was centrifuged at 400g, 4°C for 5 min. Two samples of 50 µl of supernatant were collected and placed in a black plate with 50 µl of reaction buffer (0.1 M acetate sodium pH4 buffer, 0.02% triton X100, 5 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosamine). After 1 h of incubation at 37°C in the dark, the reaction was stopped with 50 µl of stop solution (0.1 M EGTA, 3 M glycine, pH 10.4). The fluorescence was monitored with the multilabel plate reader Wallac 1420 (excitation 355 nm, emission 460 nm). Results were expressed as a percentage of the maximal fluorescence produced with HL60 cells lysed with 0.02% triton X100. Background release of unstimulated cells was subtracted. DMSO, the solvent of BTP2, did not stimulate β-glucosaminidase release.

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Production of superoxide anions by neutrophils and differentiated HL60 cells.

Reactive oxygen species (ROS) were quantified by measuring luminol-dependent chemiluminescence every 6 s at 37°C with a multilabel plate reader Wallac 1420 using white BSA coated polypropylene 96-well plates (Nunc, Roskilde, Denmark) (15). Human neutrophils or HL60 cells (5.10^4 cells) were resuspended in ES buffer or Ca^{2+}-deprived ES buffer with 10 µg/ml luminol and 4 U/ml horseradish peroxidase in a final volume of 200 µl per well. Cells were stimulated with 1 µM fMLF or 100 nM PMA and superoxide anion production was measured for 15 to 60 min. For quantitative comparisons, total ROS production was assessed by integrating photon counts over the entire measurement period. The solvent control, DMSO, did not stimulate ROS production.

Phagocytosis of bioparticles by human neutrophils.

The phagocytic capacity of PMN was evaluated by monitoring the internalization of fluorescent bioparticles (Molecular Probes, Eugene, Oregon) following supplier's instructions. Fluorescein labeled killed *E.coli* (K-12 strain) or zymosan (*Saccharomyces cerevisiae*) were opsonized by rabbit polyclonal IgG antibodies against *E.coli* or zymosan for 1 hour at 37°C. After 3 washings with ice-cold PBS buffer, PMN were resuspended in RPMI 1640 medium without phenol red (Gibco/Invitrogen, UK) supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine. 3.10^6 neutrophils/ml were coincubated with either 10 *E coli* particles or 5 zymosan particles for 1 PMN in 96-well culture plates at 37°C with 5% CO_2. After an incubation of 30 min for *E coli* and 90 min for zymosan, the phagocytosis was stopped by transfer into ice-cold PBS buffer. The addition of ice-cold trypan blue quenched the fluorescence of extracellular particles to visualize only internalized particles. The percentage

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of phagocytosing neutrophils was quantified by flow cytometry (Coulter EPICS® XL-MCL) after gating with neutrophils incubated without bioparticles.

Measurement of intra-phagosomal NADPH oxidase activity

Heat killed *Saccharomyces cerevisiae* were labeled with an oxidant sensitive probe: dichlorodihydrofluorescein ($DCFH_2$). This probe is poorly fluorescent before oxidation by ROS and becomes significantly brighter when the yeast is engulfed in a phagosome. These labeled yeasts were shown to be a tool to measure the oxidase activity directly in the phagosome (16). For $DCFH_2$ labeling, yeasts were washed two times in a carbonate buffer (100 mM, pH 8.3) and resuspended at a concentration of 10 mg/ml. Succimidyl ester of 2'7'dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, Oregon) was dissolved in anhydrous DMSO (100 µl, 1 mg/ml) and added drop wise to the yeast (1 ml). The solution was allowed to react one hour at room temperature with a continuous stirring under a nitrogen flow in the dark. In order to de-acylate the product and to generate the oxidant sensitive probe, 100 µl of a 1.5 M solution of hydroxylamine pH 8.5 was added. After one hour with a continuous stirring under a nitrogen flow in the dark, yeasts were washed 2 times with degassed PBS, resuspended at a density of 10^8 yeasts/ml in PBS and stored in sealed vials at -20°C for several months. Before use, yeast were opsonized with a polyclonal rabbit anti yeast antibody (1/100, 1 hour, 37°C), washed two times with PBS and resuspended in ES buffer supplemented with 5% heat-inactivated FBS.

For the measurement of the oxidase activity level, opsonized $DCFH_2$-labelled yeasts were added to the neutrophil suspension with a yeast / neutrophil ratio of 5 to 1 for 1 h at 37°C. The phagocytosis was stopped by addition of cold buffer (4°C). The fluorescence of neutrophils that had engulfed $DCFH_2$-yeasts was analyzed by flow cytometry with or without a previous 24 h incubation of neutrophils with 10 µM BTP2. The fluorescence of these neutrophils

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reflected the number of ingested particles and their oxidation state, which increased with phagosomal ROS production. To control the degree of phagocytosis, the same yeast particles were labelled with FITC instead of $DCFH_2$, oponized and phagocytosed under the same conditions as $DCFH_2$-labelled yeasts. No effect of BTP2 on the number of ingested particles was observed by flow cytometry (data not shown). Thus any changes in fluorescence of $DCFH_2$-labelled yeast reflect their oxidation state.

Killing of *P. aeruginosa* by human neutrophils

The capacity of PMN to kill *Pseudomonas aeruginosa* strain PAO1 was evaluated by monitoring bacterial plate counts during and after coincubation (17). Bacteria were cultured overnight in Luria-Bertani (LB) liquid medium, collected by centrifugation, and washed once in modified HEPES buffer (15 mM HEPES, 8 mM glucose, 4 mM KCl, 140 mM NaCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$). Then, bacteria were opsonized for 5 min with pooled normal human serum (NHS). After purification, 5.10^6 PMN were mixed with 5.10^7 colony forming units of opsonized *P. aeruginosa* (multiplicity of infection [MOI] of 10) in 1 ml of culture medium containing 10% NHS. This mixture was shaken for 1h at 37°C. During and after the incubation period, 50 µl samples of the coincubation mixture were collected, serially diluted in LB medium, and plated on Pseudomonas Isolation Agar plates. Bacterial colonies were counted after 24h of incubation at 37°C.

Data analyses and statistics

To evaluate the impact of each treatment, data were subjected to ANOVA analyses followed by the post hoc test of Newmann-Keuls with a threshold lower than 5%. This statistical

treatment allowed multiple comparisons of several means calculated after 3 to 5 repetitions of each experiment. Data are shown as mean ± standard error of mean (SEM).

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RESULTS

We analyzed the effect of BTP2 on two types of granulocytes; neutrophils extracted from human peripheral blood and HL60 cells differentiated into granulocytes. This second cell type was mainly used to confirm results observed with PMN but also to study the effect of a long-term incubation with BTP2.

Impact of BTP2 on calcium influx of neutrophils.

The effect of BTP2 on intracellular Ca^{2+} influx was investigated on three types of responses. Firstly, we analyzed the impact of BTP2 on store operated Ca^{2+} influx induced by thapsigargin (TG) and readdition of calcium. Secondly, we investigated the action of BTP2 on the entry of Mn^{2+} by SOC channels after a TG stimulation. And finally, we studied the impact of BTP2 on the calcium response induced by fMLF. The concentrations of BTP2 and incubation times tested were similar to those that were efficient in T lymphocytes (4).

The Store Operated Ca^{2+} Entry (SOCE) in human neutrophils was induced with thapsigargin (TG) in a Ca^{2+}-deprived buffer followed by the addition of $CaCl_2$ (**Fig. 1A**). Indeed, TG induces a depletion of intracellular calcium stores and an activation of store operated channels. Therefore, when calcium is added in the buffer, there is an entry of extracellular calcium into the cells. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined from the fluorescence ratio, F405/F485, of the indo-1 probe. Fluorescence data were calibrated and the intracellular free calcium concentration was calculated. The basal calcium level was subtracted to analyze the increase due to thapsigargin and calcium readdition treatment ($\Delta\ [Ca^{2+}]_i$, **Fig. 1B**). These

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figures clearly demonstrate that BTP2, when used at 10µM, reduced the Ca^{2+} entry into neutrophils by 81%.

To confirm these results we investigated the impact of BTP2 on the manganese entry. Mn^{2+} also enters through store-operated channels (13), and can be detected by fura-2 fluorescence quenching at the calcium independent excitation wavelength of 360 nm. As described before (13), the beginning of the quenching response was linear allowing a quantitative assessment of the rate of manganese entry. In neutrophils, we demonstrated that 10 µM BTP2 reduced by 74% the thapsigargin induced manganese entry and 1 µM of BTP2 reduced this entry by 38% (**Fig. 1C** and **1D**).

Chemoattractants such as the bacterial peptide fMLF induce a transient rise in $[Ca^{2+}]_i$. This calcium peak can be divided in three steps: 1- an important and abrupt increase that corresponds to the depletion of calcium stores, 2- a slow decrease followed by 3- a stabilization of the signal at a concentration higher than the basal level (18). This plateau is due to the opening of the store operated channels that maintain the elevated intracellular calcium concentration. We wanted to determine whether the complex calcium response to chemoattractant stimulation, under physiological calcium concentrations, was affected by BTP2. To this end, we stimulated the calcium response of neutrophils with 1 µM fMLF (**Fig. 2A**). Interestingly, when BTP2 was added to the medium, the plateau of the calcium response (evaluated 3 min after the peak) was situated at a lower concentration while the height of the peak was not significantly affected (**Fig. 2B** and **2C**). Indeed, if neutrophils were incubated for 4 minutes with 10µM BTP2, we noticed that the intracellular calcium concentration three minutes after the peak was reduced by 60 % in neutrophils (**Fig. 2C**). However, these values are different from the control condition where cells were placed in calcium deprived medium

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that reduced calcium influx by 97% (**Fig. 2C**), suggesting that calcium entry was not completely inhibited.

BTP2 had similar effects in differentiated HL60 cells. Indeed, 1µM BTP2 was able to reduce by 79% the calcium entry induced by TG and 10 µM BTP2 reduced this entry by 94% (**Fig. 3A**). Furthermore, we found that 1 µM of BTP2 inhibited manganese entry by 61% and 10 µM BTP2 by 83% (**Fig. 3B**). These results confirmed that BTP2 is able to inhibit store-operated divalent cation entry. In addition, the peak of the fMLF-stimulated calcium response was not significantly affected by 10 µM BTP2 (**Fig. 3C**). However, at this concentration, the compound reduced significantly the plateau three minutes after the peak (**Fig. 3D**). The calcium responses of neutrophils and HL60 cells have a similar sensibility to BTP2 (**Fig. 2C** and **3D**). However, these two cell types have a different sensibility to calcium free conditions (**Fig. 2B** and **3C**). Indeed, the peak of the fMLF response is reduced by 41% in neutrophils and by 96% in HL60 cells.

Long term effect and reversibility of the action of BTP2 on calcium influx of differentiated HL60 cells.

In previous studies (4, 8), it has been shown that BTP2 could have an impact on calcium entry at a much lower concentration if the incubation time was extended to 24h. We used HL60 cells differentiated into granulocytes to study the long term impact of BTP2. 24h incubation with 0.2 µM BTP2 inhibited the calcium influx by 97% after thapsigargin and $CaCl_2$ addition (**Fig. 4B**) and by 68% after fMLF addition (**Fig. 4D**).

During previous studies, a constant BTP2 concentration had been maintained throughout the entire experiment, including washing steps and periods of dye loading (4). We wondered if the action of this compound was reversible. To address this issue, we cultured HL60 cells

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with 0.2 µM BTP2 only for 21h, afterwards, BTP2 was removed by centrifugation and cells were placed in dye loading buffer without BTP2 for 3h. Interestingly, this treatment produced an inhibition of the calcium influx very similar to that observed when cells were incubated for 24h with 0.2 µM BTP2 (**Fig. 4B, 4C** and **4D**), thereby indicating that the action of BTP2 was irreversible for at least 3h.

Impact of BTP2 on bactericidal functions of human PMN.

Having shown that BTP2 is able to reduce substantially the capacitive calcium entry in neutrophils, we investigated if BTP2 could have an impact on microbicidal functions of neutrophils.

At first we looked at the impact of 10 µM BTP2 after a short incubation of 5 min. In these conditions, removal of extracellular calcium did not reduce ROS production after stimulation with fMLF. Consequently, a short incubation of 5 min with 10 µM BTP2 was insufficient to significantly inhibit the superoxide anion secretion by NADPH oxidase after stimulation with fMLF (**Fig. 5A** and **5B**). Then we investigated the impact of BTP2 on *E.coli*-FITC phagocytosis (**Fig. 5C**) and capacity of neutrophils to kill *P. aeruginosa* bacteria (data not shown). The percentage of phagocytosis was evaluated by flow cytometry. The effect of BTP2 on bacterial killing was determined by calculating the percentage of bacteria that survived when they were co-incubated with PMNs in the presence or absence of BTP2. Addition of BTP2 did not have a significant effect on bacteria phagocytosis and killing.

These results raised the question whether longer incubation with BTP2 would affect neutrophils functions. We tested the effect of BTP2 on neutrophil calcium signaling after 24h of incubation (**Fig. 4A**). Our results show that in these conditions, BTP2 was able to inhibit the capacitive calcium entry induced by TG stimulation and calcium readdition. Moreover, after 24h, the inhibition induced by 1 µM of BTP2 is as strong as the one induced by 10 µM

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of BTP2 (**Fig. 4A**). Increasing the incubation time with BTP2 could also act on the ROS production by human neutrophils. Whyte et al. have shown that the functional ability of neutrophils after 24h in culture was strongly reduced among the apoptotic cells whereas non-apoptotic cells were capable of ROS production and phagocytosis (19). We used propidium iodide staining to detect signs of necrosis and annexin V staining to reveal early signs of apoptosis on neutrophils after 24h in tissue culture medium. We found less than 0.5% of cells labeled by propidium iodide while annexin V binding was detected on 58.5 ± 0.7% and 61.8 ± 0.7% of control neutrophils and BTP2-treated neutrophils, respectively. Thus, any functional differences in these cells were not due to differential apoptosis and most likely reflect the effect of BTP2 on the remaining 40% non-apoptotic cells.

Consequently, we also tested the impact of BTP2 on fMLF-stimulated NADPH oxidase activity after 24h of incubation. Under these conditions, ROS production was inhibited by BTP2 to the same level as in the absence of extracellular calcium, corresponding to a decrease of 74% in the total amount of ROS secretion (**Fig. 6A** and **6B**). The total ROS production in cultured neutrophils was lower than in freshly isolated neutrophils (**Fig. 5B**), presumably due to the loss of function in apoptotic cells. To know if the effect of BTP2 was specific for the calcium dependence of oxidase activation, we tested the impact of BTP2 on ROS production stimulated by PMA on neutrophils incubated for 24h in presence of 10 µM of BTP2. Our results demonstrate that BTP2 had no significant impact on PMA-stimulated ROS production (**Fig. 6C** and **6D**).

Would BTP2 also compromise the phagocytosis of antibody opsonized *E. coli* or zymosan particles? No significant inhibition of phagocytosis was observed after 24h of incubation with 10 µM BTP2 (**Fig. 7A** and **7B**). Similarly, when PMN were cultured for 24h in the presence of 10 µM BTP2, bacterial killing was not significantly reduced (**Fig. 7D**). This is in apparent contrast to the observation that longer incubation with BTP2 inhibited chemoattractant-

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induced ROS production in the absence of phagocytosis (**Fig. 6B**), which may be similar to the overreaction of PMN in chronic inflammation. ROS-production is known to be essential to efficient bacterial killing by PMN. However, our previous experiments on ROS production did not determine the phagosomal ROS production. But it's the phagosomal production which is important for the anti-bactericidal functions of neutrophils. Dewitt et al. (16) recently demonstrated a method for microscopic detection of intraphagosomal ROS production. The technique is based on yeast particles labeled with an oxidant sensitive probe: dichlorodihydrofluorescein ($DCFH_2$). Using this technique with flow cytometry, we found no substantial difference of intraphagosomal ROS production due to 24h BTP2 treatment (**Fig. 7C**). Thus, 24h treatment with BTP2 had a substantial impact on store-operated calcium influx and global ROS production while phagocytosis, intraphagosomal ROS production and bacterial killing were not impaired.

Impact of BTP2 on bactericidal functions of differentiated HL60 cells.

BTP2 had similar effects on calcium signaling in human neutrophils and HL60 cells. While neutrophils undergo apoptosis during prolonged incubation periods, cell lines like HL60 can be incubated for days, an advantage in the investigation of potential drugs. Therefore, we investigated the impact of BTP2 on calcium dependent functions of these cells, namely ROS production and exocytosis of the primary granule marker β-glucosaminidase. Our data show that 10 μM of BTP2 inhibited fMLF-induced activity of the NADPH oxidase by 87%, similar to the lack of extracellular calcium, which reduced this production by 75% (**Fig. 8A**). The kinetics of the two reduced responses were identical (data not shown). Moreover, the effect of BTP2 was specific for the calcium dependence of oxidase activation since it had no impact on ROS production induced by the addition of PMA (**Fig. 8B**). Concerning β-glucosaminidase exocytosis, after stimulation by 1 μM fMLF, 10 μM of BTP2 inhibited exocytosis by 32%

while the lack of extracellular calcium reduced this function by 94% (**Fig. 8C**). These experiments indicated that BTP2 is able to reduce two major functions of differentiated HL60 cells.

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DISCUSSION

Store operated calcium entry (SOCE) plays a crucial role in lymphocytes, neutrophils and other hematopoietic cells. Store operated calcium channels are potential yet unexploited anti-inflammatory drug targets (10). Exploration of the calcium entry pathway would be substantially facilitated by specific inhibitors. We report here that the 3,5-bis(trifluoromethyl)-pyrazol derivative BTP2 completely inhibited thapsigargin-induced store operated calcium and manganese influx in human neutrophils and HL60 cells as shown before for T lymphocytes (**Fig. 1** and **3**).

With short incubation times, micromolar levels of BTP2 were required. In HL60 cells, complete inhibition could also be achieved with 50 times less BTP2, when the drug was given for 24 h instead of 5 min. Moreover washing away the drug after 21 h of treatment did not abolish the effect. Similarly, in human neutrophils, 24 h incubation markedly increased the inhibitor efficiency of 1μM BTP2 (**Fig. 4A**).

Currently it is not clear why the long-term inhibition is much more potent. Previous studies on lymphocytes and other cells also reported a slow action of BTP2, although the time course and the required concentration differed between experimental systems (4, 8). BTP2 seems to act from the outside of the cell most likely on the channel itself and does not require potentially slow entry through the membrane (4, 8). In T-lymphocytes, the CRAC channels themselves seem to be the target of BTP2 (4). Their identity is not known, but members of the TRP protein family are likely to be involved in store operated calcium entry. He et al. suggested that BTP2 acts on TRPC channels, in particularly on TRPC3 (8). Very recently, Takezawa et al. reported that BTP2 activates the non-selective cation channel TRPM4, which may counteract SOCE (6). However, this model required low nanomolar concentrations of

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BTP2 and short incubation periods (6), which had no effect in PMN or HL60 cells. Our data are consistent with the hypothesis that BTP2 acts directly on SOCE, potentially via TRP channels. However, other targets for BTP2 may be relevant for the observed effects.

A further clue to the mechanism of action of BTP2 came from the question of whether its action is readily reversible. We show here, and for the first time, that the long-term inhibition is irreversible for at least 3 h (**Fig. 4**). This may suggest a covalent interaction between BTP2 and its target. Alternatively, the compound may induce degradation of the channel itself or of a partner necessary to its function. If BTP2 were to be used as a pharmaceutical drug, the apparent irreversibility of its effect may be a major advantage to maintain the effect between two administrations. However, it would also slow down recovery of the immune response after treatment. In one preclinical study of BTP in primates, the animals showed less than 50% recovery in 4 days (20). It should be pointed out that this study used plasma concentration of 2 to 5 µM for 14 days to achieve strong and persistent suppression of IL-2 production. Moreover, serum components in the animal as well as in cell culture experiments may bind BTP2 and thereby alter the concentration of free BTP2.

It is generally accepted that store operated calcium influx represents the main pathway of calcium entry stimulated by G-protein coupled receptors of chemoattractants like fMLF. However, recent data suggest that fMLF stimulates more than one calcium entry pathway and some calcium entry may be store independent (21, 22). In neutrophils, BTP2 completely inhibited store operated calcium influx induced by thapsigargin whereas fMLF-stimulated calcium entry was not completely inhibited (**Fig. 2**). This raises the possibility that BTP2 inhibits only the store operated channels. Their inhibition by BTP2 could be partially

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compensated by store independent pathways allowing some calcium entry after fMLF-stimulation.

The role of calcium signaling in the immune response has been often emphasized. It has been demonstrated that degranulation and superoxide production by neutrophils and HL60 cells are strongly supported by changes of intracellular calcium concentration (14, 23, 24). The effects of BTP2 described above let us think that this molecule could be useful to determine the implication of this signaling pathway in some microbicidal functions of neutrophils.

We first showed that BTP2 had no impact on ROS production by human neutrophils when it was used at 10µM during 5 min (**Fig. 5**). The removal of extracellular calcium had only minor effects on the fMLF-induced ROS production in agreement with previous publications (25, 26). However, intracellular calcium buffering with chelators strongly reduced ROS production (25) suggesting that calcium release rather than calcium influx is needed (27). Short incubation in calcium free buffer affected the peak calcium response after stimulation with fMLF in HL60 cells much more than in neutrophils (**Fig. 3C** versus **2B**), suggesting that the intracellular calcium stores are more readily depleted in HL60 cells. This may explain why HL60 needed extracellular calcium for ROS production and were sensitive to short treatment with BTP2 while freshly isolated neutrophils did not (**Fig 8A** versus **5B**). In neutrophils, 24h treatment with BTP2 presumably prevents the refilling of intracellular stores for which a calcium entry from extracellular medium is necessary. Consequently, the ROS production was blocked in 24h cultured neutrophils. In support to this hypothesis we observed, in calcium free buffer, a decrease in TG-induced calcium release in neutrophils cultured in the presence of BTP2 for 24h. This effect was not observed on freshly purified neutrophils (data not shown). Moreover, it appears that BTP2 does not act directly on the NADPH oxidase

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itself or its assembly since PMA-induced ROS production was not significantly affected by 24h treatment with BTP2 (**Fig. 6D**). This is a first example which shows that BTP2 can act like an immediate inhibitor of immune function in contrast to slower effects on gene expression in lymphocytes shown previously (1, 2, 4).

Similarly, Han *et al.* also noted that the ROS production could be affected by an inhibitor of TNF-induced calcium signaling called "neucalcin" (28). The mechanism of TNF-induced calcium increase is not well understood, therefore, it is unclear whether neucalcin could act on the same target as BTP2. Alternatively, different stimuli could be linked to ROS production by multiple calcium entry pathways (22). If neucalcin and BTP2 turned out to be specific inhibitors for different pathways they would be very useful to analyze each pathway separately.

IgG-dependent and complement-dependent phagocytosis in PMN are thought to have different calcium requirements (29). Our data indicate that phagocytosis of IgG-opsonized *E. coli* or zymosan particles is insensitive to BTP2 even at high concentration and after prolonged incubation (**Fig. 7A** and **7B**). Furthermore, BTP2 did not affect intraphagosomal ROS production (**Fig. 7C**). In contrast, fMLF induces mainly extracellular ROS production (30), which seemed to require BTP2 sensitive calcium entry under conditions where intracellular calcium release was insufficient. Apparently, intraphagosomal ROS production does not depend on the same calcium entry mechanism as extracellular ROS production. Finally, BTP2 had no significant effect on bacterial killing (**Fig. 7D**), which involves phagocytosis, ROS production and granule release into the phagosome. These events are calcium dependent, but the source of calcium is not certain. Stendahl et al. (31) have shown accumulation of intracellular calcium stores around the phagosome. Lundqvist-Gustafsson et

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al. (32) have suggested that calcium release from the phagosome could also contribute to the increase in periphagosomal calcium. Our data suggest that these pathways are insensitive to BTP2 and that SOCE was unnecessary for these bactericidal functions under our experimental conditions. A much larger screen will be required to determine whether phagocytosis via other receptors and destruction of other particles, in particular pathogens, are also insensitive to BTP2.

In conclusion, neutrophils play a key role in the host defense against bacterial infections. However, excessive neutrophil activation contributes to tissue damage in acute and chronic inflammatory diseases such as inflammatory bowel disease or cystic fibrosis. The destructive role of neutrophils involves extracellular ROS production. The ideal modulator of neutrophil activation would inhibit these extracellular actions and preserve the phagocytic capacity and bactericidal properties of neutrophils. Since phagocytosis appears to be less dependent on calcium influx than degranulation and ROS production, inhibitors of calcium influx are excellent drug candidates (10). In this study, we showed that BTP2 inhibits store-operated calcium influx in human neutrophils and HL60 cells and several calcium-depend neutrophil functions. However, this inhibition is incomplete, presumably due to parallel calcium entry mechanisms. This new molecule may help to distinguish these pathways and identify their respective role. Moreover, BTP2 did not reduce the phagocytosis and killing functions thereby suggesting that it could potentially reduce damaging neutrophil reactions without impairing their bactericidal activity.

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Figures legends

Fig. 1.

Effect of BTP2 on calcium and manganese influx of neutrophils stimulated by thapsigargin (TG).

Calcium and manganese entry were monitored with indo-1 (A, B) and fura-2 (C, D) respectively. The intracellular calcium concentration, $[Ca^{2+}]_i$, was calculated with the ratio of fluorescence at 405 and 485 nm: F_{405}/F_{485} (A, B). Loaded cells were placed in a Ca^{2+}-free buffer and calcium store depletion was induced by adding 200 nM of TG. About four minutes later, 5 mM of $CaCl_2$ were added. After this external calcium addition, the intracellular calcium reached a plateau before slowly returning to basal levels. The difference of intracellular calcium concentration, $\Delta [Ca^{2+}]_i$, was defined as the average value at the beginning of the plateau minus the basal level prior to TG addition. Cells were incubated with various concentrations of BTP2 before adding TG. To follow manganese influx (C, D), neutrophils were placed in a Ca^{2+}-free buffer. Four min after the beginning of the recording, 200 nM of TG were added to induce the opening of store operated channels. Four minutes later, 2 mM $MnCl_2$ were added. Fura-2 fluorescence appears as a line whose slope is proportional to the rate of Mn^{2+} influx. Panels A and C are representative of 3 independent experiments. Panels B and D indicate the mean variations ± SEM of the intracellular calcium concentration or manganese entry. * indicates statistically significant differences. # indicates differences that are not statistically significant.

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Fig. 2.

Effect of BTP2 on calcium influx of neutrophils stimulated by fMLF.

Neutrophils were placed in a buffer with or without 2 mM of Ca^{2+} and stimulated with 1µM of fMLF. Cells were incubated with various concentrations of BTP2 for 4 minutes before the addition of fMLF. Changes of indo-1 fluorescence were monitored to follow the intracellular calcium concentration. Results presented in panel A are representative of 3 independent experiments. Variations of the intracellular Ca^{2+} concentrations were calculated at the maximum of the peak (B) and 3 minutes later (plateau-C). The Ca^{2+} concentration corresponding to the basal level was subtracted to determine Δ $[Ca^{2+}]_i$. Panels B and C show the mean ± SEM of three experiments. * indicates statistically significant differences. # indicates differences that are not statistically significant.

Fig. 3.

Effect of BTP2 on calcium and manganese influx of differentiated HL60 cells stimulated by TG (A, B) or by fMLF (C, D).

The store operated calcium influx was monitored with indo-1 loaded HL60 cells placed in a Ca^{2+}-free buffer. Cells were stimulated with 200 nM of TG and the readdition of 5 mM $CaCl_2$. Panel A shows the Δ $[Ca^{2+}]_i$ defined as the average value obtained at the beginning of the plateau minus the basal level prior to TG addition. Results presented in panel B show the mean slope which correspond to manganese influx following the addition of 2 mM of Mn^{2+} on fura-2 loaded HL60 cells stimulated with 200 nM of TG in a Ca^{2+}-low buffer (100 µM $CaCl_2$). Panels C and D correspond to mean values of Δ $[Ca^{2+}]_i$ obtained by stimulating indo-1 loaded HL60 cells with 1 µM of fMLF in a buffer with or without 2 mM of $CaCl_2$. Variations of the intracellular Ca^{2+} concentrations were calculated at the maximum of the peak (C) and 3 minutes later (plateau-D). Panels A-D show the mean ± SEM of at least three experiments.

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* indicates statistically significant differences. # indicates differences that are not statistically significant

Fig. 4.

Long term effect of BTP2 on calcium signaling in neutrophils and differentiated HL60 cells.

Neutrophils (A) or HL60 cells (B, C, D) were incubated either without BTP2, with 0.2, 1 or 10 µM of BTP2 for 24h or for 21h and washed 3h before monitoring the fluorescence variations. Cells were stimulated by TG in calcium free buffer and calcium addition 5 min later (A, B), or by fMLF in a buffer with or without calcium (C, D). Kinetics of the calcium responses had the same profiles as in figures 1A and 2A. Δ $[Ca^{2+}]_i$ was obtained after subtraction of the basal level. Values were read at the plateau after calcium addition (A, B), or at the peak (C) and at the plateau (D) induced by fMLF. Data represent the mean ± SEM of three independent experiments. * indicates statistically significant differences. # indicates differences that are not statistically significant.

Fig. 5.

Short term effect of BTP2 on ROS production and *E.coli* phagocytosis by neutrophils.

The dynamics of ROS production were studied by following the variations of luminescence of the luminol that reacts with ROS. Neutrophil responses were induced by 1 µM fMLF, with or without 10 µM of BTP2 added 5 min before the fMLF addition. Kinetics of the responses shown in panel A are representative of four independent experiments. Panel B represents mean values ± SEM of the total production during 15 min after stimulation. Panel C presents the mean percentage ± SEM of neutrophils which ingested *E. coli* bioparticles labeled with fluorescein and opsonized with polyclonal antibodies against *E. coli*. The phagocytosis was

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stopped after 30 min of coincubation of cells with bioparticles at 37°C. The fluorescence of extracellular particles was quenched with trypan blue and the percentage of PMN having internalized bioparticles was evaluated by flow cytometry. * indicates statistically significant differences. # indicates differences that are not statistically significant.

Fig. 6.

Long term effect of BTP2 on ROS production of human neutrophils.

The dynamics of ROS production were studied by following the variations of luminescence of the luminol. BTP2 was added 24h before the addition of fMLF (A-B) or PMA (C-D) where indicated. Neutrophil responses were induced by 1 µM fMLF (A-B), in a buffer with or without 2 mM $CaCl_2$. Panels C and D show results obtained by stimulating with 100 nM PMA. The kinetics of the responses shown in panels A and C are representative of three and four independent experiments, respectively. Panels B and D represent mean values ± SEM of the total ROS production during 15 min (B) or 60 min (D) after stimulation.

Fig. 7.

Long term effect of BTP2 on phagocytosis (A, B), intra-phagosomal ROS production (C) and bacterial killing activity (D) of human neutrophils.

Human neutrophils were preincubated with or without 10 µM BTP2 for 24 hours before being coincubated at 37°C with *E.coli* or zymosan bioparticles labeled with fluorescein and opsonized with polyclonal antibodies against *E. coli* (A) or zymosan (B). Phagocytosis was stopped after 30 min (*E. coli*) or 90 min (zymosan) of coincubation and trypan blue was added to quench the fluorescence of unphagocytozed particles. The number of PMN having internalized bioparticles was evaluated by flow cytometry. Results are expressed as percentage of phagocytosing neutrophils (A, B). Intra-phagosomal NADPH oxidase activity

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was evaluated with ROS sensitive $DCFH_2$-labeled yeast (C). The mean fluorescence of PMNs that had engulfed bioparticles , after an incubation of 1 hour was measured by flow cytometry and compared to the mean fluorescence of bioparticles before internalization. Killing activity of human neutrophils was tested on *Pseudomonas aeruginosa* strain PAO-1 after 24 h treatment with 10 µM BTP2 (D). This activity was evaluated by following the decrease of colony numbers of *P. aeruginosa* 24 hours after incubation of PMNs with bacteria for 0 to 45 min. Panels show mean values ± SEM of three (B-D) or four (A) independent experiments. # indicates differences that are not statistically significant.

Fig. 8.

Effect of BTP2 on the NADPH oxidase activity (A, B) and β-glucosaminidase secretion (C) of differentiated HL60 cells.

ROS production was followed by monitoring the variations of luminol luminescence. Cells were incubated with different concentrations of BTP2 for 5 min and then stimulated by the addition of 1µM fMLF (A) or 100 nM PMA (B). Panels A and B show the total ROS production during 15 (A) or 30 min (B) after stimulation. Panel C presents the percentage of β-glucosaminidase secretion induced by the addition of 1µM of fMLF to the medium after 5 min of incubation with various concentrations of BTP2. Values were expressed as a percentage of the maximal response induced by triton X100 addition. Results represent the mean ± SEM of three independent experiments. * indicates statistically significant differences. # indicates differences that are not statistically significant.