Ca\(^{2+}\) Signaling in Identified T-lymphocytes from Human Intestinal Mucosa

RELATION TO HYPOREACTIVITY, PROLIFERATION, AND INFLAMMATORY BOWEL DISEASE*

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Ca\(^{2+}\) entry across the plasma membrane is necessary for the activation and proliferation of T-lymphocytes. Human intestinal lamina propria lymphocytes physiologically exhibit minimal proliferation in response to antigen receptor stimulation when compared with peripheral blood T-lymphocytes. This hyporeactivity is partially abolished in inflammatory bowel disease. We hypothesized that differences in Ca\(^{2+}\) signaling could be related to the disease. To test this possibility, we measured Ca\(^{2+}\) signals in identified lymphocytes from human blood and human intestinal mucosa. Ca\(^{2+}\) signals in lamina propria T-lymphocytes from non-inflamed tissue were drastically reduced when compared with Ca\(^{2+}\) signals of blood T-lymphocytes from the same persons. However, Ca\(^{2+}\) signals in T-lymphocytes from inflamed intestinal mucosa were much higher than the ones from non-inflamed mucosa and almost reached levels of Ca\(^{2+}\) signals in peripheral blood T-cells. Furthermore, Ca\(^{2+}\) influx was closely linked to cell proliferation in both peripheral blood T-lymphocytes and lamina propria lymphocytes. We conclude that differences in Ca\(^{2+}\) signaling can explain the differences of T-lymphocyte reactivity in blood versus lamina propria and, importantly, also between T-lymphocytes from inflamed and non-inflamed intestinal mucosa. Ca\(^{2+}\) channels in the plasma membrane of T-lymphocytes might thus prove an excellent target to screen for immunosuppressiva to potentially treat the symptoms of inflammatory bowel disease.

Ca\(^{2+}\) influx across the plasma membrane following stimulation of Jurkat T-cells or peripheral blood T-lymphocytes is a necessary signal for T-cell activation (1). Following cross-linking of the TCR, a number of signaling cascades are activated, one of which results in Ins-1,4,5-P\(_3\) production. Ins-1,4,5-P\(_3\) initiates Ca\(^{2+}\) signaling in T-cells by two coupled processes: Ca\(^{2+}\) release from the endoplasmic reticulum through the Ins-1,4,5-P\(_3\) receptor channel and subsequent activation of plasma membrane Ca\(^{2+}\) channels. Activation of the Ca\(^{2+}\) channels is dependent on the Ca\(^{2+}\) filling state of the endoplasmic reticulum.

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‡ The abbreviations used are: TCR, T-cell receptor; Ins-1,4,5-P\(_3\), inositol 1,4,5-trisphosphate; CRAC, Ca\(^{2+}\) release-activated Ca\(^{2+}\); IBD, inflammatory bowel disease; LP, lamina propria; PB, peripheral blood; FACS, fluorescence-activated cell sorter; HBSS, Hank’s-buffered salt solution; LPL, LP lymphocytes; PBL, PB lymphocytes; TG, thapsigargin; OKT-3, an anti-CD3 antibody; [Ca\(^{2+}\)]\(_{int}\), internal Ca\(^{2+}\) concentration; [Ca\(^{2+}\)]\(_{ext}\), external Ca\(^{2+}\) concentration; PMA, phorbol 12-myristate 13-acetate.
the proliferative response of the cells. Our results explain the differences of reactivity found in intestinal T-lymphocytes from inflamed and non-inflamed tissues.

EXPERIMENTAL PROCEDURES

Isolation of Lamina Proxima Lymphocytes (LPLs) and Peripheral Blood Lymphocytes (PBLs)—LPLs were obtained from 6–8 mucosa biopsies taken from colonoscopic examinations. Biopsies were washed three times in HBSS (PAA Laboratories GmbH, catalog number H15-009). The mucosa was cut into little pieces and shaken for 3 h at 37 °C in collagenase medium of the following composition: 500 ml of RPMI 1640 medium (Invitrogen, catalog number 21875-034); 10% fetal bovine serum (Invitrogen), catalog number 10270-106; 2.5 ml of gentamycin (Biochrom, catalog number A2710); 1 ml of amphotericin B (500x, Roche Applied Science, catalog number 170262); 5 ml of trypsin inhibitor (10 mg/ml, Sigma, trypsin inhibitor from Glyxine max (soybean), type I-S, product number T6522); 5 ml of DNase I (10 mg/ml, Roche Applied Science, catalog number 1284932); 50 units/ml penicillin; and 50 μg/ml streptomycin (Invitrogen, catalog number 15140-122). Following digestion, the suspension was carefully sucked through a syringe to disrupt the remaining tissue. Cells were separated from larger mucosa remnants by filtration through a 70 μm cell sieve. The suspension was then centrifuged for 10 min at 360 × g at room temperature. The pellet was resuspended in 30% Percoll (Amersham Biosciences, catalog number 17-0891-01, diluted with 0.9% NaCl) and layered over 70% Percoll (Ficoll-Paque Plus, Amersham Biosciences, catalog number 17144002) in 50 ml of Leucosep tubes (Greiner, catalog number 227290). The peripheral blood mononuclear cell layer was washed in HBSS. Remaining red blood cells were removed by the addition of 3 ml of lysis buffer (155 mN HN4Cl, 10 mN KHCO3, 0.1 mM EDTA, pH 7.3) for 3 min. After lysis, peripheral blood mononuclear cells were washed with HBSS (200 × g for 10 min at room temperature). Viability of the cells was checked by staining with trypan blue. Cells were further purified by adhering to plastic for 24 h in RPMI 1640 complete medium at 37 °C (1.5 × 10^5 cells/ml). Non-adherent cells, mostly PBLs, were collected and used for proliferation assays.

**Ca^{2+} Imaging**—Cells were loaded at 22–23 °C for 20 min with 1 μM Fura-2/AM (Molecular Probes) in medium with 10 mM HEPES added, washed with fresh medium, stored at room temperature for 10 min, and immediately used. Cells were allowed to adhere to poly-L-ornithine-coated (0.1 mg/ml, Sigma) glass coverslip chambers on the stage of an Olympus IX 70 microscope equipped with a ×40 Uplan/Apo (numerical aperture 1.0) objective. Cells were alternately illuminated at 340 and 380 nm with the Polychrome IV monochromator (TILL Photonics). The fluorescence emissions at λ > 440 nm were captured with a CCD camera (TILL Imago), digitized, and analyzed using TILL Vision software (TILL Photonics). Ratio images were recorded at intervals of 5 s. [Ca^{2+}] was estimated from the relation [Ca^{2+}] = R/([Rmax] - R) where the values of [R], Rmax, and Rmin were determined from an in situ calibration of Fura-2/AM in Jurkat T-cells as described previously (18).

**Identification of T-lymphocytes**—Identification of subtypes of lymphocytes, cells were stained with anti-CD3-FITC, anti-CD4-PE, anti-CD8-APC. Identification of T-lymphocytes following TG stimulation. A, infrared picture of the cells. B, color-coded picture of the intracellular calcium concentration of the same cells at one time point. Warmer (red) colors indicate high [Ca^{2+}], while colder colors (blue) indicate low [Ca^{2+}]. C, kinetic analysis and quantification of [Ca^{2+}], for two cells during the entire experiment. D–F, identification of T-lymphocytes after the Ca^{2+}-imaging experiment by staining the same cells on the stage of the microscope with fluorescent-labeled antibodies. Antibodies were perfused into the chamber and washed out after 5 min of incubation time.
CD4-Cy5, and anti-CD8-fluorescein isothiocyanate antibodies (diluted 1:12 in 0.9% NaCl containing 3% fetal bovine serum, Dako) on the stage of the microscope following the Ca^{2+}-imaging experiment. After 5–10 min, the antibody solution was washed away with Ringer’s solution. Standard HQ filter sets (AHF Analysetechnik) were used for identification of T-cells, which were excited with the Polychrome IV monochromator at 480 nm (fluorescein isothiocyanate), 540 nm (PE), or 620 nm (Cy5).

**Proliferation Assays**—Proliferation experiments using the EZ4U assay were carried out in 96-well cell culture plates (BD Biosciences, catalog number 353072, flat bottom), and data points were measured as triplicates. 50,000 PBLs were cultured in a total volume of 200 μl in each well. Cells were stimulated using 5 μl phorbol 12-myristate 13-acetate (PMA, Sigma, catalog number P1885) and 0.5 μM ionomycin (Sigma, catalog number I0634). Plates were incubated for 72 h at 37°C, 5% CO₂, and 95% humidity. After incubation time, the number of living cells was determined by the reduction of the tetrazolium salt EZ4U to formazan derivatives (Biozol, catalog number BI-5000). 20 μl of EZ4U reagent was added to each well, and plates were incubated for another 4 h. Optical density was measured in a ELx800UV universal microplate reader (BIO-TEK Instruments) at wavelength settings of 465–630 nm.

For proliferation experiments using [3H]thymidine incorporation, 5 × 10⁴ purified cells were added in 0.2 ml of RPMI 1640 supplemented with 10% fetal bovine serum, 2% L-glutamine, and antibiotics. After 96-h incubation at 37°C, 1 μCi of [3H]thymidine was incorporated over a 6-h period before harvesting the cells (Inotec, Wohlen, Switzerland). [3H]Thymidine incorporation was measured in a liquid scintillation spectrometer (Beckmann, Munich, Germany). All of the assays were performed in triplicates, and the results differed by <15%. To analyze the impact of [Ca^{2+}]i, on cell proliferation, the Ca^{2+} concentration in the medium was modified by adding extra Ca^{2+} or EGTA.

**Data Analysis**—Data were analyzed using TILL Vision, Igor Pro (Wavefronts), and Microsoft Excel. Averages are presented as the mean ± S.E. For statistical analysis, an unpaired two-sided Student’s t test was used. Data were considered significantly different if p was less than 0.01.

**RESULTS**

To allow Ca^{2+} measurements in identified T-lymphocytes from intestinal mucosa and peripheral blood without potential pre-activation of the cells, separation steps of the cells with antibodies through magnetic beads or FACS were avoided. A protocol was developed that allowed parallel Ca^{2+} imaging and identification of the same cells with up to three different antibodies after the experiment. Fig. 1 shows a typical example of such a LPL experiment with an infrared picture (A), [Ca^{2+}]i, measurements of cells at one time point (B), and resulting [Ca^{2+}]i, traces for two of the cells over time (C). To enable identification of T-cell subtypes, cells were stained on the stage in the plasma membrane, also referred to as CRAC channels in the medium was modified by adding extra Ca^{2+} or EGTA.

To activate store-operated Ca^{2+} channels, cells were stimulated with 1 μM TG in Ca^{2+}-free Ringer’s solution. TG fully inhibits the SERCA Ca^{2+}-ATPases of the endoplasmic reticulum, causing a small transient rise in [Ca^{2+}], (Fig. 1C) due to the unopposed leakage of Ca^{2+} from stores followed by extrusion across the plasma membrane. Depletion of Ca^{2+} stores by this method maximally activates store-operated Ca^{2+} channels in the plasma membrane, also referred to as CRAC channels in T-lymphocytes (4, 5). No Ca^{2+} entry through the channels is observed during TG stimulation when the extracellular solution does not contain any Ca^{2+}. Changing the extracellular Ca^{2+} concentration to 1 mM following complete store depletion, then allows Ca^{2+} influx through store-operated Ca^{2+} channels, giving rise to long-lasting Ca^{2+} signals in T-cells (Fig. 1C).

To compare LP T-cells, which are hyporeactive, with “normal” reactive PB T-cells, we isolated PBLs from the same patients. PBLs were analyzed using exactly the same protocol applied for the LPLs in Fig. 1. Fig. 2 compares the Ca^{2+} signals of LPLs and PBLs grouped into CD4⁺ and CD8⁺ T-cells. Fig. 2A and B, shows representative experiments, and the statistical analysis of all of the experiments is presented in Fig. 2C. PB

**Fig. 2**. Comparison of Ca^{2+} signals in PB and LP T-lymphocytes. Experiments were carried out as described in Fig. 1. A, comparison of Ca^{2+} signals in CD4⁺ LP and PB T-cells. Two representative experiments are shown. B, same experiments, only that CD8⁺ LP and PB T-cells are shown. C, analysis of all cells from all of the patients. Average amplitudes of Ca^{2+} peak and Ca^{2+} plateau (measured at the end of the Ca^{2+} re-addition) are shown for CD4⁺ and CD8⁺ LP and PB T-cells. Numbers in the bars indicate the total number of cells taken from 22 experiments with LPL cells (14 patients) and 14 experiments with PBL cells (12 patients). Error bars represent mean ± S.E.

T-cells display larger Ca^{2+} signals following activation of store-operated Ca^{2+} entry through CRAC Ca^{2+} channels than LP T-cells (p < 0.0001). This is true for CD4⁺ and CD8⁺ cells, whereas in general, CD4⁺ cells respond slightly but significantly better than CD8⁺ cells (p < 0.01). The larger Ca^{2+} signals in PB T-cells correlate well with their reactivity and proliferation following activation of the cells, whereas the smaller Ca^{2+} signals in LP T-cells correlate with their previously described hyporeactivity (11). We did not observe differences in resting Ca^{2+} levels between LP and PB T-cells, which have been reported by another group (19). The reason for this discrepancy was not further investigated. It is noteworthy, however, that we observed higher LPL resting Ca^{2+} signals when the cell preparation was not optimal, which was evident by a very low yield of cells and many debris and “degranulated” cells.

 Whereas LP T-cells of healthy individuals are hyporeactive, this is completely changed in T-cells from inflamed tissue of patients with active IBD. In the latter case, the T-cells proliferate quite well and almost reach rates comparable with “normal” reactive PB T-cells. Considering the importance of intracellular Ca^{2+} signals for T-cell proliferation, we hypothesized that Ca^{2+} signals in T-cells from inflamed tissue could be increased, thus explaining the higher proliferation rates. Fig. 3 summarizes our evidence that TG-induced Ca^{2+} signals are indeed increased in LP T-cells from inflamed mucosa. In Fig. 3A, LP T-cells from inflamed and non-inflamed intestine of the same patient were compared with his PB T-cells. All of the CD8⁺-positive cells of this patient were pooled, revealing a
clear difference of Ca$^{2+}$ signals between LP T-cells of non-inflamed and inflamed mucosa, the latter of which reached Ca$^{2+}$ concentrations almost as high as the respective PB CD8$^+$ T-cells. The same was found for the CD4$^+$ cells of the patient (data not shown). In Fig. 3B, the CD3$^+$ T-cells from all of the patients are compared. Ca$^{2+}$ signals due to Ca$^{2+}$ influx are clearly enhanced in LP T-cells from inflamed tissue versus non-inflamed tissue and reach levels of Ca$^{2+}$ signals in PB T-cells. Ca$^{2+}$ signals in PB T-cells did not differ between cells from IBD and non-IBD individuals. Fig. 3, C and D, depicts the statistical analysis of the Ca$^{2+}$ peak and plateau for the different T-cell populations analyzed. In CD4$^+$ and CD8$^+$ T-cells as well as in CD3$^+$ CD4$^-$ CD8$^-$ T-cells, we found the same pattern. Whereas Ca$^{2+}$ peak and plateau in all of the LP T-cell subpopulations from non-inflamed tissue are significantly lower than the signals from the respective PB T-cell subpopulations ($p < 0.0001$), Ca$^{2+}$ signals in LP T-cells from inflamed tissue are significantly increased compared with the ones from non-inflamed tissue ($p < 0.01$ for Ca$^{2+}$ peak and plateau) and almost reach levels of Ca$^{2+}$ signals in PB T-cells. These findings correlate well with the hyperreactivity of LP T-cells from inflamed intestine of individuals with active IBD.

TG induces a complete depletion of Ca$^{2+}$ stores and thus allows us to study the maximal Ca$^{2+}$ entry through store-operated Ca$^{2+}$ channels independent of the activation of TCR-dependent signaling cascades. Activation of the TCR through OKT-3, which binds to CD3, also induces Ca$^{2+}$ signaling in T-lymphocytes but only partially depletes Ca$^{2+}$ stores because the released Ca$^{2+}$ can be pumped back into the Ca$^{2+}$ stores by the SERCA ATPases. TCR stimulation by 10 μg/ml OKT-3 in Ca$^{2+}$-free Ringer's solution induces Ca$^{2+}$ release transients because of the generation of Ins-1,4,5-P$_3$, which releases Ca$^{2+}$ from endoplasmic reticulum stores and subsequent re-uptake of Ca$^{2+}$ into the stores or clearance of Ca$^{2+}$ across the plasma membrane. Fig. 4A shows examples of LP and PB T-cells to illustrate typical response patterns. In cells that do not show release transients in response to OKT-3 (one of the LP T-cells, thin solid line), only very little influx is observed. The influx is clearly enhanced when OKT-3 stimulation induces a Ca$^{2+}$ release spike, thereby partially depleting Ca$^{2+}$ stores that then results in activation of store-operated Ca$^{2+}$ entry. The release transients due to OKT-3 stimulation can occur at different times (Fig. 4A) and are therefore not visible in the Ca$^{2+}$ traces when averaging cells (Fig. 4B). We compared LP and PB T-cells from the same individuals. Approximately 44% PB T-cells from healthy individuals or patients with IBD showed clear Ca$^{2+}$

![Fig. 3. Comparison of Ca$^{2+}$ signals in PB and LP T-lymphocytes from patients with active IBD and healthy individuals. Experiments were carried out as described in Fig. 1. A, comparison of Ca$^{2+}$ signals in CD8$^+$ LP and PB T-cells of the same patient with active IBD. LP T-cells from inflamed and non-inflamed parts of the intestinal mucosa were compared. B, Ca$^{2+}$ signals of CD8$^+$ cells from all of the patients are averaged. The averages were not weighted for the cell numbers of each experiment, which results in an identical contribution of all patient data to the average. C and D, analysis of all cells from all of the patients. Average amplitudes of Ca$^{2+}$ peak and Ca$^{2+}$ plateau (measured at the end of the Ca$^{2+}$ re-addition) are shown for the different LP and PB T-cell subpopulations. In this case, individual cells equally contributed to the average. PBn and LPn reflect cells from healthy individuals while PBi and LPi represent data from inflamed tissue of patients with active IBD. Cells were taken from 22 experiments/14 patients (LPn), 14 experiments/12 patients (PBn), 7 experiments/5 patients (LPi), and 12 experiments/11 patients (PBi). Data from three more patients with active IBD (LPi) and two more healthy individuals (LPn) gave similar results but were not included because not all of the antibody stainings were performed. Error bars represent mean ± S.E. Note that in B the averages of each experiment were pooled, whereas in C and D, each cell was individually analyzed, which can result in small differences regarding peak and plateau amplitudes between B and C and D. PBn, PB in normal individuals; PBi, PB in inflamed patients; LPn, LP in normal individuals; LPi, LP in inflamed patients.](Image 79x403 to 543x737)
release transients during the OKT-3 application and were counted as responders and used for the average in Fig. 4B. In contrast, only 16% of the LP T-cells from healthy individuals or patients with IBD responded to OKT-3 stimulation. Comparing the Ca²⁺ influx signals of PB and LP responders, we again observed that the Ca²⁺ peak and plateau following Ca²⁺ influx in responding LP T-cells was lower than that in the responding PB T-cells (p < 0.01, Fig. 4B, statistics on the calcium plateau in 4C). Similar to the TG stimulation protocol, Ca²⁺ peak and plateau following influx were enhanced in LP T-cells from patients with active IBD compared with LP T-cells from non-IBD individuals (p < 0.01). Surprisingly, we also found an influx increase in PB T-cells from patients with active IBD when compared with PB T-cells from healthy individuals (p < 0.01), which was not observed following TG stimulation. The reason for this difference is currently unknown but could result from the T-cells of the IBD patients being already activated.

To link the observed differences in Ca²⁺ signaling between LP T-lymphocytes from persons with and without active IBD to T-cell function, we analyzed the importance of Ca²⁺ entry for cell proliferation. Proliferation of PBL and LPL cells were analyzed while varying the net Ca²⁺ influx rate through changes in the extracellular Ca²⁺ concentration (details are described in the figure legend). Fig. 5A illustrates the strong dependence of PBL proliferation rates on the external Ca²⁺ concentration. Free [Ca²⁺]_{ext} below 100 μM is not sufficient to induce the full proliferative response of PBLs, even when a strong stimulus (PMA, ionomycin) was used. Using TG or OKT-3 as a stimulus, cell proliferation was also completely blocked by low micromolar [Ca²⁺]_{ext} (Fig. 5B), whereas it could be slightly enhanced by adding 1 mM Ca²⁺ to the medium, indicating that proliferation in PBL depends on the external Ca²⁺ concentration and therefore on net Ca²⁺ entry. The same pattern was found in LPL cells using TG as stimulus (Fig. 5C), whereas only very little Ca²⁺ signals were causally related to cell hyporeactivity LPL cells from healthy individuals, whereas the enhanced Ca²⁺ signals might well account for the pathological hyperreactivity of LPLs from patients with active IBD.

**DISCUSSION**

Several groups have shown that T-lymphocytes of the intestinal mucosa are hyporeactive and respond with low proliferative response and low transcriptional activity upon antigen presentation (11, 17, 20, 21). In contrast, T-lymphocytes from peripheral blood are able to react with strong proliferative activity upon antigen stimulation. The hyporeactivity of intestinal T-cells is one mechanism likely to avoid pathologic inflammatory responses in the intestine, which is continuously exposed to antigen. The molecular mechanism for the different reactivity of LP and PB T-cells is unclear. Because elevations of [Ca²⁺], have been closely linked to activity and proliferation of T-lymphocytes (1, 7, 10), we compared Ca²⁺ signals in resting and activated LP and PB T-cells. We found resting [Ca²⁺], levels to be similar in LP and PB T-cells following stimulation; however, PB T-cells reached much higher [Ca²⁺], levels than LP T-cells, which correlate well with their proliferative responses. Ca²⁺ signals in LP T-cells from inflamed tissue were drastically increased, which could explain the enhanced T-cell
proliferation described in those cells. The increased responsiveness of LP T-cells from inflamed tissue could be attributed to infiltration of the lamina propria by PB T-cells or to increased Ca²⁺ influx of the resident LP T-cells themselves. It has been shown that lymphocytes, which are primed and differentiated in Peyer’s patches and in mesenteric lymph nodes, migrate into the bloodstream and preferentially return to Peyer’s patches or the lamina propria of the gut (22, 23). Tissue-specific accumulation and proliferation of T-cells and/or proliferation of resident T-cells may both modulate chronic inflammation such as IBD. At present, we cannot distinguish whether the increased Ca²⁺ responses observed in LP T-cells from inflamed tissue are caused by infiltrating PB T-cells or resident LP T-cells. The only hint that we measure LP T-cells and not infiltrated PB T-cells in the inflamed tissue comes from the observation that the same amount of LP T-cells (16%) responds to OKT-3 stimulation in inflamed and non-inflamed tissue.

De Maria et al. (19) postulated that elevated resting levels of [Ca²⁺], are responsible for the hyporeactivity of LP T-cells. They used FACS analysis to measure [Ca²⁺], which does not allow kinetic measurements of individual cells, limits resolution, and requires pre-labeling of cells that might pre-activate the cells. Using high resolution Ca²⁺ imaging, our kinetic analysis of individual T-cells did not reveal differences of resting [Ca²⁺] between LP and PB T-cells from the same individuals. Pre-activation of the cells was also excluded by identification of the cells as T-lymphocytes after the Ca²⁺ measurements. We conclude that differences in resting Ca²⁺ levels cannot explain the differences of higher basal activity of LP T-cells versus PB T-cells.

Similar to De Maria et al. (19), Qiao et al. (24) showed by FACS analysis that in contrast to PB T-cells, LP T-cells did not respond with [Ca²⁺] elevations following stimulation of the cells. Analyzing single identified T-cells, we came to a different...
conclusion. Whereas Ca\textsuperscript{2+} release from stores was on average comparable in PB and LP T-cells following TG stimulation, only very few LP T-cells (10% compared with 44% PB T-cells) responded to TCR stimulation with clear Ca\textsuperscript{2+} release transients during the 500-s OKT-3 exposure. Ca\textsuperscript{2+} entry following activation of plasma Ca\textsuperscript{2+} channels by OKT-3 or thapsigargin was present in all of the LP T-cells, but it was clearly reduced when compared with PB T-cells. Because activity of the plasma membrane Ca\textsuperscript{2+} channels is closely correlated with T-cell activation and proliferation (7), the reduction of Ca\textsuperscript{2+} entry could explain the hyporeactivity of LP T-cells upon antigen stimulation.

Recent publications highlight the importance of Ca\textsuperscript{2+} signals for the induction of peripheral tolerance in T-cells. Macian et al. (25) reported that TCR stimulation without co-stimulation strongly favors Ca\textsuperscript{2+}-dependent signal transduction via nuclear factor of activated T-cells over other TCR-dependent signal transduction pathways, thereby inducing T-cell anergy, which is a tolerance mechanism in which T-cells are functionally inactivated (26). On the other hand, oral tolerance can also be associated with an impairment of Ca\textsuperscript{2+}-dependent signal transduction (27). At first sight, our results would better fit in with the latter observation. However, one can easily imagine that both mechanisms (25, 27) could even co-exist in the same cell because Ca\textsuperscript{2+} can have very different specific cellular functions depending on kinetics, amplitude, and localization of the [Ca\textsuperscript{2+}], elevation (1, 28, 29). How the molecular mechanism of mucosal T-cell hyporeactivity exactly works is still unknown, but it has been speculated that the microenvironment of the mucosal T-cell hyporeactivity exactly works is still unknown, but it has been speculated that the microenvironment of the intestinal lamina propria is thought to play an important role in IBD such as Crohn’s disease or ulcerative colitis (12–17) with contrast to T-cells from non-inflamed tissue, T-cells from inflamed tissue have been found to be hyperreactive. Following TCR stimulation, the cells show abnormally high proliferation rates that are thought to be one reason for the constant intestinal inflammation. If our hypothesis that Ca\textsuperscript{2+} entry is closely linked to proliferation in LP T-cells were correct, we would expect to find increased Ca\textsuperscript{2+} entry in LP T-cells from inflamed tissue. Indeed, this was the case. Whereas the number of responding cells following TCR stimulation was not increased in T-cells from inflamed intestine, there was a clear increase of Ca\textsuperscript{2+} signals because of Ca\textsuperscript{2+} influx across the plasma membrane following TCR or thapsigargin stimulation when compared with LP T-cells from non-inflamed tissue. This increase of [Ca\textsuperscript{2+}]\textsubscript{i}, following stimulation could explain the hyperreactivity of LP T-cells from inflamed intestine of patients with active IBD. Because enhanced proliferation of activated T-cells is believed to be one of the problems during IBD, decreasing Ca\textsuperscript{2+} influx could potentially also decrease inflammation in the intestine. An important question is how the higher Ca\textsuperscript{2+} signals in LP T-cells from patients with active IBD are generated. There are two principal possibilities to account for the differences. One is an increase of net influx, the other is a decrease of net efflux of Ca\textsuperscript{2+} from the cytosol. A change in the net efflux is not very likely because Ca\textsuperscript{2+} clearance rates were not different between PB and LP T-cells from healthy individuals or patients with active IBD. This leaves increased Ca\textsuperscript{2+} entry as the best explanation, which could be achieved by higher expression of Ca\textsuperscript{2+} channels or expression of a different type of Ca\textsuperscript{2+} channels with higher Ca\textsuperscript{2+} permeability in the plasma membrane. Alternatively, an increase in the net Ca\textsuperscript{2+} influx rate could also be mediated by a more negative membrane potential of the cells. In any case, influx of Ca\textsuperscript{2+} through store-operated CRAC Ca\textsuperscript{2+} channels is increased in LP T-cells from inflamed tissue and reaches levels of PB T-cells, thus abolishing the hyporeactivity of the LP T-cells. Inhibition of store-operated Ca\textsuperscript{2+} entry in LP T-cells of patients with active IBD could decrease activation and proliferation of the cells leading to a reduction of intestinal inflammation. Their localization in the plasma membrane makes the CRAC Ca\textsuperscript{2+} channels in T-cells a good target for future immunosuppressives to potentially treat the symptoms of IBD.

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