Upregulation of KCa3.1 K⁺ channel in mesenteric lymph node CD4⁺ T lymphocytes from a mouse model of dextran sodium sulfate-induced inflammatory bowel disease

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Am J Physiol Gastrointest Liver Physiol 306: G873–G885, 2014. First published March 27, 2014; doi:10.1152/ajpgi.00156.2013.—The intermediate-conductance Ca²⁺-activated K⁺ channel KCa3.1/KCNN4 constitutes an attractive therapeutic target for diseases of the immune system (25, 49). Recent reports have suggested that KCa3.1 is also an attractive target for therapeutic strategies to IBD (11, 38). Di et al. (11) have shown that KCa3.1 knockdown causes the impairment of both Ca²⁺ influx and cytokine production in mice CD4⁺ T lymphocytes and thereby protected mice from developing severe colitis in IBD models. Enlargement of mesenteric lymph nodes (MLNs) is observed in most CD patients (30). Furthermore, Biswas et al. (4) have recently shown that MLNs are markedly enlarged with an expansion of Th1 lymphocytes in Nod-2-deficient, CD model mice.

KCa3.1 plays an important role in T lymphocyte Ca²⁺ signaling by maintaining a negative membrane potential, which provides an electrochemical gradient to drive Ca²⁺ influx through a Ca²⁺ release-activated Cu²⁺ channel (3, 6, 16, 21), and thereby regulating cell proliferation and differentiation in T lymphocytes (26). Recent reports have shown that the potential KCa3.1 regulators [phosphoinositide-3-kinase, class 2, beta polypeptide (PI3K-C2β), nucleoside diphosphate kinase B (NDPK-B), phosphohistidine phosphatase 1 (PHPT-1), and myotubularin related protein 6 (MTMR-6)] play critical roles in T cell receptor-mediated Ca²⁺ influx and T cell proliferation (10, 41–44).

The goal of the present study was to evaluate the following issues by using MLN CD4⁺ T lymphocytes of a DSS-induced mice IBD model: 1) the involvement of KCa3.1 in the MLN enlargement and 2) the effect of pharmacological blockade of KCa3.1 in vivo on inflammation-induced changes in the expression levels of KCa3.1 and its regulatory molecules.

MATERIALS AND METHODS

Preparation of DSS-induced mouse IBD model. Male C57BL/6J (5–6 wk of age) mice were purchased from Japan SLC (Shizuoka, Japan) and were acclimatized for 1 wk before the experiment. They were given distilled water containing 5% (wt/vol) dextran sulfate sodium 5000 (DSS) (Wako Pure Chemical, Osaka, Japan) ad libitum. Control mice were given drinking water only. Clinical assessment of inflammation included daily monitoring of weight-loss. Seven days after DSS administration, mice were euthanized, tissue samples were taken, and colitis and inflammation was assessed macroscopically, on...
the basis of weight change, colon length and weight, stool quality, and colonic tissue appearance (12). The macroscopic appearance stool consistency (diarrhea score) and visible fecal blood was scored separately on a scale of 0–3. Diarrhea scores were designated as 0, normal pellets, 1, slightly loose feces; 2, loose feces; 3, watery diarrhea. Visible fecal blood scores were designated as 0, normal; 1, slightly bloody; 2, bloody; 3, blood in whole colon. The mucosal lesion area in colon was evaluated by Alcian blue staining (29). Briefly, isolated colon was fixed with 10% buffered formalin for 30 min and stained 1% Alcian blue (Muto Pure Chemicals, Tokyo, Japan) for 20 min. After destaining with 3% acetic acid, the lesion area was determined by using Image J software (ver. 1.42, NIH). A subcutaneous (sc) injection was given in the loose skin around the neck, and the injection volume was 100 μl. In the “vehicle” group, the same volume of dimethyl sulfoxide (DMSO) was administered. All experiments were carried out in accordance with the guiding principles for the care and use of laboratory animals in Nagoya City University and Kyoto Pharmaceutical University, and also with the approval of the presidents of both universities.

**Histological scoring.** For histological assessment, the 1-cm tissue of the distal colon was fixed in 10% buffered formalin, embedded in paraffin block, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E). Inflammation and crypt damage in the H&E-stained sections were assessed as described by Dieleman et al. (13). The inflammation score was determined as the multiplication of the grade of the inflammation severity (grade 0, none; grade 1, slight; grade 2, moderate; grade 3, severe) and the extent (grade 0, none; grade 1, mucosa; grade 2, mucosa and submucosa; grade 3, transmural). Also, the crypt damage score was determined the multiplication of the grade of the crypt (grade 0, none; grade 1, basal 1/3 damage; grade 2, basal 2/3 damage; grade 3, only surface epithelium intact; grade 4, entire crypt and epithelium lost) and the percent area score (grade 1, 1–25%; grade 2, 25–50%; grade 3, 51–75%; grade 4, 76–100%). Data are obtained from three sections of colon measured at least 200 μm apart per animal from four individual mice per group.

**Measurement of compound concentration in plasma.** Under anesthesia, blood was collected into heparinized tubes at 0, 24, 48, 72, and 96 h after subcutaneous administration of 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34; 1 and 10 mg/kg) and was kept on ice. The plasma was obtained by centrifugation and stored at −80°C until use. Methanol was added to the plasma and the mixture was centrifuged (14,000 × g) for 10 min. After the supernatant was evaporated under nitrogen at 55°C, the residue was dissolved in the mobile phase. The solution was loaded to Acuity UPLC system (Waters, Milford, MA) with InertSustain C18 column (2.1 × 50 mm, GL sciences, Tokyo, Japan). The mobile phase was 0.2% aqueous formic acid and acetonitrile by gradient elution. The injection volume was 5 μl and the flow rate 0.3 ml/min. The Quattro Premier XE triple quadrupole mass spectrometer (Waters) equipped with electrospray ionization was used for MS/MS analysis (34). TRAM-34 was analyzed in positive ion multiple-reaction monitoring mode.

**Isolation of CD4+ T lymphocytes from mesenteric lymph node.** CD4+ T lymphocytes were isolated from MLN cell suspension using Dynabeads FlowComp mice CD4+ according to the experimental manual supplied by Invitrogen (31). Flow cytometric analysis confirmed 95% of purified T lymphocytes to be CD4+.

**RNA extraction, RT-PCR, and real-time PCR.** Total RNA extraction and RT-PCR from mice MLN CD4+ T lymphocytes were performed as previously reported (31). The reverse transcription cDNA products were amplified with gene-specific primers and the primers designated by use of Primer Express software (ver. 1.5, Applied Biosystems, Foster City, CA). Quantitative, real-time PCR was performed with the use of Sybr Green chemistry (SYBR Premix Ex Taq II) (TaKaRa Bio, Osaka, Japan) on the ABI PRISM 7700 and ABI 7500 real-time PCR instruments (Applied Biosystems) as previously reported (31). The following PCR primers for mice clones were used for the real-time PCR: KCa3.1a (GenBank accession number: NM_008433, 343–452), 110 bp; KCa3.1b (AB128930, 308–419), 112 bp; PI3K-C2β (NM_001099276, 2293–2422), 130 bp; NDPK-B (NM_008705, 467–597), 131 bp; HPT-1 (NM_029293, 69–189), 121 bp; MTMR-6 (NM_144843, 722–832), 111 bp; tripartite motif-containing protein 27 (TRIM-27) (NM_009054, 1530–1650), 121 bp; interferon (IFN)-γ (NM_008337, 222–323), 102 bp; interleukin-2 (IL-2) (NM_008366, 84–122), 39 bp; IL-4 (NM_021283, 126–246), 121 bp; IL-6 (NM_031168, 532–660), 129 bp; IL-10 (NM_010548, 245–355), 111 bp; IL-17 (NM_010552, 162–277), 113 bp; β-actin (ACTB) (NM_031144, 419–519), 101 bp. Regression analyses of the mean values of three multiplex RT-PCRs for the log10-diluted cDNA were used to generate standard curves. Unknown quantities relative to the standard curve for a particular set of primers were calculated, yielding transcriptional quantitation of gene products relative to the endogenous standard, ACTB. To confirm the nucleotide sequences, amplified PCR products were sequenced with an ABI PRIZM 3100 genetic analyzer (Applied Biosystems). RT-PCR with ACTB primers (2384–2402, 498 bp) that were designated to span two exons and an intron controlled for genomic DNA contamination in the source RNA. There was no contamination with genomic DNA (an intron-containing amplicon = 708 bp), as confirmed by RT-PCR assay.

**Western blotting.** Protein lysates were prepared from mouse MLNs as previously reported (31). The same amount of proteins was subjected to 10% SDS-PAGE. The blots were incubated with anti-KCa3.1 (Alomone Labs) and then incubated with anti-rabbit horseradish peroxidase-conjugated IgG (Millipore). An enhanced chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK) was used for the detection of the bound antibody. The resulting images were analyzed with LAS-1000 and LAS-3000 devices (Fujifilm, Tokyo, Japan).

**Flow cytometric analysis.** Cell surface markers were analyzed with a FACSscan flow cytometer (BD LSR, BD Pharmingen, Franklin Lakes, NJ) acquiring at least 10,000 events, and gated according to forward- and side-scatter (31). Data were analyzed with CellQuest software (Becton-Dickinson). The lymphocyte gate was established by analysis of forward angle vs. right angle light scatter. The percentage of positive-staining cells was determined by comparing the test histograms with those obtained by using fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-CD4 (FITC/PE-CD4) (BD Pharmingen, Berkeley, CA) and anti-IFN-γ (PE-IFN-γ), PE-conjugated anti-interleukin (IL-2) (PE-IL-2), PE-conjugated IL-7 (PE-IL-7), and anti-KCa3.1 (labeled with FITC-conjugated anti-rabbit IgG antibody) (Becton Coulter, Brea, CA) antibodies (FITC-KCa3.1) antibodies. Lymphocytes were fixed and permeabilized using the CytoFix/Perm-kit (BD Pharmingen) before they were stained intracellularly with FITC/PE-labeled antibodies. After incubation with the primary and secondary antibodies for 1 h at room temperature, respectively, excess antibodies were removed by repeated washing with PBS. In the flow cytomtery (FCM) analysis, PE/FITC-conjugated rat IgG1 and 2b were used as isotype controls.

**Measurement of membrane potential using fluorescent voltage-sensitive dyes.** Mice were euthanized and MLNs were isolated and processed individually. Single-cell suspensions were prepared by pressing the lymph node with a frosted slide grass and then filtering through a nylon mesh. Cells were collected on medium containing RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and antibiotics (0.1% penicillin and 0.1% streptomycin). Measurement of the membrane potential was performed using the voltage-sensitive dye bis-(1,3-dibutylbarbituric acid)trimethine xonon [DiBAC4(3)], as previously reported (17, 31). Prior to the fluorescence measurements with DiBAC4(3), isolated cells were incubated in normal HEPES buffer containing 100 nM DiBAC4(3) for 20 min at room temperature. The cells were continuously incubated with 100 nM DiBAC4(3) throughout the experiments. For membrane potential imaging, the fluorescence emission was collected by using a 505-nm dichroic mirror and a long-pass filter (>520 nm). Data collection and analysis were performed via an
ARUGUS-HiSCA imaging system (Hamamatsu Photonics, Hamamatsu, Japan). The staining cells were also analyzed with a FACScan flow cytometer.

Electrophysiological recordings. A whole-cell patch clamps was applied to single CD4+ T lymphocytes by using a CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan) at room temperature (23 ± 1°C). FITC-CD4-positive T lymphocytes were detected under fluorescence microscopy. The external solution was (in mM) 160 Na-aspartate, 4.5 KCl, 2 CaCl2, 1 MgCl2, and 5 HEPES, pH 7.4. The pipette solution was (in mM) 145 K-aspartate, 2 MgCl2, 10 HEPES, 10 EGTA, and 8.5 CaCl2, pH 7.2, with an estimated free Ca2+ concentration of 1 μM. The procedures used for electrophysiological recordings and data acquisition/analysis for whole-cell recording have been reported previously (31). Currents were measured in voltage-clamp mode and induced by ramp depolarization from −120 to +60 mV, 200 ms duration, every 10 s at −80 mV holding potential. The 1 μM TRAM-34-sensitive current density at +40 mV was calculated.

Chemicals. The sources of pharmacological agents were as follows: ionomycin (Sigma-Aldrich, Tokyo, Japan), DiBAC4(3) (Invitrogen), 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazole-2-one (DC-EBIO; TOCRIS Bioscience, Ellisville, MO); TRAM-34 (Santa Cruz Biotechnology, Santa Cruz, CA; Toronto Research Chemicals, Toronto, Canada), and 2,2-bis(4-fluorophenyl)-2-phenyl-acetamide (ICA-17043, Chemistick, Shanghai, China). The others were obtained from Sigma-Aldrich or Wako Pure Chemical Industries (Tokyo, Japan).

Statistical analysis. Statistical significance between two and among multiple groups was evaluated by Student’s t-test, Welch’s t-test after normality testing, and ANOVA. Data that were normally distributed were analyzed using Mann-Whitney’s U-test. Data are presented as means ± SE.

RESULTS

Effect of sc administration of the KCa3.1 blocker the TRAM-34 on the enlargement of MLN during acute colitis in the DSS-induced mouse IBD model. Enlargement of the lymph nodes is a common symptom in many immune diseases, and enlargement of MLNs is observed in CD patients and CD model mice (4, 30). 7 days after DSS administration, the mice were euthanized and tissue samples were taken. In this study, control mice (control) were given drinking water only. As shown in Fig. 1A, acute colitis caused a significant increase in the relative MLN weight: control, 0.51 ± 0.05 mg/g body wt (n = 8); IBOD model, 0.81 ± 0.02 mg/g body wt (n = 17). In parallel, the number of viable MLN lymphocytes determined by the Trypan blue dye exclusion assay was significantly increased in the IBOD model compared with the control mice: control, 2.28 ± 0.23 (× 107 cells/tissue) (n = 14, P < 0.01); IBOD model, 3.86 ± 0.46 (× 107 cells/tissue) (n = 17). A viable lymphocyte gate was set on the basis of a forward light scatter channel (FSC) and side light scatter channel (SSC) (high-FSC/low-SSC) as reported by Chrest et al. (9). The FACS profiles of the gated CD4+ lymphocytes showed that there was no significant difference in FSC (cell size) distribution detected between MLN CD4+ lymphocytes of the groups, and the CD4+ lymphocytes in the IBOD model were significantly increased (~140%) compared with the control mice (not shown).

Di et al. (11) have suggested that KCa3.1 blockers may have a unique therapeutic profile for inhibiting pro-colitis-inducing Th1 and Th2 cells. We therefore examined the effect of a single sc administration of a potent and specific KCa3.1 blocker, TRAM-34 (1 and 10 mg/kg sc), on day 3 in terms of the effect on the enlargement of MLNs. We measured the total TRAM-34 concentration in plasma after the administration (1 and 10 mg/kg sc). The TRAM-34 concentration fell from 0.56 ± 0.07 μM (n = 10) after 1 day (on day 4) to 0.27 ± 0.07 μM (n = 9) after 4 days (on day 7) (Fig. 1B). Even 4 days after a single sc administration, the plasma concentration of TRAM-34 was still over 10 times higher than the in vitro IC50 (less than 20 nM) (1, 25). As shown in Fig. 1C, the relative MLN weight was significantly reduced by the administration of TRAM-34 (1 and 10 mg/kg sc): 0.67 ± 0.04 and 0.64 ± 0.03 mg/g body wt (n = 8 for each, P < 0.05) compared with vehicle control (0.81 ± 0.04 mg/g body wt, n = 8). The decrease in the number of viable MLN lymphocytes was prevented by the administration of TRAM-34 (10 mg/kg sc) at a similar level as in control mice: vehicle control, 4.35 ± 0.63 (× 107 cells/tissue) (n = 18); TRAM-34, 2.48 ± 0.35 (× 107 cells/tissue) (n = 18, P < 0.01). The relative MLN weight was also significantly reduced by the administration of 1 and 3 mg/kg sc ICA-17043, the KCa3.1 blocker that has been evaluated in multiple preclinical and clinical studies: 0.62 ± 0.04 (n = 9, P < 0.05) and 0.61 ± 0.04 mg/g body wt (n = 9, P < 0.05) compared with vehicle control (0.84 ± 0.05 mg/g body wt, n = 9) (Fig. 1D). Cytotoxic or proapoptotic actions poten-
tially arising from the administration of TRAM-34 and ICA-17043 were not observed (not shown). In control mice, the administration of 10 mg/kg sc TRAM-34 induced the significant increase (~15%) in the relative spleen weight (“splenomegaly”) as reported by Grigic et al. (18): vehicle, 3.41 ± 0.25 mg/g body wt (n = 5); TRAM-34, 3.96 ± 0.34 mg/g body wt (n = 5, P < 0.05); however, no significant changes in the MLN weight were observed: vehicle, 0.48 ± 0.04 mg/g body wt (n = 5); TRAM-34, 0.50 ± 0.05 mg/g body wt (n = 5, P > 0.05). These findings support the notion that KCa3.1 is associated with MLN enlargement due to an enhancement of T lymphocyte proliferation during acute colitis.

**Effect of sc administration of TRAM-34 on clinical symptoms during acute colitis in DSS-induced IBD model mice.**

Seven days after DSS administration, we also assessed the effect of sc administration of TRAM-34 on clinical symptoms: loose feces (watery diarrhea) and fecal blood (Fig. 2, A–D). Macroscopically, the inflammation was generally located in the distal colon, as previously reported by Okayasu et al. (33).

Loose feces and fecal blood were macroscopically not detected in control mice, resulting that the average score for both diarrhea and visible fecal blood was 0 (n = 8) (Fig. 2, A and C). In the IBD model, severe clinical symptoms were observed as previously reported (33) (Fig. 2, A and C), and the average scores of diarrhea and visible fecal blood were 2.2 ± 0.1 and 2.6 ± 0.1, respectively (n = 17, P < 0.01 vs. control). The administration of TRAM-34 (1 and 10 mg/kg sc) suppressed the severity of these clinical symptoms (Fig. 2, B and D). The diarrhea scores were significantly reduced by the administration of TRAM-34 (1 and 10 mg/kg sc): 1.2 ± 0.2 and 0.7 ± 0.2, respectively [n = 17 for each, P < 0.01 vs. vehicle control (2.3 ± 0.2, n = 17)]. The visible fecal blood scores were also significantly reduced by the administration of TRAM-34 (1 and 10 mg/kg sc): 1.5 ± 0.2 and 0.8 ± 0.3, respectively [n = 17 for each, P < 0.01 vs. vehicle control (2.5 ± 0.2, n = 17)]. Similar results were obtained by the administration of 3 mg/kg sc ICA-17043 (not shown). Additionally, colonic inflammation and crypt damage were assessed by colon weight-to-length ratio measurements, Alcian blue staining and histological scoring. As shown in Fig. 2E, colon weight-to-length ratio was significantly increased in the IBD model compared with control (n = 5, P < 0.01 vs. control), and recovered by the administration of TRAM-34 (10 mg/kg sc) (n = 5, P < 0.01 vs. vehicle control). Alcian blue staining revealed a significant decrease in the lesion area by the administration of TRAM-34 (10 mg/kg sc) compared with vehicle control (n = 8 for each, P < 0.01) (Fig. 3, A and B). Histological assessment of the inflammation and crypt damage also showed that both the colonic inflammation and the crypt damage scores in TRAM-34 (10 mg/kg sc)-administered group were significantly lower than in the vehicle-administered group (n = 4 for each, P < 0.05) (Fig. 3, C–E). In control mice, no significant changes in both scores were observed by the administration of 10 mg/kg sc TRAM-34 (both scores of 0).

**Increase in KCa3.1a expression in the MLN CD4+ T lymphocytes of IBD model mice.** We have previously reported that lymphoid cells express the dominant-negative isoform of KCa3.1, KCa3.1b, which suppresses the membrane trafficking of functional KCa3.1a (32). We examined the quantitative analysis of the transcripts of these KCa3.1 splice variants. Quantitative, real-time PCR analysis showed that the expression level of the KCa3.1a transcripts alone was significantly increased in the MLN CD4+ T lymphocytes of IBD model compared with control mice (Fig. 4A), whereas that of KCa3.1b was almost identical between the groups (Fig. 4B). The expression levels of KCa3.1a relative to ACTB (in arbitrary units)
were 0.011 ± 0.002 and 0.027 ± 0.002 in MLN CD4^+ T lymphocytes of the control (n = 4) and IBD model (n = 6, P < 0.01), respectively. Western blot analysis also showed the upregulation of KCa3.1a protein in the MLN membrane protein fraction of the IBD model compared with that of the control mice without any effect on KCa3.1b protein levels (Fig. 4C, top), correlating with the results obtained by real-time PCR examinations. When the optical density for the KCa3.1a protein band signal in the MLNs of the control mice was expressed as 1.00, the relative optical density in IBD model was 2.35 ± 0.34 (n = 4 for each, P < 0.05) (Fig. 4C, bottom). The expression of KCa3.1b protein in the plasma membrane was very low in MLNs of both groups compared with that of KCa3.1a protein. The two band signals specific for an anti-KCa3.1 antibody with molecular weights of ~50 (for KCa3.1a) and 25 (for KCa3.1b) kDa were observed (Fig. 4C, top). As shown in our previous report (32), these signals were not seen before preincubation with excess antigen, but the nonspecific signals observed in Fig. 4C, top, were unchanged by it. Subsequently, a fluorescent dual-color dot plot of FITC-CD4 vs. PE-KCa3.1 was analyzed in fixed and permeabilized lymphocytes by use of FCM. PE-conjugated rat IgG was used as an isotype control. Significantly higher populations of KCa3.1^+ were detected in the MLN CD4^+ T lymphocytes of the IBD model than the control mice (Fig. 4D). The percentages of CD4^+KCa3.1^+ T lymphocytes to the total CD4^+ ones were determined in the control and the IBD model: 12.8 ± 2.4% (n = 13) and 33.7 ± 5.7% (n = 16, P < 0.05 vs. control), respectively (Fig. 4D). The KCa3.1^+ cell population was markedly decreased (less than 1% of cell populations) by preincubation of an anti-KCa3.1 antibody with the excess antigen (not shown). These results suggest that upregulation of KCa3.1a may be associated with the proliferation of MLN CD4^+ T lymphocytes in the IBD model. Enhancement of TRAM-34-induced depolarization responses and TRAM-34-sensitive current density in MLN CD4^+ T lymphocytes of IBD model mice. We examined the measurement of depolarization responses by TRAM-34 in isolated MLN CD4^+ T lymphocytes using the membrane potential-sensitive dye DiBAC_4(3) and a fluorescence imaging system. TRAM-34 (1 μM)-induced depolarization (ΔF_{TRAM-34}) was measured after activating the KCa3.1 channel by application of ionomycin (0.5 μM) and DC-EBIO (10 μM) (39), a potent KCa3.1 channel activator. Figure 5A shows the KCa3.1 activator and blocker-induced changes in the fluorescence intensity of DiBAC_4(3) in
the MLN CD4⁺ T lymphocytes of the IBD model. The summarized data are expressed as the ratio of ΔF_{TRAM-34} to 140 mM high-K⁺-induced depolarization (ΔF_{140 K}). After measurement of 140 mM high-K⁺-induced depolarization, DiBAC₄(3) was washed out for 30 min. Then the MLN lymphocytes were stained with FITC-CD4 and the data for the CD4⁺ T lymphocytes alone were analyzed. In the MLN CD4⁺ T lymphocytes of the control mice, 1 μM TRAM-34-induced depolarization responses were relatively small (20.9 ± 2.0%, n = 74); however, significantly larger responses were observed in the MLN CD4⁺ T lymphocytes of the IBD model (34.1 ± 2.7%, n = 114, P < 0.01) (Fig. 5B). Similarly, the fluorescence intensity of DiBAC₄(3) in the MLN T lymphocytes of the control and the IBD model was measured by FCM analysis. Experiments were performed with the same drug application protocol. Changes in the fluorescence intensity of DiBAC₄(3) (F_{DBAC}) was plotted in a single dimension so as to produce a histogram. When F_{DBAC}^{high} was defined as the region including over 95% of the cell populations after an application of the 140 mM high-K⁺ solution (the other region was defined as F_{DBAC}^{low}), the percentages of the cell populations transferring from F_{DBAC}^{low} to F_{DBAC}^{high} by application of 1 μM

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**Fig. 4.** Expression of KCa3.1 in MLN CD4⁺ T lymphocytes of DSS-induced mouse IBD model. A and B: quantitative, real-time PCR assay for KCa3.1a (A) and KCa3.1b (B) in MLN CD4⁺ T lymphocytes of the control and the IBD model mice. ACTB, β-actin. C: whole tissue lysates from MLN were probed by immunoblotting with an anti-KCa3.1 antibody. Molecular mass standards are shown in kilodaltons at top right. Arrowheads indicate the migrating positions of KCa3.1a (50 kDa) and KCa3.1b (25 kDa). The summarized results were obtained as the optical density of KCa3.1a band signal in the IBD model relative to that in control mice. D: expression patterns of KCa3.1 in MLN CD4⁺ T lymphocytes of the control and IBD groups. MLN T lymphocytes were double stained with FITC-anti-KCa3.1 and phycoerythrin (PE)-anti-CD4⁺ antibodies. The percentage of T lymphocytes with CD4⁺KCa3.1⁺ population was analyzed by flow cytometry (FCM), acquiring at least 10,000 events. Results are expressed as means ± SE. Numbers used for the experiments are shown in parentheses. *,** P < 0.05, 0.01 vs. control.

**Fig. 5.** Depolarization responses by pharmacological blockade of KCa3.1 in MLN CD4⁺ T lymphocytes of the DSS-induced mouse IBD model and effect of the administration of TRAM-34 on them. A and B: measurement of TRAM-34-induced depolarization responses in MLN CD4⁺ T lymphocytes using the voltage-sensitive fluorescent dye bis-(1,3-dibutylbarbituric acid)trimethyl ethine oxonol [DiBAC₄(3)]. KCa3.1 was activated by the preincubation with ionomycin (iono; 0.5 μM) and 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one (EBIO; 10 μM), and then TRAM-34 (1 μM) was applied. Measurement of the fluorescence intensity of DiBAC₄(3) was performed via an ARUGUS-HiSCA imaging system. A shows the time course of the changes in fluorescence intensity of DiBAC₄(3) (F_{DBAC}) in the MLN CD4⁺ T lymphocytes of the IBD model. C: FCM analysis of the percentages of the cell populations transferring from F_{DBAC}^{low} to F_{DBAC}^{high} (where F_{DBAC}^{low} was defined as the region including over 95% of the cell populations after an application of the 140 mM high-K⁺ solution and the other region was defined as F_{DBAC}^{low}) by the application of 1 μM TRAM-34 in the MLN CD4⁺ T lymphocytes of control and IBD mouse models. D: measurement of the depolarization responses induced by KCa3.1 blockade in the MLN CD4⁺ T lymphocytes of the IBD model administered vehicle or TRAM-34 (10 mg/kg sc). E: FCM analysis of the percentages of the cell populations transferring from F_{DBAC}^{low} to F_{DBAC}^{high} by the application of 1 μM TRAM-34 in the MLN CD4⁺ T lymphocytes of the IBD model administered vehicle or TRAM-34 (10 mg/kg sc). B and D, the data are shown as the ratio (ΔF_{TRAM-34}/ΔF_{140 K}) of TRAM-34-induced fluorescence change (ΔF_{TRAM-34}) to 140 mM K⁺-induced fluorescence change (ΔF_{140 K}). In C and E, the data are shown as the percentages of the cell populations transferring from F_{DBAC}^{low} to F_{DBAC}^{high} by the application of 1 μM TRAM-34 in the MLN CD4⁺ T lymphocytes. Results are expressed as means ± SE. Numbers used for the experiments are shown in parentheses. **P < 0.01 vs. control; # P < 0.05 vs. vehicle control.
TRAM-34 were 2.0 ± 0.8% (n = 10) and 9.6 ± 1.7% (n = 21, P < 0.01) in the MLN CD4+ T lymphocytes of the control and the IBD model, respectively (Fig. 5C). We further examined the effects of 1 µM TRAM-34 on the whole-cell membrane currents that were recorded in MLN CD4+ T lymphocytes of the control and IBD model by the ramp pulse protocol (Fig. 6A) (35). In the MLN CD4+ T lymphocytes of the IBD model, significantly larger levels of current density (Fig. 6B) were observed: 20.6 ± 5.3 (n = 14, control) and 40.3 ± 6.6 pA/pF (n = 12, IBD model, P < 0.05). No significant differences of cell capacitance were observed between the MLN CD4+ T lymphocytes of the control and the IBD model: 9.62 ± 0.36 (n = 14, control) and 9.33 ± 0.58 pF (n = 12, IBD model, P > 0.05).

Effects of the in vivo administration of TRAM-34 on KCa3.1a expression. We examined the effects of sc administration of TRAM-34 on the expression of KCa3.1 in the MLN CD4+ T lymphocytes of the IBD model. As shown in Fig. 7A, the expression of the KCa3.1a transcripts was significantly downregulated by the administration of TRAM-34 (1 and 10 mg/kg sc) (n = 6 for each, P < 0.05, 0.01 vs. vehicle control) and was almost identical to that in control mice. There was no significant change in the expression of KCa3.1b transcripts by the administration of TRAM-34 (1 and 10 mg/kg sc) (Fig. 7B). In control mice, the administration of 10 mg/kg sc TRAM-34 induced no significant changes in the expression levels of KCa3.1 transcripts in the MLN CD4+ T lymphocytes: vehicle, 0.009 ± 0.002; TRAM-34, 0.011 ± 0.002 (n = 5 for each, P > 0.05). In correlation with the result by real-time PCR, the administration of TRAM-34 (10 mg/kg sc) caused downregulation of the KCa3.1a protein in the MLNs of the IBD model compared with vehicle control (n = 4 for each, P < 0.01 vs. vehicle control) (Fig. 7C). When the optical densities for KCa3.1a in the MLNs of control mice (see Fig. 4C) were calculated as 1.0, the relative optical densities for them were 2.28 ± 0.22 and 0.86 ± 0.14 in the vehicle control and TRAM-34 (10 mg/kg sc)-administered IBD model, respectively (Fig. 7C). No significant changes in the expression of KCa3.1b protein were found. Furthermore, significantly lower populations of KCa3.1+ were detected in the MLN CD4+ T lymphocytes of TRAM-34 (10 mg/kg sc)-administered mice than the vehicle control (Fig. 7D). The percentages of CD4+KCa3.1+ T lymphocytes to total CD4+ ones were determined in vehicle control and TRAM-34-treated group: 27.9 ±
5.1% (n = 10) and 14.6 ± 2.8% (n = 14, P < 0.05 vs. vehicle control), respectively (Fig. 7D).

Additionally, the changes in KCa3.1 activity induced by the administration of TRAM-34 (10 mg/kg sc) was demonstrated in MLN CD4+ T lymphocytes. A 1 μM TRAM-34-induced depolarization response (ΔFTRAM-34/ΔF140K) was significantly inhibited by the administration of TRAM-34 (10 mg/kg sc: 13.1 ± 1.8%, n = 52, P < 0.01 vs. vehicle control: 36.4 ± 1.1%, n = 117) (Fig. 5D). Similarly, FCM analysis showed that the percentages of the cell populations transferring from FDBAClow to FDBACHigh by the application of 1 μM TRAM-34 were 9.4 ± 2.2% (n = 10) and 2.0 ± 0.7% (n = 12, P < 0.01) in the MLN CD4+ T lymphocytes of the vehicle control and TRAM-34 (10 mg/kg sc)-treated groups, respectively (Fig. 5E). These results were consistent with the result of changes in the expression patterns of KCa3.1 genes and proteins by pharmacological blockade of KCa3.1.

Changes in the expression levels of the positive and negative regulators of KCa3.1 in T lymphocytes of IBD model mice. In addition to the upregulation of KCa3.1, the upregulation of the positive regulators of KCa3.1 and/or the downregulation of the negative regulators of KCa3.1 may be responsible for the higher KCa3.1 activity in MLN CD4+ T lymphocytes of IBD model, as shown in Fig. 4B. Srivastava et al. (41–44) have identified the positive (PI3K-C2β and NDPK-B) and negative regulators (PHPT-1 and MTMR-6) of KCa3.1 in T lymphocytes. Therefore, the transcriptional expression patterns of the positive and negative regulators of KCa3.1 were compared between the MLN CD4+ T lymphocytes of the control and the IBD model. In the MLN CD4+ T lymphocytes of the IBD model, the transcriptional expression levels of both positive regulators (PI3K-C2β and NDPK-B) were significantly higher than in the control (Fig. 8, A and C). The sc administration of TRAM-34 (10 mg/kg) in the IBD model elicited the restoration of the increase in both transcripts (Fig. 8, B and D). On the other hand, no significant differences in the expression levels of either of the negative regulators of KCa3.1 (PHPT-1 and MTMR-6) were found between the MLN CD4+ T lymphocytes of the control and the IBD model (Fig. 8, E and G).

TRIM-27, a RING (Really Interesting New Gene) family protein with E3 ubiquitin ligase activity, function as a negative regulator of KCa3.1 through the polyubiquitination of PI3K-C2β in the inflammatory colon sections in a dose-dependent manner (n = 4 for each). In control mice, protein expression pattern of IL-6 was not observed, and the administration of 10 mg/kg sc TRAM-34 also induced no significant change in the expression levels of IFN-γ, IL-2, and IL-17A in the MLN CD4+ T lymphocytes (not shown).

IL-6 signaling during the development of IBD plays a crucial role in immunological reactions, and the serum IL-6 levels correlate with many of the clinical features in both CD and UC patients (27, 35). KCa3.1 activity is in part associated with IL-6 transcription and secretion in mast cells and macrophages (20, 40). As shown in Fig. 10G, the administration of TRAM-34 resulted in a substantial decrease (~55%) in the protein expression of IL-6 (~20 kDa) in the inflammatory colon sections in a dose-dependent manner (n = 4 for each). In control mice, protein expression pattern of IL-6 was not observed, and the administration of 10 mg/kg sc TRAM-34 also induced no significant change (not shown). These findings suggest that the enhancement of IL-6 signaling during the development of IBD is suppressed by a pharmacological blockade of KCa3.1.

DISCUSSION

Recently, Di et al. (11) have shown that genetic deletion and pharmacological blockade of KCa3.1 reduce disease severity in two different IBD models and have suggested that a KCa3.1 inhibitor may be useful in treating patients with IBD. The enlargement of MLNs is observed in most CD patients (30) and CD model mice (4), but it remains unclear whether KCa3.1 is responsible for the enlargement of the MLN in IBD. In agreement with the results from previous reports (4, 11), the present regulation of PI3K-C2β may be canceled out by the upregulation of TRIM-27 in the MLN CD4+ T lymphocytes of the IBD model.
study showed that the pharmacological blockade of KCa3.1 inhibited clinical symptoms, such as diarrhea and fecal blood (Fig. 2), colonic inflammation and crypt damage (Figs. 2E and 3), and the enlargement of MLN (Fig. 1, C and D) in DSS-induced IBD model mice. The main findings in the present study are as follows: 1) the stimulation of MLN CD4+ T lymphocyte proliferation through an upregulation of KCa3.1a and NDPK-B in the DSS-induced mouse IBD model; 2) the prevention of this upregulation by pharmacological blockade of KCa3.1; and 3) the pharmacological blockade of KCa3.1-induced inhibition of interleukin-6 (IL-6) production in the inflamed tissues. These results corroborated the proposal concept that pharmacological blockade of KCa3.1 is effective for prophylactic and definitive treatments in the pathogenesis of
IBD. Di et al. (11) have treated mice with 100 mg·kg\(^{-1}\)·day\(^{-1}\) of TRAM-34 for 6 days (1 day before TNBS treatment until day 5 after treatment); however, in our study, a much lower dose (a single sc administration of 1 mg/kg TRAM-34 at 3 days) induced a significant therapeutic effect on clinical symptoms in the IBD model (Fig. 2, A–D). In the present study, DSS-induced IBD model was prepared with 5- to 6-wk-old male mice. An upregulation of \(K_{Ca3.1}\) in MLN CD4\(^+\) T lymphocytes and the prevention of it by pharmacological blockade of \(K_{Ca3.1}\) were observed when 9- to 12-wk-old male mice were used in the experiments (not shown).

We have recently identified the dominant-negative isoform of \(K_{Ca3.1}\), i.e., \(K_{Ca3.1b}\), lacking the NH\(_2\)-terminal region from both human and rodent lymphoid tissues, and have shown that...

Fig. 9. Effects of sc administration of TRAM-34 (10 mg/kg) on the protein expression levels of PI3K-C2\(\beta\), NDPK-B and TRIM-27 in the MLNs of DSS-induced IBD model mice. The blots were probed with anti-PI3K-C2\(\beta\) (A), anti-NDPK-B (B), and anti-TRIM-27 (C) antibodies, respectively. The same blots were also probed with anti-ACTB antibody. Molecular mass standards are shown in kilodaltons (kDa) at the left side of top. Arrowheads indicate the migrating positions of PI3K-C2\(\beta\) (180 kDa), NDPK-B (20 kDa), and TRIM-27 (60 kDa).

Fig. 10. Effects of TRAM-34 administration on the expression patterns of IFN-\(\gamma\), IL-2, and IL-17 transcripts in the MLN CD4\(^+\) T lymphocytes of the control and DSS-induced IBD model and on IL-6 proteins in the inflammatory tissues of them. Quantitative, real-time PCR assay for IFN-\(\gamma\) (A and B), IL-2 (C and D), and IL-17 (E and F) expression in the MLN CD4\(^+\) T lymphocytes of the control (\(n = 4\)) and IBD model mice (\(n = 5\)) (A, C, E) and in those of the IBD model administered vehicle control (DMSO) (\(n = 5\)) or TRAM-34 (10 mg/kg sc) (\(n = 5\)) (B, D, F). G: proteins from inflamed colonic tissue lysates were probed by immunoblotting with anti-IL-6 antibody. The summarized results were obtained as the optical density of each band signal relative to that of the band signal in the IBD model (\(n = 4\) for each). Results were expressed as means ± SE. **\(P < 0.01\) vs. control, ##\(P < 0.01\) vs. vehicle control.
KCa3.1 activity was significantly enhanced in MLN CD4⁺ T lymphocytes of the IBD model (Fig. 4B). Alternative splicing of large-conductance Ca²⁺-activated K⁺ channel (KCa1.1) is regulated by pharmacological blockade (36). The present study showed there was no significant change in KCa3.1b expression in MLN CD4⁺ T lymphocytes by the sc administration of a KCa3.1 blocker (Fig. 7B). The molecular mechanism underlying the alternative splicing of KCa3.1 remains to be determined, but the elucidation of this mechanism in T lymphocytes may provide a new insight into the therapeutic significance of KCa3.1b for immune diseases, including IBD.

In CD4⁺ T lymphocytes, two positive (PI3K-C2β and NDPK-B) and two negative (PHPT-1 and MTMR-6) regulators of KCa3.1 activity play important roles in proliferation and cytokine production (10, 12, 41–44). We observed a significant increase in the expression of both PI3K-C2β and NDPK-B in CD4⁺ T lymphocytes of IBD model mice, and these elevated expression levels were restored to the control level by the administration of TRAM-34 (10 mg/kg sc) (Figs. 8 and 9). Of interest, TRIM-27, which suppresses KCa3.1 activity through the polyubiquitination of PI3K-C2β and leads to decreased proliferation and cytokine production in CD4⁺ T lymphocytes (7), was significantly increased in the MLN CD4⁺ T lymphocytes of the IBD model (Fig. 8). Similarly, a recent study reported the enhancement of TRIM-27 expression in CD patients (51). Upregulation of TRIM-27 may be a compensatory mechanism underlying the excess CD4⁺ T lymphocyte activation in IBD patients. On the other hand, NDPK-B forms the clustering at the immunological synapse (IS) together with KCa3.1, and the recruitment of NDPK-B with KCa3.1 to the peripheral zone, p-SMAC (peripheral supramolecular activating complex) in the IS is critical for KCa3.1 activation (42). Elucidation of the molecular mechanism underlying the formation of the KCa3.1/NDPK-B complex in the p-SMAC of the IS may enable novel drug design for the treatment of IBD.

Hyperpolarization of the cell membrane by K⁺ channel activation promotes Ca²⁺ influx during the G1 phase of the cell cycle, and the effect of K⁺ channel inhibitors are attributable to cell accumulation in the G0/G1 phase (48). A number of reports have shown that pharmacological blockade of KCa3.1 suppresses cell proliferation through G0/G1 arrest in renal fibroblasts (19), preadipocytes (50), mesenchymal stem cells (47), and airway smooth muscle cells (37). Additionally, in cancer cells, KCa3.1 activity is involved in the transition to the S phase and entry into the G2/M phase in cancer cells, and TRAM-34 blocks the cell cycle at the G2/M phase (2, 8, 24). Our previous study reported that, in delayed-type hypersensitivity model mice, the suppression of cell proliferation in CD4⁺ T lymphocytes by the administration of TRAM-34 is related to the arrest of the G1/S and G2/M transitions during cell cycle progression (31). The present study also indicated that upregulation of KCa3.1a promotes cell-cycle progression at the G1/S and G2/M transitions in MLN lymphocytes of the IBD model and that suppression of cell proliferation in IBD model MLN CD4⁺ T lymphocytes by the administration of TRAM-34 includes the arrest of the G1/S and G2/M transitions (not shown). KCa3.1b may be a promising target for modulating the cell-cycle progression of CD4⁺ T lymphocytes in IBD.

Cytokines are important in the pathogenesis of IBD, and their manipulation has been shown to successfully reduce...
disease severity, whereas the clinical symptoms that manifest during acute colitis are associated with the changes in proinflammatory and anti-inflammatory cytokine expression (46). The IL-6 signaling pathway plays a central role in several different immunological reactions during the development of IBD, and the serum IL-6 levels correlate with the clinical features in both CD and UC patients (27, 35). In mast cells and macrophages, KCa3.1 activity is associated with the IL-6 transcription and secretion (20, 40). The present study showed that the KCa3.1 inhibitor significantly suppressed the IL-6 protein levels in inflamed tissues of IBD model mice (Fig. 11G). Of interest, voltage-gated K+ channel inhibitor-induced depolarization inactivates Ca2+ release-activated Ca2+ channel activity and thereby suppresses the expression of IL-6 in immune cells (22, 28). Therefore, pharmacological blockade of KCa3.1 may have inhibitory effects on the IL-6 signaling pathway and thereby suppress the inflammatory responses in IBD. Also, the upregulation of the Th1 cytokine IFN-γ in MLN CD4+ T lymphocytes was recovered by the KCa3.1 blockade (Figs. 10B and 11A). Further study will be necessary to clarify the molecular mechanisms underlying the suppression of proinflammatory cytokine expressions by the KCa3.1 blockade. KCa3.1 is involved in the regulation of cell migration in various types of cells including T lymphocytes, and pharmacological blockade of KCa3.1 suppresses it (23). CD4+ T lymphocyte migration to inflammatory tissues plays an important role in the pathogenesis of IBD. Therefore, upregulation of KCa3.1 and NDKP-B in MLN CD4+ T lymphocytes may be involved in the migration of Th1 cells to inflammatory colonic tissue. Real-time PCR analysis showed that both KCa3.1a and NDKP-B transcripts were highly expressed in CD4+ T lymphocytes enzymatically isolated from colonic lamina propria of IBD model (not shown); however, the effect of KCa3.1 blockade on their migration remains unclear in this study. Further study using these cells will be necessary to clarify the pathophysiological significance of KCa3.1 and its regulators in the migration of T lymphocytes in IBD.

The increase in KCa3.1 activity through the upregulation of KCa3.1a and NDKP-B might constitute an important initiation step for MLN CD4+ T lymphocyte proliferation and migration in acute colitis and play a key role in the pathogenesis of IBD. Conversely, pharmacological blockade of KCa3.1 activity was associated with significant suppression of severe IBD clinical symptoms, substantial reduction of MLN CD4+ T lymphocyte proliferation, and downregulation of KCa3.1a and NDKP-B. Our findings also suggest that membrane depolarization by pharmacological blockade of KCa3.1 results in a significant decrease in the proinflammatory cytokine (IL-6) expression of which has been critically correlated to clinical features in IBD patients. In addition to KCa3.1, NDKP-B and TRIM-27 may be potential therapeutic targets to decrease the risk of IBD development. These findings should prove to be useful in both the prevention and treatment of IBD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


