Inhibition of Interleukin 10 Transcription through the SMAD2/3 Signaling Pathway by Ca\textsuperscript{2+}-Activated K\textsuperscript{+} Channel K\textsubscript{Ca}3.1 Activation in Human T-Cell Lymphoma HuT-78 Cells\textsuperscript{[S]}

Miki Matsui, Junko Kajikuri, Hiroaki Kito, Kyoko Endo, Yuki Hasegawa, Shinya Murate, and Susumu Ohya

Department of Pharmacology, Kyoto Pharmaceutical University, Kyoto, Japan (M.M., K.E., Y.H.) and Department of Pharmacology, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan (M.M., J.K., H.K., E.M., S.O.)

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ABSTRACT

The hyperpolarization induced by intermediate-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel (K\textsubscript{Ca}3.1) activation increases the driving force for Ca\textsuperscript{2+} influx, which generally promotes cell proliferation, migration, and cytokine production in immunocompetent cells. Interleukin-10 (IL-10) from tumor-infiltrating lymphocytes and macrophages, lymphoma, and carcinoma cells facilitates escape from cancer immune surveillance; however, the role of K\textsubscript{Ca}3.1 in IL-10 production remains unclear. The objective of the present study was to elucidate the involvement of K\textsubscript{Ca}3.1 in IL-10 expression and production using the human T-cell lymphoma HuT-78 cells. In HuT-78 cells, IL-10 gene expression and production were reduced by treatment with the K\textsubscript{Ca}3.1 activator, as 6-hour Western blotting showed that the protein expression ratio of phosphorylated Smad2 (P-Smad2)/Smad2, but not P-Smad3/Smad3, was decreased by the treatment with K\textsubscript{Ca}3.1 activator in HuT-78 cells. Concomitant with this, the nuclear translocation of P-Smad2 was inhibited by K\textsubscript{Ca}3.1 activator. Furthermore, the K\textsubscript{Ca}3.1 activator–induced transcriptional repression of IL-10 disappeared with pretreatment with the calmodulin kinase II (CaMKII) inhibitor KN-62 for 1 hour, and K\textsubscript{Ca}3.1 activator–induced decreases in the nuclear translocation of P-Smad2 were also prevented by pretreatment with KN-62. Taken together, the K\textsubscript{Ca}3.1 activator–induced transcriptional repression of IL-10 is due to the inhibition of the nuclear translocation of P-Smad2 in HuT-78 cells, resulting in the prevention of P-Smad2/3 complex formation in nuclei, and the activation of CaMKII induced by K\textsubscript{Ca}3.1 activators suppresses the constitutive activation of P-Smad2/3 in HuT-78 cells. Therefore, K\textsubscript{Ca}3.1 activators have potential as a therapeutic option to suppress the tumor-promoting activities of IL-10.

Introduction

The anti-inflammatory cytokine interleukin-10 (IL-10) is an immunosuppressive factor involved in tumorigenesis and plays a crucial role in escape from cancer immune surveillance (Hamidullah et al., 2012). IL-10 expression is associated with lymphoproliferative disorders, such as lymphocytic leukemia, lymphoma, and myeloma (Benjamin et al., 1992; Finke et al., 1993; Lu et al., 1995). Increased IL-10 levels in patients with lymphoma are associated with poor survival due to the facilitation of tumor immune escape (Blay et al., 1993). IL-10 is frequently upregulated in various types of cancers (Sato et al., 2011), and the inhibition of IL-10 accelerates apoptosis in breast cancer cells (Alotaibi et al., 2018). The T-cell lymphoma cell line HuT-78, which is forkhead box P3 (Foxp3)/CD25-negative and constitutively produces IL-10, has been used to study the underlying mechanisms of IL-10 expression in T cells (Mori and Prager, 1997; Tiffon et al., 2011). Mori and Prager (1997) showed the involvement of the constitutive activation of nuclear factor-κB (NF-κB) in the high-level expression of IL-10 in HuT-78 cells. IL-10–producing naturally occurring regulatory T (Treg) cells are Foxp3-positive CD4\textsuperscript{+}CD25\textsuperscript{+} T cells and control autoimmunity (Itoh et al., 1999); however, recent studies showed that tumor-infiltrating Foxp3-negative CD4\textsuperscript{+}CD25\textsuperscript{-} T cells are naturally present as IL-10–secreting T cells (Burugu et al., 2017; Ma et al., 2017).

Ca\textsuperscript{2+}-activated K\textsuperscript{+} (K\textsubscript{Ca}) channels are classified into three subfamilies based on their unitary conductance: K\textsubscript{Ca}1.1, K\textsubscript{Ca}2.x (2.1–2.3), and K\textsubscript{Ca}3.1. In lymphoid and myeloid cells, membrane hyperpolarization by K\textsuperscript{+} channel opening (i.e., K\textsubscript{Ca}3.1, voltage-gated K\textsubscript{v}1.3, and two-pore domain K\textsubscript{Tp}5.1) increased the activity of Ca\textsuperscript{2+} release–activated Ca\textsuperscript{2+} channels and transient receptor potential Ca\textsuperscript{2+} channels (Cahalan and Chandy, 2009; Esjte et al., 2015; Ohya and Kito, 2018).

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Abbreviations: AM, 2-acetoxymethyl ester; ACTB, β-actin; Blimp, B-lymphocyte-induced maturation protein; CaMK, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase; DCEBIO, 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one; E4BP, E4 promoter–binding protein; Foxp, forkhead box P; IFN, interferon; IL, interleukin; K\textsubscript{Ca}, Ca\textsuperscript{2+}-activated K\textsuperscript{+} (channel); mTOR, mammalian target of rapamycin; NDKP-B, nucleoside diphosphate kinase B; NF-κB, nuclear factor-κB; PCR, polymerase chain reaction; P-Smad, phosphorylated Smad; PI3K, phosphoinositide 3-kinase; STAT, signal transducers and activator of transcription; TGF, transforming growth factor; Th, helper T (cell).
Ca^{2+}-dependent regulation of transcription factors [nuclear factor of activated T cells, NF-κB, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and mitogen-activated protein kinase] is involved in the activation of immune responses in T cells. KCa3.1 plays a pivotal role in the proliferation, differentiation, and migration of immune cells and is a potential therapeutic target for autoimmune and inflammatory disorders. In addition, the enhancement of KCa3.1 activity promotes proinflammatory cytokine production and secretion by regulating Ca^{2+} signaling in CD4^+ T cells (Di et al., 2010; Ohya et al., 2014). In CD8^+ T cells with the capacity to kill malignant cells, K^+ efflux–mediating KCa3.1 activation increases antitumor function by promoting interferon (IFN)-γ production (Eil et al., 2016). The following positive and negative KCa3.1 function-modifying molecules have been identified in T cells: class II phosphoinositide-3 kinase B (PI3K-C2B), nucleoside diphosphate kinase B (NDPK-B), protein histidine phosphatase-1, phosphatidylinositol 3-phosphate myotubulin-related protein, phosphoglycerate mutase family member 5, and tripartite motif (family) 27 (Ohya and Kito, 2018). In T cells, KCa3.1 accumulates in the uropod, but not the leading edge, and is associated with oscillations in intracellular Ca^{2+} levels (Kuras et al., 2012). The pathologic significance of KCa3.1 in IL-10–producing T cells and cancerous cells remains to be elucidated.

Smad2 and Smad3 (Smad2/3) are essential transcription factors in the development and maintenance of T reg cells (Sekiya et al., 2016). Phosphorylated Smad2/3 (P-Smad2/3) translocate to the nucleus, together with Smad4, and activate the transcription of downstream target genes (Derynck and Zhang, 2003). The constitutive activation of Smad2/3 signaling is evoked by constitutive transforming growth factor (TGF)-β signaling in resting human CD4^+ T cells (Classen et al., 2007) and is maintained even in the presence of a TGF-β antagonist. CaMKII directly controls Smad2/3 function (Zimmerman et al., 1998; Wicks et al., 2000). The nuclear translocation of Smad2 is negatively regulated by Ca^{2+}-dependent CaMKII signaling, which disrupts the P-Smad2/3 complex formation (Ming et al., 2010). Limited information is currently available on the role of Smad2/3 in proinflammatory and anti-inflammatory cytokine expression.

Herein, we examined the effects of KCa3.1 activators on the transcriptional repression of IL-10 in HuT-78 cells. Our results indicated that IL-10 is a downstream target gene of the Smad2/3 signaling pathway, and KCa3.1 activators inhibit the nuclear translocation of P-Smad2, which is constitutively active in HuT-78 cells. Our results also provide evidence for the importance of CaMKII signaling in the negative regulation of the nuclear translocation of Smad2 in HuT-78 cells.

Materials and Methods

Cell Culture. The human T-cell lymphoma cell line HuT-78 was supplied by the RIKEN BioResource Center (Tsukuba, Japan). The human leukemic T-cell lymphoblast cell line Jurkat clone E6, the human B lymphoma cell line Daudi, the human chronic myeloid leukemia cell line K562, and the human acute monocytic leukemia cell line THP-1 were supplied by the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and Cell Resource Center for Biomedical Research (Sendai, Japan). Cells were maintained at 37°C in 5% CO2 with RPMI 1640 medium (Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and a penicillin-streptomycin mixture (Wako Pure Chemical Industries).

Real-Time Polymerase Chain Reaction Assay. Total RNA extraction and reverse transcription from HuT-78, Jurkat, Daudi, K562, and THP-1 cells were performed as previously reported (Endo et al., 2015). cDNA products were amplified with gene-specific polymerase chain reaction (PCR) primers, designated using Primer Express software (version 3.0.1; Thermo Fisher Scientific, Waltham, MA). Quantitative real-time PCR was performed using SYBR Green chemistry on an ABI 7500 Fast real-time PCR system (Thermo Fisher Scientific). The following gene-specific PCR primers of human origin were used for real-time PCR: KCa1.1 (GenBank accession number NM_001014797, 1120-1239), alpophilin = 120 bp; KCa2.1 (NM_002248, 649-764), 116 bp; KCa2.2 (NM_021614, 1492-1612), 121 bp; KCa2.3 (NM_002249, 2042-2146), 105 bp; KCa3.1 (NM_002250, 1475-1595), 121 bp; NDPK-B (NM_002512, 288-408), 121 bp; PI3K-C2B (NM_002646, 4052-4172), 121 bp; protein histidine phosphatase-1 (NM_00135861, 783-903), 121 bp; phosphatidylinositol 3-phosphate myotubulin-related protein (NM_046855, 1476-1607), 132 bp; phosphoglycerate mutase family member 5 (NM_001170543, 277-396), 120 bp; tripartite motif (family) 27 (NM_006519, 403-522), 120 bp; IL-1A (NM_002198, 380-500), 120 bp; IL-5 (NM_000679, 327-446), 120 bp; IL-13 (NM_002188, 166-285), 120 bp; IL-10 (NM_000572, 393-458), 120 bp; IL-32 (NM_00102631, 408-527), 120 bp; E4 promoter–binding protein (E4BP4; NM_001228999, 1219-1339), 123 bp; GATA3 (NM_001022955, 1335-1454), 124 bp; cMaf (NM_0000360, 1697-1818), 120 bp; B-lymphocyte-induced maturation protein (Blimp1; NM_001198, 1425-1544), 120 bp; CD25 (NM_000417, 750-869), 120 bp; Foxp3 (NM_014009, 1253-1372), 120 bp; lymphocyte-activation gene-3 (NM_002286, 1289-1408), 120 bp; early growth response-2 (NM_000399, 590-711), 120 bp; and β-actin (ACTB) (NM_001198, 411-511), 101 bp. Unknown quantities relative to the standard curve for a particular set of primers were calculated as previously reported (Endo et al., 2015), yielding the transcriptional quantification of gene products relative to the endogenous standard, ACTB.

Measurement of IL-10 Production by an Enzyme-Linked Immunosorbent Assay. Human IL-10 levels in culture supernatant samples were measured with a human IL-10 enzyme-linked immunosorbent assay kit (Thermo Fisher Scientific), according to the manufacturer’s protocol. Six hours after the treatments with KCa3.1 activators, culture medium was exchanged with new medium, and an additional 18 hours after the treatments, culture supernatant samples were collected.

Western Blotting. Protein lysates were prepared from HuT-78 cells using radio-immunoprecipitation assay (RIPA) lysis buffer for Western blotting. Protein expression levels were measured 6 hours after the compound treatments. After the quantification of protein concentrations using the Bio-Rad DC protein assay, protein lysates were subjected to SDS-PAGE (10%). Blots were incubated with anti-Smad2/3, P-Smad2 (Ser465/467), anti-P-Smad3 (Ser423/425; Cell Signaling Technology Japan, Tokyo, Japan), and anti-ACTB (Medical & Biologic Laboratories, Nagoya, Japan) antibodies, then incubated with anti-rabbit horseradish peroxidase–conjugated IgG (Merck, Darmstadt, Germany). An enhanced chemiluminescence detection system (Nacalai Tesque, Kyoto, Japan) was used to detect the bound antibody. The resulting images were analyzed using Ameraham Imager 600 (GE Healthcare Japan, Tokyo, Japan). The light intensities of the Smad2/3, P-Smad2, and P-Smad3 protein band signals relative to that of the ACTB signal were calculated using ImageJ software (version 1.42; National Institutes of Health, Bethesda, MD), and the respective ratios of P-Smad2/Smad2 and P-Smad3/Smad3 were obtained. In summarized results, relative protein expression levels in the vehicle control were expressed as 1.0.

Measurement of Membrane Potential and Intracellular Ca^{2+} Concentrations by a Voltage-Sensitive Fluorescent Dye and Fluorescent Ca^{2+} Indicator Dye Imaging. Membrane potential was measured using the fluorescent voltage-sensitive dye DiBAC(3) (as previously reported (Endo et al., 2015). Cells were seeded onto
fibronectin-coated glass-bottomed dishes (Matsumi, Osaka, Japan). In brief, prior to fluorescence measurements, HuT-78 cells were incubated in normal HEPES buffer containing 100 mM DiBAC4(3) at room temperature for 20 minutes and then continuously incubated in 100 mM DiBAC4(3) throughout the experiments. In addition, intracellular Ca2+ concentrations were measured using the fluorescent Ca2+ indicator dye Fura 2-acetoxyethyl ester (AM). Cells were incubated with 10 μM Fura 2-AM in normal HEPES solution for 30 minutes at room temperature. Cells loaded with Fura 2-AM were alternatively illuminated at wavelengths of 340 and 380 nm. Fluorescence images were recorded on the ORCA-Flash2.8 digital camera (Hamamatsu Photonics, Hamamatsu, Japan). Data collection and analyses were performed using an HCImage system (Hamamatsu Photonics). Images were measured every 5 seconds.

Electrophysiological Recording. A whole-cell patch clamp was applied to single HuT-78 cells using the CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan) at room temperature (23°C ± 1°C). The procedures used for electrophysiological recordings and data acquisition/analysis have been reported previously (Ohya et al., 2014). Membrane potential values and whole-cell current densities were measured in the current- and voltage-clamp modes, respectively. Whole-cell currents were induced by ramp depolarization from −120 to +60 mV, 200 ms duration, every 15 seconds at +80 mV holding potential. The data were expressed as the current density (pA/pF). The external solution was as follows: 137 mM NaCl, 2.2 mM KCl, 1.2 mM MgCl2, 14 mM glucose, and 10 mM HEPES (pH 7.4). The pipette solution was as follows: 140 mM KCl, 4 mM MgCl2, 3.2 mM CaCl2, 5 mM EGTA, 10 mM HEPES, and 2 mM Na2ATP (pH 7.2), with an estimated free Ca2+ concentration of 300 nM (pCa 6.5).

Confocal Imaging of the Nuclear Translocation of P-Smad2 and P-Smad3. HuT-78 cells were fixed and permeabilized using the CytoFix/Perm kit (BD Pharmingen, Franklin Lakes, NJ). Anti-P-Smad2 and anti-P-Smad3 antibodies were labeled with an Alexa Fluor 488-conjugated secondary antibody (Abcam, Cambridge, UK), and nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence images were visualized using a confocal laser scanning microscope system (A1R; Nikon, Tokyo, Japan) (Endo et al., 2015). Image data were quantitatively analyzed using ImageJ software.

Chemicals. 5,6-Dichloro-1-ethyl-1,3-dihydro-2H-benzimidazole-2-one (DCEBIO, PubChem compound identifier 657675; Torcis Bioscience, Bristol, UK); SKA-31 (656734; Sigma-Aldrich), DiBAC4(3) (643531; Dejin, Kumamoto, Japan); TRAM-34 (656734; Santa Cruz Biotechnolog, Dallas, TX), TNF-α (531511; MedChemexpress, Monmouth Junction, NJ); UCL1684 (656773; Torcis Bioscience), AZD 5363 (25227436; Cayman Chemical), 5,15-DPP (diphenylpropylamine, 10895582; Abcam), everolimus (6442177; Cayman Chemical), and Bay 11-7082 (535343; Sigma-Aldrich). The other chemicals used in the present study were from Sigma-Aldrich, Wako Pure Chemical Industries, or Nacalai Tesque unless otherwise stated.

Statistical Analysis. The significance of differences among two and multiple groups was evaluated using Student’s or Welch’s t test and Tukey’s test after the F test and analysis of variance, respectively. Significance at P < 0.05 and P < 0.01 is indicated in the figures. Data are presented as the means ± S.D.

Results

Functional Expression of Kcα3.1 K+ Channels in IL-10–Expressing HuT-78 Cells. Previous studies showed that human T-cell lymphoma HuT-78 cells express the anti-inflammatory cytokine IL-10 at a relatively high level (Mori and Prager, 1997; Tiffon et al., 2011). HuT-78 cells strongly expressed IL-10 transcripts but not helper T (Th1), Th2, or Th17 cytokine transcripts (IFN-γ, IL-17A, IL-4, IL-5, and IL-13) (Supplemental Fig. S1A), whereas the other hematologic cell lines (Jurkat E6, Daudi, K562, and THP-1) rarely expressed IL-10 (Supplemental Fig. S1B). Concomitant with these results, IL-10 secretion was markedly higher in HuT-78 cells than in Jurkat E6, Daudi, K562, and THP-1 cells (Supplemental Fig. S1C). A quantitative PCR assay showed that HuT-78 cells negatively expressed CD25 and Foxp3 (Supplemental Fig. S1D). These results indicate that HuT-78 cells have a similar phenotype to CD4+ CD25+ Foxp3+ inducible regulatory T cells. IL-10–producing CD4+ CD25+ Foxp3+ inducible regulatory T cells express both the lymphocyte activation gene-3 and early growth response gene-2 (Okamura et al., 2009). HuT-78 cells expressed both genes at relatively high levels (Supplemental Fig. S1D).

Among five Ca2+-activated K+ channel subtypes, Kcα3.1 transcripts were the most abundantly expressed in HuT-78 cells (Fig. 1A). Additionally, among six Kcα3.1-function-modifying molecules, NDPK-B, which enhances Kcα3.1 activity, was the most abundantly expressed in HuT-78 cells (Fig. 1B). The depolarization responses induced by the Kcα3.1 inhibitor TRAM-34 (1 μM) were subsequently measured by voltage-sensitive fluorescent dye imaging. The application of TRAM-34 did not result in depolarization responses [P > 0.05 vs. vehicle control (0.1% dimethylsulfoxide)], suggesting that Kcα3.1 is rarely activated under normal conditions in HuT-78 cells (Fig. 1, C and D). On the other hand, the application of two different Kcα3.1 activators (1 μM DCEBIO and 1 μM SKA-31) induced hyperpolarization responses [P < 0.01 vs. vehicle control] (Fig. 1, C and D). An approximately 80% reduction in the DCEBIO-induced hyperpolarization response was observed following the application of 1 μM TRAM-34 (not shown). SKA-31 (1 μM)–induced activation of Kcα3.1 was also observed using the whole-cell patch-clamp recordings. In current-clamp mode, the magnitude of membrane hyperpolarization by 1 μM SKA-31 was −69.6 ± 2.3 mV (n = 5, P < 0.01 vs. vehicle) (Supplemental Fig. S2, A and B). In voltage-clamp mode, the currents induced by ramp depolarization from −120 to +60 mV were activated by the application of 1 μM SKA-31 (Supplemental Fig. S2C), and the current density at +60 mV in SKA-31–treated cells (286.9 ± 79.6 pA/pF, n = 7, P < 0.05) was larger than the vehicle control (170.3 ± 95.0 pA/pF, n = 7) (Supplemental Fig. S2, C and D). Both SKA-31–induced hyperpolarization and current activation were suppressed following the application of 1 μM TRAM-34 (Supplemental Fig. S2, A and C). The application of Kcα3.1 activators induced hyperpolarization-induced intracellular Ca2+ elevation (Fig. 1, E and F). The elevated Ca2+ levels (ARatio340/380) were 0.017 ± 0.017 (n = 28), 0.063 ± 0.042 (n = 22, P < 0.01), and 0.052 ± 0.044 (n = 35, P < 0.01) in vehicle-, DCEBIO-, and SKA-31–treated cells, respectively.

Inhibitory Effects of Kcα3.1 Activators on IL-10 Gene Expression and Production in HuT-78 Cells. Using IL-10–producing HuT-78 cells that functionally expressed Kcα3.1 K+ channels, we examined the effects of Kcα3.1 activators on IL-10 transcription and secretion. As shown in Fig. 2, A and C, IL-10 transcription and secretion were both reduced by treatments with 1 μM DCEBIO and 1 μM SKA-31. The expression levels of IL-10 transcripts relative to ACTB were 0.039 ± 0.002, 0.019 ± 0.002, and 0.025 ± 0.002 in vehicle-, DCEBIO-, and SKA-31–treated HuT-78 cells, respectively (n = 4 for each, P < 0.01 vs. vehicle control). The expression levels of vehicle-treated cells is arbitrarily expressed as 1.0. We further examined the effects of 30 mM
K⁺-induced depolarization on KCa3.1 activator–induced inhibition of IL-10 transcription in HuT-78 cells. The application of 30 mM K⁺-containing solution induced moderate depolarization in HuT-78 cells (Supplemental Fig. S3A). Similar to the results in Fig. 1C, 1 μM SKA-31 induced obvious hyperpolarization responses under normal K⁺ (2.2 mM) concentration; however, these responses almost disappeared with 30 mM K⁺-containing solution (Supplemental Fig. S3B). Concomitant with these results, KCa3.1 activator–induced inhibitions of IL-10 transcription disappeared by 30 mM K⁺-containing solution (Supplemental Fig. S3C). In addition, treatment with the Ca²⁺-ATPase inhibitor thapsigargin (1 nM) for 6 hours reduced IL-10 transcription (Supplemental Fig. S3D). IL-10 secretion levels (6–24 hours) were 3207 ± 22, 2206 ± 168, and 2321 ± 66 pg/ml in vehicle-, DCEBIO-, and SKA-31–treated HuT-78 cells, respectively (n = 4 for each, P < 0.01 vs. vehicle control) (Fig. 2C). Approximately 20% of IL-10 secretion was inhibited by treatments with KCa3.1 activators in HuT-78 cells. The transcriptional repression of IL-10 was not found 1 or 3 hours after treatments with KCa3.1 activators (Supplemental Fig. S4), and no changes in IL-10 secretion levels were observed in the culture medium from HuT-78 cells treated with vehicle, DCEBIO, and SKA-31 for 0–6 hours: 961 ± 62, 280 ± 16, and 232 ± 6 pg/ml (n = 4 for each, P < 0.01 vs. vehicle control) (Fig. 2D).

Fig. 1. Functional expression of KCa3.1 K⁺ channels in HuT-78 cells. (A and B) Real-time PCR assay for KCa channel subtypes (KCa1.1, KCa2.x, and KCa3.1) (A) and KCa3.1 function-modifying molecules [PI3K-C2B, NDKP-B, protein histidine phosphatase-1 (PHPT1), phosphatidylinositol 3-phosphate myotubularin-related protein (MTMR6), phosphoglycerate mutase family member 5 (PGAM5), and tripartite motif (family) 27 (TRIM27)] (B) (n = 4 for each). Expression levels were expressed as a ratio to ACTB. (C) The time course of changes in the relative fluorescent intensity of DiBAC₄(3) by the application of KCa3.1 activators (1 μM DCEBIO and 1 μM SKA-31) and a KCa3.1 inhibitor (1 μM TRAM-34). The fluorescent intensity of DiBAC₄(3) before the application of the agents at 0 seconds is expressed as 1.0. Horizontal bar shows the duration of drug application. (D) Summarized data are shown as Δ relative fluorescence intensity of DiBAC₄(3) by the application of KCa3.1 activators (1 μM DCEBIO and 1 μM SKA-31) and a KCa3.1 inhibitor (1 μM TRAM-34). Δ relative fluorescence intensity of DiBAC₄(3) before the application of the agents at 0 seconds is expressed as 1.0. Horizontal bar shows the duration of drug application. (E) Summarized data are shown as Δ relative fluorescence intensity of Fura 2 ratio (340/380 nm) in vehicle-, DCEBIO-, and SKA-31–treated HuT-78 cells. (F) The time course of changes in the intracellular Ca²⁺ concentration ([Ca²⁺]i) by the application of KCa3.1 activators. [Ca²⁺], was expressed as the Fura 2 ratio (340/380 nm). (F) Summarized data are shown as Δ Fura 2 ratio (340/380 nm) in vehicle-, DCEBIO-, and SKA-31–treated HuT-78 cells. Cell numbers used for the experiments are shown in parentheses. Results are expressed as means ± S.D. **P < 0.01 vs. vehicle control.
877 ± 114, and 872 ± 66 pg/ml, respectively (n = 4 for each, P > 0.05 vs. vehicle control). Consistent with the results shown in Fig. 2, C and D, no changes in the expression levels of IL-10 transcripts were noted following treatment with 1 μM TRAM-34 for 6 hours: 0.038 ± 0.008 relative to ACTB (n = 4, P > 0.05 vs. vehicle control: 0.037 ± 0.004). When cells were co-treated with 1 μM TRAM-34, reductions in IL-10 transcription and secretion by treatments with KCa3.1 activators were mostly prevented (Fig. 2, B and D). These results indicate that KCa3.1 is one of the molecules controlling IL-10 production in T-cell lymphoma.

Involvement of the Smad2/3 Signaling Pathway in KCa3.1 Activator–Induced Transcriptional Repression of IL-10 in HuT-78 Cells. As shown in Fig. 2A and Supplemental Fig. S4, B and C, IL-10 transcription was inhibited by KCa3.1 activation within 6 hours. These results suggest that the KCa3.1 activator–induced transcriptional repression of IL-10 is elicited through an IL-10 transcription factor–independent pathway. No changes in the transcriptional expression levels of the IL-10 transcription factors (E4BP4, GATA3, cMAF, and Blimp1) were found in HuT-78 cells (Fig. 3). Their expression levels relative to ACTB were 0.006 ± 0.001, 0.080 ± 0.010, 0.004 ± 0.001, and 0.014 ± 0.004, respectively, in vehicle-treated HuT-78 cells (n = 4 for each).

Kitani et al. (2003) showed that TGF-β1/Smad signaling positively regulated IL-10 gene expression in Th1 cells. Therefore, we assessed the expression levels of P-Smad2 and P-Smad3 by Western blotting. As shown in Fig. 4A and Supplemental Fig. S5A, decreases in P-Smad2 proteins, but not P-Smad3, were found in KCa3.1 activator–treated (for 6 hours) HuT-78 cells without changes in total Smad2 and Smad3 protein expression. After the ratios of P-Smad2/3 to total Smad2/3 were calculated in vehicle-, DCEBIO-, and SKA-31–treated HuT-78 cells, those in the vehicle control were expressed as 1.0. The relative expression of P-Smad2, but not P-Smad3, in DCEBIO- and SKA-31–treated HuT-78 cells was reduced (n = 5 for each, P < 0.01) (Fig. 4, B and C). In addition, P-Smad2 and P-Smad3 proteins were expressed in vehicle-treated HuT-78 cells, suggesting that Smad2 and Smad3 are constitutively active and TGF-β1–independently regulated in HuT-78 cells. The gene expression of IL-10 was not reduced by the treatment with the potent TGF-β1 receptor blocker LY364947 (1 μM) (Supplemental Fig. S6A).

To clarify whether KCa3.1 activators inhibit the nuclear translocation of P-Smad2 and/or P-Smad3 in HuT-78 cells, the cellular localization of P-Smad2/P-Smad3 was visualized by laser-scan confocal fluorescence microscopy. Anti-P-Smad2 and anti-P-Smad3 antibodies were labeled with an Alexa Fluor 488–conjugated secondary antibody, and nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (Fig. 5A). When the mean fluorescence intensities of the nuclear-cytoplasmic ratios of P-Smad2 and P-Smad3 were calculated, the nuclear translocation of P-Smad2, but not...
P-Smad3, was reduced by treatments with KCa3.1 activators (P < 0.01) (Fig. 5, B and C). These results suggest that the nuclear translocation of P-Smad3 is regulated by the Ca\(^{2+}\)-dependent KCa3.1 downstream signaling pathway(s), and thus, IL-10 as a target gene of Smad2/3 signaling may be transcriptionally regulated in HuT-78 cells.

**Effects of the Ca\(^{2+}\)/Calmodulin-Dependent Protein Kinase II Inhibitor on the KCa3.1 Activator–Induced Inhibition of IL-10 Expression and Secretion, P-Smad2 Protein Expression, and the Nuclear Translocation of P-Smad2 in HuT-78 Cells.** CaMKII blocks the nuclear accumulation of Smad2 and prevents Smad2-Smad3 interactions (Zimmerman et al., 1998; Wicks et al., 2000). Increases in intracellular Ca\(^{2+}\) concentrations through KCa3.1 activation enhance the CaMKII signaling pathway. Therefore, we investigated whether pretreatment with the CaMKII inhibitor KN-62 (10 μM) for 1 hour prevents: 1) KCa3.1 activator–induced inhibition of IL-10 transcription and secretion, 2) P-Smad2 protein expression, and 3) the nuclear translocation of P-Smad2 in HuT-78 cells. In Fig. 2, A and C, KCa3.1 activators inhibited IL-10 transcription and secretion. As shown in Fig. 6, the KCa3.1 activator–induced inhibition of IL-10 transcription and secretion disappeared following pretreatment with KN-62 in HuT-78 cells. The expression levels of IL-10 transcripts were 0.035 ± 0.004, 0.040 ± 0.002, and 0.040 ± 0.002 relative to ACTB in vehicle-, DCEBIO-, and SKA-31–treated groups, respectively (n = 4 for each, P > 0.05 vs. vehicle control). Thapsigargin (1 nM)-induced inhibition of IL-10 transcription also disappeared following pretreatment with KN-62 (Supplemental Fig. S3D). In Figs. 4 and 5, KCa3.1 activators decreased the protein expression of P-Smad2 and the nuclear translocation of P-Smad2. KCa3.1 activator–induced decreases in the protein expression of P-Smad2 and the nuclear translocation of P-Smad2 were prevented by pretreatment with KN-62 in HuT-78 cells (Figs. 7 and 8; Supplemental Fig. S5B). These results suggest that CaMKII is an important mediator to promote the KCa3.1 activator–induced blockade of IL-10 transcription and secretion by suppressing the nuclear translocation of P-Smad2.

**Discussion**

The intermediate-conductance Ca\(^{2+}\)-activated K\(^{+}\) channel KCa3.1 plays an important role in the control of proinflammatory cytokine production and secretion by regulating Ca\(^{2+}\) signaling in lymphoid and myeloid cells (Di et al., 2010; Ohya et al., 2014); however, it currently remains unclear whether KCa3.1 contributes to IL-10–induced escape from cancer immune surveillance. The present study showed that the pharmacological blockade of KCa3.1 by 1 μM TRAM-34 did not evoke depolarization responses in human T-cell lymphoma HuT-78 cells under resting conditions (Fig. 1, C and D). These results suggest that KCa3.1 is rarely active under resting conditions in HuT-78 cells. In support of this hypothesis, IL-10 transcription was not affected by a single treatment with TRAM-34; however, TRAM-34 blocked KCa3.1 activator–induced decreases in IL-10 expression and secretion (Fig. 2, B and D). Using a whole-cell patch-clamp recording in current- and voltage-clamp modes, SKA-31–induced hyperpolarization and current activation were almost completely inhibited following the application of 1 μM TRAM-34 (Supplemental Fig. S2). The main results of the present study are: 1) IL-10 expression and secretion were inhibited in HuT-78 cells treated with KCa3.1 activators (Fig. 2); 2) KCa3.1 activators reduced the phosphorylation of Smad2 and its nuclear translocation in HuT-78 cells (Figs. 4 and 5), resulting in the partial
During the last decade or two, several transcription factors of IL-10 have been identified in immune cells: E4BP4, GATA3, cMAF, and Blimp1 (Shoemaker et al., 2006; Motomura et al., 2011; Male et al., 2012; Heinemann et al., 2014; Rutz and Ouyang, 2016; Xu et al., 2018). As shown in Fig. 3, no changes in the expression levels of these transcripts by treatments with KCa3.1 activators for 12 and 24 hours (not shown). Alternatively, the proinflammatory cytokine IL-32, which is detected in sera from patients with Crohn disease and rheumatoid arthritis, increases IL-10 transcription (Kang et al., 2011; Male et al., 2012; Heinemann et al., 2014; Rutz and Ouyang, 2016). In HuT-78 cells, IL-32 transcripts were expressed at a high level (approximately 10-fold that of IL-10); however, it was not affected by longer treatments (Yanginlar and Logie, 2018). However, the expression levels of histone deacetylase 11 transcripts were extremely low in HuT-78 cells (less than 0.0001 relative to ACTB; not shown). These results suggest that these transcriptional and post-translational modulators are not involved in the KCa3.1 activator–induced transcriptional repression of IL-10.

Recent studies showed that KCa3.1 blockade attenuated pulmonary, renal, and corneal fibrosis by mediating Smad2/3 signaling (Huang et al., 2013, 2018; Roach and Wulff, 2014; Roach et al., 2015; Anumanthan et al., 2018). The resultant decreases in Ca\textsuperscript{2+} influx prevented the phosphorylation of Smad2 proteins in fibroblasts, and thus, the expression levels of histone deacetylase 11 transcripts were extremely low in HuT-78 cells (less than 0.0001 relative to ACTB; not shown). These results indicate that KCa3.1 is a negative regulator of Smad2/3 signaling in fibroblasts. In fibroblasts, and thus, the expression levels of the downstream candidate for the transcriptional repression of IL-10 (Yanginlar and Logie, 2018). However, the expression levels of histone deacetylase 11 transcripts were extremely low in HuT-78 cells (less than 0.0001 relative to ACTB; not shown). These results suggest that these transcriptional and post-translational modulators are not involved in the KCa3.1 activator–induced transcriptional repression of IL-10.

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Effects of the CaMKII inhibitor KN-62 (10 \mu M) on KCa3.1 activator–induced reductions in the nuclear translocation of P-Smad2 in HuT-78 cells. (A) Whole protein lysates of DCEBIO (1 \mu M)- and SKA-31 (1 \mu M)-treated HuT-78 cells in the presence of KN-62 were probed by immunoblotting with anti-Smad2/Smad3 (a, upper panel), anti-P-Smad2 (b, upper panel), anti-P-Smad3 (c, upper panel), and anti-ACTB antibodies (a–c, lower panels). (B and C) Summarized results of the relative expression of P-Smad2/Smad2 and P-Smad3/Smad3 were obtained from the optical density of Smad2/3, P-Smad2, P-Smad3, and ACTB band signals (n = 5 for each). Results are expressed as means ± S.D.

Fig. 7. Effects of the CaMKII inhibitor KN-62 (10 \mu M) on KCa3.1 activator–induced reductions in the phosphorylation of Smad2 proteins in HuT-78 cells. (A) Whole protein lysates of DCEBIO (1 \mu M)- and SKA-31 (1 \mu M)-treated HuT-78 cells in the presence of KN-62 were probed by immunoblotting with anti-Smad2/Smad3 (a, upper panel), anti-P-Smad2 (b, upper panel), anti-P-Smad3 (c, upper panel), and anti-ACTB antibodies (a–c, lower panels). (B and C) Summarized results of the relative expression of P-Smad2/Smad2 and P-Smad3/Smad3 were obtained from the optical density of Smad2/3, P-Smad2, P-Smad3, and ACTB band signals (n = 5 for each). Results are expressed as means ± S.D.

Fig. 8. Effects of the CaMKII inhibitor KN-62 (10 \mu M) on KCa3.1 activator–induced reductions in the nuclear translocation of P-Smad2 in HuT-78 cells. (A) Confocal fluorescent images of Alexa Fluor 488–labeled P-Smad2 and P-Smad3 in vehicle-, DCEBIO (1 \mu M)-, and SKA-31 (1 \mu M)-treated HuT-78 cells in the presence of KN-62. Nuclear morphologies were shown by 4′,6-diamidino-2-phenylindole (DAPI) images. Thick and thin dashed lines show the nuclear boundary and plasma membrane, respectively. (B and C) Summarized results of the expression levels of P-Smad2 (B) and P-Smad3 (C) in the nuclei of HuT-78 cells. The mean fluorescent intensities of nuclear-cytoplasmic ratios were calculated. Results are expressed as means ± S.D. Cell numbers used for the experiments are shown in parentheses.
contributes to the constitutive facilitation of Smad2/3 signaling (Mori and Prager, 1997; Tiffon et al., 2011); however, resting KCa3.1 activity was very low (Fig. 1, C and D). Consistent with our results, previous studies reported that the inhibition of Smad2/3 signaling was strongly dependent on CaMKII activation, and CaMKII blocked the nuclear accumulation of Smad2 and prevented Smad2-Smad3 interactions (Zimmerman et al., 1998; Wicks et al., 2000). The present results provide novel evidence to suggest that KCa3.1 activators successfully inhibit constitutively active Smad2/3 signaling via CaMKII activation in IL-10-producing tumor-infiltrating lymphocytes and lymphoma.

Signal transducers and activators of transcription 3 (STAT3) and PI3K/AKT/mammalian target of rapamycin (mTOR) signaling pathways are both involved in the regulation of IL-10 expression (Braunschweig et al., 2011; Male et al., 2012; Zhu et al., 2015; Huang et al., 2016). STAT3 and mTOR inhibition reduced IL-10 expression in natural killer cells (Male et al., 2012) and peripheral dendritic cells (Weichhart et al., 2008), respectively. KCa3.1 blockade has been shown to inhibit the TGF-β1 signaling pathway through PI3K/AKT/mTOR signaling pathways (Huang et al., 2016), whereas PI3K/AKT/mTOR signaling positively and negatively regulated STAT3 and NF-κB, respectively (Weichhart et al., 2008). Furthermore, a positive feedback loop may operate to maintain strong STAT3 activity and IL-10 expression. IL-10 expression was reduced by treatment with the NF-κB inhibitor Bay 11-7082 (1 μM) (Supplemental Fig. S6B); however, no changes were observed in its expression following a treatment with the STAT3 inhibitor 5,15-DPP (20 μM), the mTOR inhibitor everolimus (10 nM), and the AKT inhibitor AZD 5363 (1 μM) for 6 (Supplemental Fig. S6C), 12, and 24 hours (not shown) in HuT-78 cells. These results suggest that STAT3 and PI3K/AKT/mTOR signaling pathways do not play an essential role in KCa3.1 activator–induced transcriptional repression in HuT-78 cells.

In conclusion, the present study provided a new approach to IL-10 regulation in cancer immunotherapy. The results obtained clearly showed that IL-10 is a downstream gene of Smad2/3 signaling in HuT-78 cells, and its production is negatively regulated by KCa3.1 activation, which blocks the nuclear accumulation of constitutively active Smad2 through CaMKII signaling. KCa3.1 activators are a possible therapeutic option to suppress the tumor-promoting activities of IL-10.

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Authorship Contributions

**Participated in research design:** Matsui, Ohya.
**Conducted experiments:** Matsui, Kajikuri, Kito, Endo, Hasegawa, Murata, Ohya.
**Performed data analysis:** Matsui, Kajikuri, Kito, Endo, Hasegawa, Murata, Ohya.
**Wrote or contributed to the writing of the manuscript:** Matsui, Kajikuri, Kito, Ohya.

**References**


Address correspondence to: Dr. Susumu Ohya, Department of Pharmacology, Graduate School of Medical Sciences, Nagoya City University, Nagoya 467-8601, Japan. E-mail: sohya@med.nagoya-cu.ac.jp