Protein kinase C–dependent activation of $Ca_V1.2$ channels selectively controls human T_H2-lymphocyte functions

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Background: In addition to calcium release–activated calcium channel/ORAI calcium channels, the role of voltage-gated calcium (Ca_v1) channels in T-cell calcium signaling is emerging. Ca_v1 channels are formed by α1 ($Ca_V1.1$ to $Ca_V1.4$) and auxiliary subunits. We previously demonstrated that mouse T_H2 cells selectively overexpressed $Ca_V1.2$ and $Ca_V1.3$ channels. Knocking down these channels with Ca_v1 antisense (AS) oligonucleotides inhibited T_H2 functions and experimental asthma.

Objective: We investigated the expression profile and role of Ca_v1 channels in human T-cell subsets, with a focus on T_H2 cells.

Methods: We compared the profile of Ca_V1 channel subunit expression in T-cell subsets isolated *ex vivo* from the blood of healthy donors, as well as *in vitro*–polarized T-cell subsets, and tested the effect of the Ca_v1 inhibitors nicardipine and $Ca_v1.2$AS on their functions.

Results: $Ca_V1.4$ expression was detectable in $CD4^+$ T cells, *ex vivo* T_H1 cells, and T_H17 cells, whereas $Ca_v1.2$ channels predominated in T_H2 cells only. T-cell activation resulted in $Ca_v1.4$ downregulation, whereas $Ca_v1.2$ expression was selectively maintained in polarized T_H2 cells and absent in T_H1 or T_H9 cells. Nicardipine and $Ca_V1.2$AS decreased Ca^{2+} and cytokine responses in T_H2, but not T_H1, cells. Protein kinase C (PKC) α/β inhibition decreased Ca^{2+} and cytokine responses, whereas both calcium and cytokine responses induced by PKC activation were inhibited by nicardipine or $Ca_v1.2$AS in T_H2 cells.

Conclusion: This study highlights the selective expression of $Ca_v1.2$ channels in human T_H2 cells and the role of PKC-dependent $Ca_v1.2$ channel activation in T_H2 cell function. Blocking PKC or $Ca_v1.2$ channel activation in T_H2 cells might represent new strategies to treat allergic diseases in human subjects. (J Allergy Clin Immunol 2014;133:1175-83.)

Key words: *Chemoattractant receptor-homologous molecule expressed on T_H2 cells, asthma, calcium, voltage-dependent calcium channels, T_H2 cytokines, protein kinase C α/β*

T-cell receptor (TCR) engagement induces the formation of inositol 1, 4, 5-triphosphate[1] binding to its receptor at the membrane of the endoplasmic reticulum (ER), leading to the release of Ca^{2+} from the ER into the cytosol. The ER Ca^{2+} sensor stromal interaction molecules sense the decreased ER Ca^{2+} concentration and activate the ORAI calcium channels at the plasma membrane.[2] ORAI channels then open and sustain the Ca^{2+} influx needed for full-blown T-cell activation.[3-5] However, other calcium channels[1] are likely to contribute to Ca^{2+} entry in T lymphocytes, including voltage-dependent Ca_v1 channels also defined as dihydropyridine receptors. These consist of the Ca^{2+}-forming pore α1 subunit encoded by 4 genes ($Ca_v1.1$ to $Ca_v1.4$) and the $Ca_vβ$ ($Ca_vβ1$ to $Ca_vβ4$) and $Ca_vα2$-δ ($Ca_vα2$-δ1 to $Ca_vα2$-δ4) auxiliary subunits.[6] The inhibitory effect of dihydropyridine antagonists on the Ca^{2+} response, the detection of Ca_v1 mRNA–related products in T cells, and the immune phenotype observed in mice with genetic ablation of $Ca_v1.4$,[7] $Ca_vβ3$, or $Ca_vβ4$ subunits[8,9] support a role for Ca_v1 channels in T-cell function.[10,11] $Ca_v1.1$-, $Ca_v1.2$-, and/or $Ca_v1.3$-related channels were also reported as implicated in calcium signaling of T lymphocytes, raising the question of the respective contribution of each isoform in human and murine T-cell activation.[12-14] Furthermore, patients with Timothy syndrome caused by mutated $Ca_v1.2$, which is responsible for a gain of function of the channel and display, among other disorders, increased susceptibility to recurrent infections.[15,16] Correct cell functions depend on a tight regulation of intracellular Ca^{2+} levels, and both loss and gain of function of Ca_v1 were shown to induce alterations in cell function.[17] Our group previously showed that mouse T_H2 lymphocytes producing IL-4, IL-5, and IL-13 and orchestrating allergic diseases overexpress $Ca_v1.2$ and $Ca_v1.3$ channels compared with T_H1 or $CD4^+$ T cells.[18] T_H2 cells transfected with Ca_v1 antisense (AS) oligodeoxynucleotides (ODNs; Ca_v1AS) displayed impaired TCR-driven Ca^{2+} responses and cytokine production. In addition, Ca_v1AS inhalation prevented the development of experimental asthma.[18] We then looked for the expression profile of Ca_v1 channel isoforms in human $CD4^+$ T-cell subsets with a focus on T_H2 cells. We show that human T_H2, but not T_H1, T_H17, or T_H9, cells overexpress $Ca_v1.2$ channels, which play a critical role in calcium signaling and T_H2 cytokine production.

METHODS

Methods for human blood cell subset isolation, generation of T_H subset cells, RNA preparation and reverse transcription, cell proliferation, intracellular cytokine staining, cytokine determination, and fluorescence microscopy are described in the Methods section in this article's Online Repository at www.jacionline.org.

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Abbreviations used
AS: Antisense
$[Ca]_i$: Intracellular calcium concentration
Ca_v1: Voltage-dependent calcium channels
CFSE: Carboxyfluorescein succinimidyl ester
CRTH2: Chemoattractant receptor-homologous molecule expressed on T_H2 cells
ER: Endoplasmic reticulum
FITC: Fluorescein isothiocyanate
NFAT: Nuclear factor of activated T cells
ODN: Oligodeoxynucleotide
PE: Phycoerythrin
PKC: Protein kinase C
PMA: Phorbol 12-myristate 13-acetate
qPCR: Quantitative PCR
TCR: T-cell receptor

Real-time quantitative PCR

Primer sequences are listed in Table E1 in this article's Online Repository at www.jacionline.org. Transcripts were measured by using real-time quantitative PCR (qPCR) with the LightCycler 480 Instrument (Roche Diagnostics, Mannheim, Germany) and expressed as arbitrary units relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrognase ($2^{-[Ct\ interest\ gene\ -\ Ct\ GAPDH]}$) $\times\ 10^{-6}$ or 10^{-5}. Ct is defined as the numbers of cycle for which fluorescence is detectable, and a value of less than 1.5 was the limit of PCR product detection.

Antisense transfection experiments

Cells were transfected with 8 μmol/L $Ca_V1.2$AS or sense oligonucleotides in the presence of Turbofect (Fermentas; Thermo Fisher Scientific, Uppsala, Sweden), according to the manufacturer's recommendations.

Intracellular calcium measurements

The fluorescence (F_{340}/F_{380}) was measured at an emission wavelength of 510 nm for excitation at wavelengths of 340 and 380 nm at the single-cell level in Fura2-AM–loaded cells, as previously described.[19] The F_{340}/F_{380} ratio was indicative of intracellular calcium ($[Ca]_i$). Resting cells were recorded for approximately 1 minute before adding α-CD3/α-CD28–coated beads (5 beads per cell) or phorbol 12-myristate 13-acetate (PMA; 200 ng/mL). The response was then recorded for 15 minutes before the addition of ionomycin (10 μmol/L), which was used as a positive control. Images were analyzed with MetaFluor imaging software. Data are always expressed as the F/F0 ratio ($F = F_{340}/F_{380}$ in stimulated T cells and $F0 = F_{340}/F_{380}$ under basal conditions) and represent the mean of at least 25 cells. The area under the curve over the mean + 2 SDs of the baseline ratio was measured with GraphPad Prism (GraphPad Software, La Jolla, Calif). Cells for which the F_{340}/F_{380} ratio was superior to the mean + 2 SDs of basal levels were considered responsive cells.

Statistical analysis

Results were expressed as the mean + 1 SD. The significance of differences was calculated by using paired or unpaired nonparametric or parametric tests depending on the number of samples. A P value of less than .05 was considered significant.

RESULTS

Resting and activated human T lymphocytes express all the subunits required to form functional Ca_v1 channels

Analyzing the expression of $Ca_v1\alpha1$ subunits in immune cells from the peripheral blood of healthy donors revealed that $Ca_v1.4$ predominated in lymphocytes at steady state (Fig 1, *A*). $Ca_v1.2$ transcripts were expressed in $CD4^+$ and, to a lesser degree, $CD8^+$ T lymphocytes. $Ca_v1.3$ was detected in neutrophils and $CD4^+$ and $CD8^+$ T lymphocytes and highly expressed in eosinophils (Fig 1, *A*). $Ca_v1.1$ transcripts were not detected in any hematopoietic cell subsets (data not shown).

Among $CD4^+$ T cells, expression of $Ca_v1.4$ was higher in $CD4^+CD45RA^+$ naive than in $CD4^+CD45RA^-$ memory T cells, whereas $Ca_v1.2$ and $Ca_v1.3$ were weakly expressed in both subsets (Fig 1, *B*). The stimulation of $CD4^+$ T cells with α-CD3/α-CD28–coated beads downregulated $Ca_v1.4$ transcripts. By contrast, the expression of $Ca_v1.2$ and $Ca_v1.3$ transcripts was maintained or even upregulated on TCR stimulation (Fig 1, *C*).

Resting $CD4^+$ T lymphocytes expressed mainly transcripts coding for β1, β3, and α2δ2 auxiliary subunits (Fig 1, *D*), and their expression tends to increase after TCR engagement (Fig 1, *D*). These data indicate that all the subunits required to compose functional Ca_v1 channels are expressed in resting and activated $CD4^+$ T lymphocytes and that TCR engagement controls their expression levels.

Nicardipine decreases Ca^{2+} response and cytokine production without affecting $CD4^+$ T-cell proliferation

Nicardipine, a dihydropyridine antagonizing Ca_v1 channels, affects neither the survival (data not shown) nor the proliferative response induced by TCR stimulation of $CD4^+$ cells, as shown by using a carboxyfluorescein succinimidyl ester (CFSE) dilution assay (Fig 2, *A*) or based on thymidine uptake (data not shown). TCR cross-linking induced a rapid $[Ca]_i$ increase, followed by a plateau in $CD4^+$ T cells, which was strongly diminished by nicardipine (Fig 2, *B*). The residual TCR-driven $[Ca]_i$ increase in nicardipine-treated cells was only 26% of the values observed in untreated $CD4^+$ cells (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). Nicardipine also reduced the frequency of IL-2–, IFN-γ–, and IL-4–producing cells (Fig 2, *C* and *D*) and cytokine secretion (Fig 2, *E*) after TCR stimulation. These data suggest that Ca_v1 channels participate in the calcium response and cytokine production of $CD4^+$ T cells.

Ex vivo sorted T_H1, T_H2, and T_H17 cell subsets express distinct sets of Ca_v1 channels that might contribute to T-cell functions

On the basis of the expression of cell-surface markers, T_H1 ($CD4^+CD45RA^-CXCR3^+CCR6^-$), T_H17 ($CD4^+CD45RA^-CCR6^+$), and T_H2 ($CD4^+$ chemoattractant receptor-homologous molecule expressed on T_H2 cells $[CRTH2]^+$) cells were *ex vivo* sorted from healthy donor PBMCs (Fig 3, *A* and *B*). These subsets represented 8% to 20%, 15% to 30%, and 0.2% to 3% of $CD4^+$ T cells and were characterized by the selective expression of *IFNG*, *IL17*, and *IL4* transcripts, respectively (Fig 3, *C*). $Ca_v1.4$ was the sole isoform detected in *ex vivo* T_H1 and T_H17 subsets (Fig 3, *D*). Interestingly, $CD4^+CRTH2^+$ cells predominantly expressed $Ca_v1.2$ and, to a lesser degree, $Ca_v1.3$ channels, which were not detected in T_H1 and T_H17 lymphocytes (Fig 3, *D*). Nicardipine reduced subset-specific cytokine production by $CRTH2^+$, $CCR6^+$ T_H17, and $CXCR3^+CCR6^-$ T_H1 (Fig 3, *E*) cells, suggesting a functional role of Ca_v1 channels in these cells.