Original Paper

AMPK α 1-Sensitivity of Orai1 and Ca²⁺ Entry in T - Lymphocytes

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Key Words

AMP-activated protein kinase • Calcium • Orai1 • T lymphocytes

Abstract

Background/Aims: T-lymphocyte activation and function critically depends on Ca²⁺ signaling, which is regulated by store operated Ca²⁺ entry (SOCE). Human and mouse T lymphocytes express AMP activated kinase AMPK α 1, which is rapidly activated following elevation of cytosolic Ca²⁺ concentration ([Ca²⁺]_i) by treatment of the cells with Ca²⁺ ionophore or following inhibition of endosomal Ca²⁺ ATPase with thapsigargin. AMPK is further activated by triggering of the T cell antigen receptor (TCR). The present study explored whether AMPK influences Ca²⁺ entry and Ca²⁺-sensitive regulation of T-lymphocyte function. **Methods:** T-lymphocytes were isolated and cultured from AMPK α 1-deficient (*ampk*^{-/-}) mice and from their wild-type (*ampk*^{+/+}) littermates. The phenotype of the cells was analysed by flow cytometry, [Ca²⁺]_i estimated from Fura-2 fluorescence, SOCE from increase of [Ca²⁺]_i following thapsigargin treatment (1 μ M), and cell function analysed by measuring cytokine secretion and western blotting. **Results:** Expression of surface markers in CD4⁺ and CD8⁺ T-cells were similar in *ampk*^{-/-} and *ampk*^{+/+} T-lymphocyte blasts. Moreover, total STIM1 protein abundance was similar in *ampk*^{-/-} and *ampk*^{+/+} T-lymphocyte blasts. However, Orai1 cell membrane protein abundance was significantly higher in *ampk*^{-/-} than in *ampk*^{+/+} T-lymphocyte blasts. SOCE and increase of [Ca²⁺]_i following TCR activation by triggering TCR with anti-CD3 and cross-linking secondary antibody were both significantly more pronounced in *ampk*^{-/-} than in *ampk*^{+/+} T-lymphocyte blasts. The difference of Ca²⁺ entry between *ampk*^{-/-} and *ampk*^{+/+} T-lymphocytes was abrogated by Orai1 inhibitor 2-aminoethoxydiphenyl borate (2-APB, 50 μ M). Proliferation of unstimulated *ampk*^{-/-} lymphocytes was higher than proliferation of *ampk*^{+/+} T-lymphocytes, a difference reversed by Orai1 silencing. **Conclusions:** AMPK downregulates Orai1 and thus SOCE in T-lymphocytes and thus participates in negative feed-back regulation of cytosolic Ca²⁺ activity.

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Introduction

AMP-activated protein kinase (AMPK), a kinase sensing cellular energy status, is activated by increased intracellular AMP/ATP ratio. Several functions of AMPK restore energy balance by inhibiting ATP-consuming processes and stimulating ATP-generation [1]. The stimulation of AMPK by an increase in the AMP/ATP ratio requires the phosphorylation of Thr-172 by the kinase LKB1 [2, 3]. AMPK is further stimulated by Sirtuins [4] and adenosine [5]. Moreover, AMPK is activated by Ca^{2+} /calmodulin-dependent kinase kinase- β (CaMKK) following K^{+} -induced depolarization or treatment of cells with Ca^{2+} ionophore [6-8]. AMPK participates in the response to ischemia [9], cardiac pressure overload, [10], lymphocyte metabolism and function [11] and modulation of inflammation [12].

AMPK α 1 but not AMPK α 2 is expressed in human and mouse T lymphocytes, where it is rapidly activated in response to triggering of the T cell antigen receptor (TCR) [13]. TCR stimulation of AMPK was dependent on the adaptors LAT and SLP76 and could be mimicked by the elevation of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) with Ca^{2+} ionophores or thapsigargin [13].

$[\text{Ca}^{2+}]_i$ plays a pivotal role in the regulation of lymphocyte function [14] and survival [15-17]. During T lymphocyte activation, TCR stimulation is followed by phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate with generation of inositol-1,4,5-trisphosphate (IP3) and polyunsaturated diacylglycerol (DAG) [14]. IP3 triggers the release of Ca^{2+} from intracellular stores, which is followed by activation of store operated Ca^{2+} entry and increase in $[\text{Ca}^{2+}]_i$ [14]. The increase of $[\text{Ca}^{2+}]_i$ is decisive during the initial phases of T cell activation, particularly for the production of effector cytokines [18, 19]. Ca^{2+} regulated functions in T-cells include the phosphatase calcineurin, which regulates NFAT transcription factors that control cytokine gene expression [19-23].

Channels accomplishing Ca^{2+} entry into lymphocytes involve Ca^{2+} -release activated Ca^{2+} channels [14], composed of the pore-forming units Orai 1, 2 or 3 [24-26] and their regulators STIM 1 or 2, which are activated by emptying of the intracellular Ca^{2+} stores [27-31]. Most recent observations revealed the ability of AMPK to downregulate Orai1 in dendritic cells [32]. AMPK has previously been shown to regulate ion channels [33, 34] including the Ca^{2+} channel Orai [32].

Phenotypic features of AMPK α 1 deficient mice include accelerated erythrocyte death [35-37], increased bone remodelling and reduced bone mass [38-40], increased skeletal muscle mass [41-43], altered endothelial and vascular functions [44-48], altered epithelial transport [49, 50], decreased insulin release [51], enhanced susceptibility to insulin resistance and obesity [52], asthenozoospermia [53] as well as altered circadian rhythm [54]. Despite altered function of AMPK α 1 deficient dendritic cells [32], AMPK α 1 deficient mice had no striking defects in immunocompetence and displayed normal cell proliferation, humoral, cytotoxic and delayed-type hypersensitivity (DTH) responses following antigen injection [55]. A role of AMPK in the regulation of Ca^{2+} entry into T-lymphocytes has, however, to the best of our knowledge, not been reported.

The present study thus explored the role of AMPK on Ca^{2+} signaling in T-lymphocytes. To this end, experiments were performed in T-lymphocytes isolated from AMPK α 1-deficient (*ampk* $^{-/-}$) mice and from their wild-type (*ampk* $^{+/+}$) littermates.

Materials and Methods

Mice

All animal experiments were conducted according to the guidelines of the American Physiological Society. CTLs were isolated from AMPK α 1-deficient (*ampk* $^{-/-}$) mice and, as control, from their wild-type (*ampk* $^{+/+}$) littermates. The *ampk* $^{-/-}$ mice have been described previously [56]. All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities.

Splenic T-lymphocyte culture

T lymphocytes were cultured from splenic T-cells as described earlier [57]. For activation of primary naive T cells, spleens from wildtype mice were disaggregated following red blood cell lysis. Cells were cultured in RPMI-1640 medium containing L-glutamine (Life Technologies GmbH, Darmstadt, Germany), 10% heat-inactivated FBS (PAA, Pasching, Austria), 50 μM β -mercaptoethanol (AppliChem, Darmstadt, Germany) and 1% penicillin-streptomycin (PAA). Single-cell suspensions from splenocytes adjusted to a density of 5×10^6 cells/ml were stimulated with monoclonal anti-CD3 (5 $\mu\text{g}/\text{ml}$; 145-2C11, R&D Systems) to 'trigger' the T-cell receptor (TCR). For generation of mature cytotoxic T-lymphoblasts, mouse T cells grown from spleen preparations cultured for 48 h in the presence of stimulus (monoclonal antibody 2C11) were washed and resuspended at a density of 4×10^5 cells per ml with IL-2 (0.02 $\mu\text{g}/\text{ml}$; 360 IU/ml, BD Biosciences, Heidelberg, Germany) for 72h to generate mature CTL blasts. After activation and clonal expansion mature cytotoxic T-cell blasts were characterized phenotypically by flow cytometry. As a result, more than 80% of the cells were positive for CD8⁺. Cells were further maintained in medium containing IL-2 (0.02 $\mu\text{g}/\text{ml}$; 360 IU/ml, BD Biosciences). Cells were counted each day, and maintained to the optimum cell density with IL-2 supplementation in the medium.

Flow cytometric analysis and phenotyping of the cells

Mature T-cell blasts were analysed with standard multicolor flow cytometry [58, 59] and commercially available specific fluorescence conjugated antibodies as follows: Fluorescein isothiocyanate (FITC) or Phycoerythrin (PE) conjugated anti-CD4, Allophycocyanin (APC) or Cyanine 5.5 (Cy5.5) conjugated anti-CD8, PE conjugated anti-CD25, PE conjugated anti-CD62L, APC conjugated anti-CD25, PE conjugated anti-CD98, PE-anti-CD71 and APC anti-TCR β (BD Biosciences). A minimum of 2×10^5 cells were washed and stained for 30 min at 4 °C with saturating concentrations of antibody in RPMI-1640 medium (Life Technologies GmbH) and 0.5% FBS (PAA). Cells were washed and resuspended in RPMI-1640 medium (Life Technologies GmbH) and 0.5% FBS (PAA) before being analysed with a FACS Calibur (BD Biosciences). A minimum of 5×10^4 relevant events were measured and stored ungated. Live cells (> 90% of total events) were gated according to their forward scatter and side scatter. Data was analyzed using Cell Quest Pro software (BD Biosciences).

Measurement of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$)

Fluorescence measurements were carried out with an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany). Cells were excited alternatively at $\lambda = 340$ or 380 nm and the light deflected by a dichroic mirror into either the objective (Fluar 40 $\times$ /1.30 oil, Zeiss, Oberkochen, Germany) or a camera (Proxitronic, Bensheim, Germany). Emitted fluorescence intensity recorded at $\lambda = 505$ nm and data acquisition accomplished by using specialized computer software (Metafluor, Universal Imaging, Downingtown, USA).

$[\text{Ca}^{2+}]_i$ increase upon T-cell receptor activation was determined in activated T-cell blasts as described earlier [60-62]. T lymphocytes were incubated with anti-CD3 (1 $\mu\text{g}/\text{ml}$; 145-2C11, R&D Systems) for 30 min on ice and subsequently loaded with Fura-2AM (2 μM , Molecular Probes, Goettingen, Germany) for 15 min at 37°C. Intracellular Ca^{2+} was measured prior to and following addition of Goat Anti-Syrian Hamster IgG (2 $\mu\text{g}/\text{ml}$, Thermo Scientific, Bonn, Germany) to the Ringer solution (see below).

To measure SOCE, changes in $[\text{Ca}^{2+}]_i$ were monitored on depletion of the intracellular Ca^{2+} stores. Experiments were carried out prior to and during exposure of the cells to Ca^{2+} -free solution (see below). In the absence of Ca^{2+} , the intracellular Ca^{2+} stores were depleted by inhibition of the vesicular Ca^{2+} pump by thapsigargin (1 μM , Molecular Probes). Readdition of Ca^{2+} allowed assessing the SOCE. Where indicated, experiments were performed in the absence or presence of SOCE inhibitor 2-Aminoethoxydiphenyl borate (2-APB, 50 μM , Sigma-Aldrich, Schnellendorf, Germany) for 30 min at 37°C. Experiments were performed in Ringer solution containing (in mM/l): 125 NaCl, 5 KCl, 1.2 MgSO_4 , 2 CaCl_2 , 2 Na_2HPO_4 , 32 HEPES, and 5 glucose at pH 7.4. Ca^{2+} -free solutions contained (in mM/l): 125 NaCl, 5 KCl, 1.2 MgSO_4 , 2 Na_2HPO_4 , 32 Hepes, 0.5 EGTA, 5 glucose, pH 7.4.

Western Blotting

The expression levels of proteins were analyzed by Western blotting. In brief, 4×10^7 cells were washed twice with ice cold phosphate-buffered saline (PBS, PAA) and cells were lysed with cell lysis buffer (20 mM tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA , 1 mM EGTA, 1% triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ leupeptin and 1 mM PMSF, added immediately prior to use). The extracts were centrifuged at 14,000 g for 10 min at 4 °C to remove insoluble material. The protein concentration

of the supernatant was determined and 1:5 Laemmli sample buffer added. 50–100 μg of protein were solubilized in Laemmli sample buffer at 95°C for 5 min and resolved by 10% SDS-PAGE. For immunoblotting proteins were electro-transferred onto a nitrocellulose membrane and blocked with 5% nonfat milk in TBS-0.1% Tween 20 (TBST) at room temperature for 1 hour. Then, the membrane was incubated with affinity purified anti-AMPK- α antibody (1:1000, Cell Signaling Technology, MA, USA) or with anti-Orai1 antibody (1:1000, Abcam, Cambridge, UK) or with STIM1 antibody (1:1000, Cell Signaling Technology) at 4°C overnight. After washing 3 times with TBST (10 min each) the blots were incubated with horseradish peroxidase conjugated secondary anti-rabbit antibody (1:2000; Cell Signaling Technology) for 1 hour at room temperature. After washing antibody binding was detected with the ECL detection reagent (Cell Signaling Technology). For loading control the same membranes were stripped with stripping buffer (Millipore, Billerica, MA, USA) and reblotted with rabbit anti- α/β -Tubulin antibody (1:1000, Cell Signaling Technology), with rabbit anti-GAPDH antibody (1:1000, Cell Signaling Technology) or with rabbit anti-beta-Actin antibody (1:1000, Cell Signaling Technology). Antibody-binding was quantified with Quantity One Software (Biorad, München, Germany).

Western Blotting of membrane proteins

Membrane proteins were isolated by membrane protein extraction kit [ThermoScientific, Rockford, IL, USA] according to the manufacturers instructions. The isolated membrane protein was subjected to western blotting as detailed above utilizing anti-Orai1 antibody (1:1000, Abcam) followed by re-blotting with anti-actin antibody (1:1000, Santa Cruz Biotech, Heidelberg, Germany) as loading control.

siRNA Transfection

Cells were electroporated using the Amaxa Mouse T Cell Nucleofector Kit (Lonza, Cologne, Germany) according to the manufacturer's instructions. Briefly, 1×10^6 cells were suspended in 100 μl of Nucleofactor solution. After addition of control siRNA (10 or 50 nM, Santa Cruz Biotech) or Orai1 siRNA (10 or 50 nM, Santa Cruz Biotech), cells were electroporated using a Nucleoporation (Lonza) Amaxa program X-001 and were immediately plated in 12-well plates containing complete media and incubated for 24h at 37°C in a humidified 5% CO_2 chamber.

Proliferation assay/MTS assay

The proliferation assay was performed in 96-well plates. Where indicated 2×10^5 cells were either stimulated with pre-coated (5 $\mu\text{g}/\text{ml}$) CD3 mAb (R&D Systems) and 2 $\mu\text{g}/\text{ml}$ CD28 mAb (BD Biosciences) [63] or non-stimulated or transfected with siRNA 24h prior to the assay. The Cell Titer 96 AQueous non radioactive assay was used for the measurement of cell proliferation using tetrazolium compounds according to the manufacturer's instructions (Promega, WI, USA). 24h after cells were placed in the 96-well plate, 20 μl of the MTS/PMS solution was added directly to each well and incubated for 4h. The absorbance was measured at 490 nm with a reference wavelength at 690 nm using an ELISA plate reader (PowerWave XS2, BioTek, VT, USA) [60-62].

Statistical analysis

Data are provided as means $\pm$ SEM, n represents the number of independent experiments. All data were tested for significance using paired or unpaired Student t-test or ANOVA. GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, USA) was used. $p < 0.05$ was considered statistically significant.

Results

As published earlier [13], human and mouse T-lymphocytes express AMPK α 1, which is rapidly activated in response to triggering of the T-cell antigen receptor (TCR). As illustrated in Fig. 1, AMPK α staining could be detected in T-lymphocytes from wildtype mice (*ampk*^{+/+}) but not in T-lymphocytes lacking AMPK α 1 (*ampk*^{-/-}). The loading control GAPDH was similarly expressed in both genotypes (Fig. 1A). The mature IL2-T-lymphocyte blasts were phenotyped by flow cytometry and % of CD4 and CD8 positive cells determined. As shown in Fig. 1B there was no difference between genotypes in the lymphocyte phenotype or in the percentage CD4 and CD8 cells (Fig. 1C).

Fig. 1. AMPK α expression, phenotype and abundance of CD8⁺ and CD4⁺ cells in *ampk*^{-/-} and *ampk*^{+/+} T-lymphocyte preparations. **A.** Original western blot of the expression of total AMPK α protein (upper panel) and of GAPDH as loading control (lower panel) in *ampk*^{-/-} and *ampk*^{+/+} T-lymphocytes. **B.** Analysis of mature cytotoxic T cell blasts with standard multicolor flow cytometry settings and commercially available specific fluorescence conjugated antibodies - Fluorescein isothiocyanate (FITC) or Phycoerythrin (PE) conjugated anti-CD4, Allophycocyanin (APC) or Cyanine 5.5 (Cy5.5) conjugated anti-CD8, PE conjugated anti-CD25, PE conjugated anti-CD62L, APC conjugated anti-CD25, PE conjugated anti-CD98, PE-anti-CD71 and APC anti-TCR β . Live cells (> 90% of total acquired events) were gated according to their forward scatter and side scatter. The cells were again gated for CD8 and CD4 positive cells and respective fluorescence calculated for cells in CD8 and CD4 gate. Data were analyzed using Cell Quest Pro software. Phenotype of CD8⁺ (left) and CD4⁺ (right) cells. Black line indicates *ampk*^{+/+} CTLs and grey filled histogram indicates *ampk*^{-/-} T-lymphocytes. **C.** Arithmetic means $\pm$ SEM (n = 6) of percent CD4⁺ and CD8⁺ cells in *ampk*^{-/-} and *ampk*^{+/+} T-lymphocyte preparations.

Fura-2 fluorescence was employed to determine intracellular Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) and store operated Ca^{2+} entry (SOCE) into T-lymphocytes from *ampk*^{-/-} and *ampk*^{+/+} mice (Fig. 2A,B). In nominally Ca^{2+} free extracellular solution the Fura-2 fluorescence ratio reflecting $[\text{Ca}^{2+}]_i$ was similar in *ampk*^{-/-} (0.41 ± 0.07 a.u., n = 9) and *ampk*^{+/+} (0.40 ± 0.06 a.u., n = 10) T-lymphocytes. The Ca^{2+} stores were depleted by sarco-/endoplasmic reticulum Ca^{2+} /ATPase (SERCA) inhibitor thapsigargin (1 μM) in the nominal absence of extracellular Ca^{2+} . Readdition of extracellular Ca^{2+} in the continued presence of thapsigargin was followed by store operated Ca^{2+} entry (SOCE) (Fig. 2A,B). Both, peak and slope of SOCE were significantly higher in *ampk*^{-/-} than in *ampk*^{+/+} T-lymphocytes. (Fig. 2A,B). Thus, lack of AMPK α 1 increased SOCE in T-lymphocytes. SGK1 sensitive SOCE was modified by Orai1 inhibitor 2-aminoethoxydiphenyl borate (2-APB, 50 μM). As illustrated in Fig. 2A,B), 2-APB significantly decreased SOCE in both, *ampk*^{-/-} and in *ampk*^{+/+} T-lymphocytes and virtually abolished the difference of SOCE between the genotypes.

Fig. 2. Capacitative Ca^{2+} entry into cytotoxic T-lymphocytes from *ampk*^{-/-} and *ampk*^{+/+} T-lymphocytes. **A.** Representative tracings showing the 340/380 nm fluorescence ratio in Fura-2/AM loaded lymphocytes from *ampk*^{-/-} (closed symbols) and *ampk*^{+/+} (open symbols) T-lymphocytes upon extracellular Ca^{2+} readmission after Ca^{2+} depletion by extracellular Ca^{2+} removal and exposure to thapsigargin (1 μM). Experiments were performed in the absence (squares) and presence (circles) of Orai1 inhibitor 2-aminoethoxydiphenyl borate (2-APB, 10 μM). **B.** Arithmetic means $\pm$ SEM of the peak (left) and slope (right) of the fluorescence ratio change for *ampk*^{+/+} (n = 6, each experiment 20-40 cells, open bars) and *ampk*^{-/-} (n = 6, each experiment 20-40 cells, closed bars) T-lymphocytes following readdition of Ca^{2+} in the absence (-2-APB) and presence (+2-APB) of Orai1 inhibitor 2-aminoethoxydiphenyl borate (50 μM). ** indicates statistically significant difference (p<0.01) from *ampk*^{+/+}, ### indicates statistically significant difference (p<0.001) from absence of inhibitor.

Further experiments analysed Ca^{2+} entry upon T-cell receptor activation in activated T-cell blasts. T-lymphocytes were incubated with anti-CD3 (10 $\mu\text{g}/\text{ml}$; 145-2C11) for 30 min on ice in Ringer solution containing 2 mM CaCl_2 (2 mM Ca^{2+}), and subsequently were cross-linked by goat anti-hamster at the indicated time (TCR stimulation). Then Fura-2 fluorescence was determined. As illustrated in Fig. 3, TCR activation was followed by an increase of the Fura-2 fluorescence ratio reflecting $[\text{Ca}^{2+}]_i$, which was significantly larger in *ampk*^{-/-} T-lymphocytes than in *ampk*^{+/+} T-lymphocytes (Fig. 3B).

Additional experiments were performed to elucidate, whether AMPK sensitivity of SOCE was secondary to AMPK sensitivity of Orai1 expression. As illustrated in Fig. 4, total Orai1 and STIM1 protein abundance was not significantly different between *ampk*^{-/-} and *ampk*^{+/+} T-lymphocytes. In contrast, the Orai1 protein abundance in the cell membrane was significantly higher in *ampk*^{-/-} than in *ampk*^{+/+} T-lymphocytes (Fig. 4).

To analyse the role of AMPK α 1 or Orai1 in the stimulation of lymphocyte proliferation following antigenic stimulation, *ampk*^{-/-} T-lymphocytes and *ampk*^{+/+} T-lymphocytes were either stimulated with 5 $\mu\text{g}/\text{ml}$ pre-coated CD3 mAB and 2 $\mu\text{g}/\text{ml}$ CD28 mAB (Fig. 5A) or transfected with 10 nM Orai1 siRNA (Fig. 5B). The specific OD (optical density) ($\lambda_{490} - \lambda_{650}$) measured after performing a MTS assay was used to determine cell proliferation. As illustrated in Fig. 5A, proliferation was significantly higher in unstimulated *ampk*^{-/-} T-lymphocytes (0.55 ± 0.03 sOD, n=5) than in unstimulated *ampk*^{+/+} T-lymphocytes (0.35 ± 0.05 sOD, n=5). Stimulation significantly increased proliferation in both, *ampk*^{+/+}

Fig. 3. Increase of cytosolic Ca^{2+} concentration in *ampk*^{-/-} and *ampk*^{+/+} T-lymphocytes following store depletion.

A. Representative tracings showing the 340/380 nm fluorescence ratio in Fura-2/AM loaded T-lymphocytes prior to and following TCR activation by cross linking with goat anti-hamster antibody at the indicated time in the graph. Prior to the experiments, cells were incubated with anti-CD3 (10 $\mu\text{M}/\text{ml}$; 145-2C11) for 30 min on ice in Ringer solution containing 2 mM CaCl_2 (2 mM Ca^{2+}), and subsequently were cross-linked by secondary antibody for TCR stimulation.

C. Arithmetic means $\pm$ SEM of the peak (left) and slope (right) of the fluorescence ratio change in *ampk*^{+/+} (n = 6, each experiment 5-15 cells, open bars) and *ampk*^{-/-} (n = 9, each experiment 2-27 cells, closed bars) T-lymphocytes following TCR activation by cross linking with goat anti-hamster at the indicated time in the graph. Prior to the experiments, cells were incubated with anti-CD3 (5 $\mu\text{g}/\text{ml}$; 145-2C11) for 30 min on ice in Ringer solution containing 2 mM CaCl_2 (2 mM Ca^{2+}), and subsequently were cross-linked by secondary antibody for TCR stimulation. * indicates statistically significant difference ($p < 0.05$) from *ampk*^{+/+}.

Fig. 4. Expression of Orai1 and STIM1 as well as membrane abundance of Orai1 in *ampk*^{-/-} and *ampk*^{+/+} T-lymphocytes. A. Original western blot of the expression of total Orai1, Stim1, Nedd4-2 and GAPDH as loading control in *ampk*^{-/-} and *ampk*^{+/+} T-lymphocytes. B. Original western blot of the expression Orai1 in membrane protein and beta-Actin as loading control in *ampk*^{-/-} and *ampk*^{+/+} T-lymphocytes. C. Arithmetic means $\pm$ SEM (n = 3) of Orai1 protein as compared to loading control actin in membrane of *ampk*^{-/-} (closed bars) and *ampk*^{+/+} (open bars) T-lymphocytes. * indicates statistically significant difference ($p < 0.05$) from *ampk*^{+/+}.

(0.56 $\pm$ 0.02 sOD, n=5) and *ampk*^{-/-} (0.66 $\pm$ 0.03 sOD, n=5) T-lymphocytes. Transfection with Orai1 siRNA decreased proliferation in *ampk*^{-/-} T-lymphocytes (0.29 $\pm$ 0.02 sOD, n=4) compared to non-transfected *ampk*^{-/-} T-lymphocytes (0.37 $\pm$ 0.02 sOD, n=4) but had no effect on transfected *ampk*^{+/+} T-lymphocytes (0.37 $\pm$ 0.02 sOD, n=4) compared to non-transfected *ampk*^{+/+} T-lymphocytes (0.28 $\pm$ 0.01 sOD, n=4) (Fig. 5B).

Fig. 5. Proliferation of *ampk*^{-/-} and *ampk*^{+/+} T-lymphocytes after antigenic stimulation or transfection with Orai1 siRNA. A. Arithmetic means $\pm$ SEM (n=5) of the specific OD (optical density) ($\lambda_{490} - \lambda_{650}$) of *ampk*^{-/-} (closed bars) and *ampk*^{+/+} (open bars) T-lymphocytes with and without antigenic stimulation with 5 $\mu\text{g}/\text{ml}$ CD3 mAB and 2 $\mu\text{g}/\text{ml}$ CD28 mAB. ** indicates statistically significant difference ($p < 0.01$) from *ampk*^{+/+} lymphocytes ($p < 0.01$), # indicates statistically significant difference from unstimulated lymphocytes ($p < 0.05$). B. Arithmetic means $\pm$ SEM (n=4) of the specific OD (optical density) ($\lambda_{490} - \lambda_{650}$) of *ampk*^{-/-} (closed bars) and *ampk*^{+/+} (open bars) T-lymphocytes transfected with either control 10 nM siRNA (left) or 10 nM Orai1 siRNA (right). ** indicates statistically significant difference ($p < 0.01$) from *ampk*^{+/+} lymphocytes, # indicates statistically significant difference from nontransfected lymphocytes ($p < 0.05$).

Discussion

The present results reveal that AMP-activated protein kinase (AMPK) is a powerful negative regulator of store operated Ca^{2+} entry (SOCE) in T lymphocytes. SOCE is higher in T-lymphocytes derived from mice lacking AMPK α 1 (*ampk*^{-/-}) than in T-lymphocytes from their wild type littermates (*ampk*^{+/+}). As AMPK is activated by increase of cytosolic Ca^{2+} activity [13], our data shows that the kinase is part of a negative feedback limiting Ca^{2+} entry. The difference of SOCE between *ampk*^{-/-} and *ampk*^{+/+} lymphocytes is virtually abrogated by Orai1-inhibitor 2-APB indicating that AMPK α 1 is largely if not exclusively effective by downregulating Orai1. The residual Ca^{2+} entry in the presence of 2-APB may reflect incomplete inhibition of Orai1 or Ca^{2+} entry through an Orai1 independent mechanism.

AMPK is known to be activated during T-cell activation [13] and to mediate T-cell activation-induced expression of FasL and COX-2 [64]. T-cell activation by T-cell receptor (TCR) engagement or by phorbol ester (PMA) plus Ca^{2+} ionophore ionomycin induces immunomodulatory FasL and cyclooxygenase-2 (COX-2) expression [64]. AMPK regulates expression of FasL and COX-2 via the PKC θ and NFAT and AP-1 pathways in activated Jurkat cells [64]. AMPK activator metformin displayed a remarkable anti-leukemic activity. Thus, AMPK may be considered an attractive therapeutic target in the treatment of T-cell acute lymphoblastic leukemia [65]. AMPK has further been implicated in the influence of lymphocytes on the assembly of tight junctions (TJs) in epithelial cells [66].

The influence of AMPK on SOCE and thus activation of T-lymphocytes may play a role in limiting T-lymphocyte activation. Engagement of the T cell receptor (TCR) with its cognate peptide/MHC initiates a cascade of signaling events resulting in T-cell activation. Limiting the extent and duration of TCR signaling ensures a tightly constrained response, protecting cells from the deleterious impact of chronic activation [67]. Unstimulated *ampk*^{-/-} lymphocytes proliferated more than *ampk*^{+/+} lymphocytes, a finding presumably do to unrestricted activation. Enhanced proliferation may contribute to the splenomegaly of *ampk*^{-/-} mice, which is, however, at least in part due to enhanced trapping of erythrocytes [35].

The present study did not address the AMPK-dependent mechanisms accounting for the downregulation of SOCE. Nevertheless, it is shown that the Orai1 protein abundance is decreased in *ampk*^{-/-} T-lymphocytes. AMPK is known to stimulate the ubiquitin ligase Nedd4-2 [49, 68-70], an enzyme recently shown to trigger the degradation of Orai1 [71]. Thus, AMPK sensitive regulation of SOCE in T-lymphocytes may at least in part be due to stimulation of Nedd4-2.

Inhibition of Orai1 by AMPK is particularly important during energy depletion, which is followed by increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) with subsequent activation of CaMKK β and CaMKK β -dependent phosphorylation of AMPK [72]. Silencing of STIM1 blunts the increase of $[\text{Ca}^{2+}]_i$ following hypoxia [72]. Without the influence of AMPK, energy depletion would presumably result in the activation of Orai1, as ATP depletion compromises the function of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) leading to depletion of intracellular Ca^{2+} stores and subsequent activation of Orai by STIM. It is noteworthy that AMPK inhibits the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and thus impedes Ca^{2+} extrusion [32]. Without inhibition of Orai1, unrestrained Ca^{2+} entry would thus lead to Ca^{2+} overload of cells.

In conclusion, the present observations disclose a novel mechanism of cytosolic Ca^{2+} regulation in T-lymphocytes. Specifically, we do show that lack of AMPK α 1 increases Orai1 cell membrane protein abundance, increases store operated Ca^{2+} entry into T-lymphocytes and increases Ca^{2+} entry following T-cell receptor activation. The AMPK α 1 sensitive Ca^{2+} entry is abrogated by Orai1 blocker 2-APB. AMPK α 1 is thus part of a negative feedback limiting Ca^{2+} entry at increased cytosolic Ca^{2+} . The mechanism has the capacity to limit the duration of T-lymphocyte activation. AMPK is thus not only rapidly activated upon TCR activation, but serves as kinase limiting Ca^{2+} -entry and T-lymphocyte activation.

Conflict of Interest

The authors of this manuscript state that they have neither financial nor any other conflicts of interests.

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