Biochimica et Biophysica Acta 1864 (2017) 977–986

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Mag-Fluo4 in T cells: Imaging of intra-organelle free Ca^{2+} concentrations☆

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ARTICLE INFO

Article history:
Received 7 October 2016
Received in revised form 21 November 2016
Accepted 22 November 2016
Available online 30 November 2016

Keywords:
Ca^{2+} signaling
Mag-Fluo4
Endoplasmic reticular Ca^{2+} concentration
Mitochondrial Ca^{2+} concentration
T cell
Signal transduction

ABSTRACT

Ca^{2+} signaling is a major signal transduction pathway involved in T cell activation, but also in apoptosis of T cells. Since T cells make use of several Ca^{2+}-mobilizing second messengers, such as nicotinic acid adenine dinucleotide phosphate, D-*myo*-inositol 1,4,5-trisphosphate, and cyclic ADP-ribose, we intended to analyze luminal Ca^{2+} concentration upon cell activation. Mag-Fluo4/AM, a low-affinity Ca^{2+} dye known to localize to the endoplasmic reticular lumen in many cell types, showed superior brightness and bleaching stability, but, surprisingly, co-localized with mito-tracker, but not with ER-tracker in Jurkat T cells. Thus, we used Mag-Fluo4/AM to monitor the free luminal mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mito}$) in these cells. Simultaneous analysis of the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and $[Ca^{2+}]_{mito}$ upon cell stimulation revealed that Ca^{2+} signals in the majority of mitochondria were initiated at $[Ca^{2+}]_i \geq$ approx. 400 to 550 nM.

In primary murine $CD4^+$ T cells, Mag-Fluo4 showed two different localization patterns: either co-localization with mito-tracker, as in Jurkat T cells, or with ER-tracker. Thus, in single primary murine $CD4^+$ T cells, either decreases of $[Ca^{2+}]_{ER}$ or increases of $[Ca^{2+}]_{mito}$ were observed upon cell stimulation. This article is part of a Special Issue entitled: ECS Meeting edited by Claus Heizmann, Joachim Krebs and Jacques Haiech.

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1. Introduction

T-lymphocytes (T cells) are major cell types of the immune system. In healthy individuals, T cells are quiescent unless pathogens invade the body and activate the T cells. T cell activation thus is the central on-switch of the adaptive immune system. Further, T cell activation requires coordinated signal transduction. One essential part of T cell signaling is Ca^{2+} signaling. T cell Ca^{2+} signaling proceeds in the context of immune synapse formation, an intimate cell-cell interaction between a T cell and an antigen presenting cell. Once the T cell receptor recognizes the antigenic peptide presented, and additional co-receptor interactions are starting, initial, very rapid local Ca^{2+} signals, so called Ca^{2+} microdomains, are formed [1]. Within tens of seconds, Ca^{2+} microdomains culminate into global Ca^{2+} signaling that, in contrast to many other cell types, last for hours (reviewed in [2]).

Initial Ca^{2+} microdomains require functional type 1 ryanodine receptor (1), likely activated by the Ca^{2+} mobilizing second messenger nicotinic acid adenine dinucleotide phosphate (NAADP; refs. [3–6]). Ca^{2+} microdomains are important as (local) co-activators of further Ca^{2+} release by D-*myo*-inositol 1,4,5-trisphosphate (IP_3) and cyclic ADP-ribose (cADPR), Ca^{2+} mobilizing second messengers formed subsequently upon T cell activation [7,8].

Given the importance of Ca^{2+} release by Ca^{2+} mobilizing second messengers in the initiation of the process of T cell activation, we set out to analyze the luminal Ca^{2+} concentration in the endoplasmic reticulum (ER). There are several genetically encoded Ca^{2+} indicators that can be targeted to organelles, such as ER [9–12]. Because of the difficulties in transfecting primary T cells, we decided to use small molecule Ca^{2+} indicators that have been shown to diffuse into the ER lumen, where they are de-esterified, such as Fluo5N, Mag-Fura2 and Mag-Fluo4 [13–16]. Certainly, de-esterification also takes place within the cytosol; however, due to the low affinity of these indicators for Ca^{2+}, any Ca^{2+} dynamics in the cytosol are unlikely to disturb visualization of processes within the ER lumen.

Here, we report selection of a bright and adequately photo stable dye, Mag-Fluo4, to be used in T cells. Surprisingly, localization of the dye within organelles depends on the specific T cell type. In human Jurkat T-lymphoma cells, a T cell line possessing an effector cell phenotype, an almost exclusive localization in mitochondria was observed. In contrast, in individual primary murine $CD4^+$ T cells, Mag-Fluo4 was

Abbreviations: AM, acetoxymethylester; $[Ca^{2+}]_{ER}$, free luminal endoplasmic reticular Ca^{2+} concentration; $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; $[Ca^{2+}]_{mito}$, free luminal mitochondrial Ca^{2+} concentration; cADPR, cyclic adenosine diphosphoribose; ER, endoplasmic reticulum; IP_3, D-*myo*-inositol 1,4,5-trisphosphate; IP_3R, D-*myo*-inositol 1,4,5-trisphosphate receptor; NAADP, nicotinic acid adenine dinucleotide phosphate; RT, room temperature; RyR, ryanodine receptors; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; VDAC, voltage-dependent anion channel.

☆ This article is part of a Special Issue entitled: ECS Meeting edited by Claus Heizmann, Joachim Krebs and Jacques Haiech.

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http://dx.doi.org/10.1016/j.bbamcr.2016.11.026
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localized either to the ER or to mitochondria opening the possibility to measure both the free ER-luminal Ca^{2+} concentration ($[Ca^{2+}]_{ER}$) or the free mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mito}$). These measurements were combined with analyses of the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$).

2. Materials and methods

2.1. Materials

Mag-Fluo4-AM, Mag-Fura2-AM, Fluo5N-AM, lyso-tracker® Red DND-99, mito-tracker® Red FM, oligomycin A and ER-tracker® Red were obtained from Life Technologies. All dyes were dissolved in DMSO, divided into aliquots, and stored at −20 °C until required for use. Anti-human CD3 mAb (clone OKT3) was generated as described previously [17]. Anti-mouse CD3 mAb was obtained from BD Biosciences and thapsigargin from Calbiochem/Merck Millipore.

2.2. Cell culture

Jurkat T cells (subclone JMP) were cultured in RPMI 1640 containing 25 mM Hepes and GlutaMAX-I (Gibco, Life Technologies), supplemented with 7.5% newborn calf serum (Biochrom, Merck Millipore), penicillin (100 U/mL), and streptomycin (100 μg/mL).

2.3. Isolation of primary CD4$^+$ WT T cells

Primary CD4$^+$ T cells were freshly isolated from lymph nodes and spleens of naïve C57Bl/6 or naïve FVB mice by negative selection with an EasySep Mouse T Cell Isolation Kit (STEMCELL Technologies Inc.) according to the manufacturer's instructions. Cell purity (which was typically >95% T cells) was assessed by immunostaining with fluorescein isothiocyanate–conjugated anti-mouse TCRb antibody (clone H57-597, BioLegend) and measured with a FACSCalibur flow cytometer (BD Biosciences). The freshly isolated CD4$^+$ T cells were directly loaded with the respective dyes.

2.4. Preparing coverslips

Coverslips were coated with bovine serum albumin (5 mg/mL, Sigma-Aldrich) and poly-L-lysine (0.1 mg/mL, Sigma-Aldrich). Chamber slides were prepared by mounting rubber O-rings onto the slides with silicon grease [3].

2.5. Assessment of Ca^{2+} indicators

Jurkat T cells were incubated with the membrane-permeable AM esters of the Ca^{2+} dyes Mag-Fluo4 (5 μM), Mag-Fura2 (10 μM) and Fluo5N (10 μM + 0,05% (v/v) Pluronic F-127, Sigma-Aldrich). Therefore, about 2×10^6 cells were centrifuged at 500g for 5 min and resuspended in 1 mL of freshly supplemented RPMI medium containing the respective

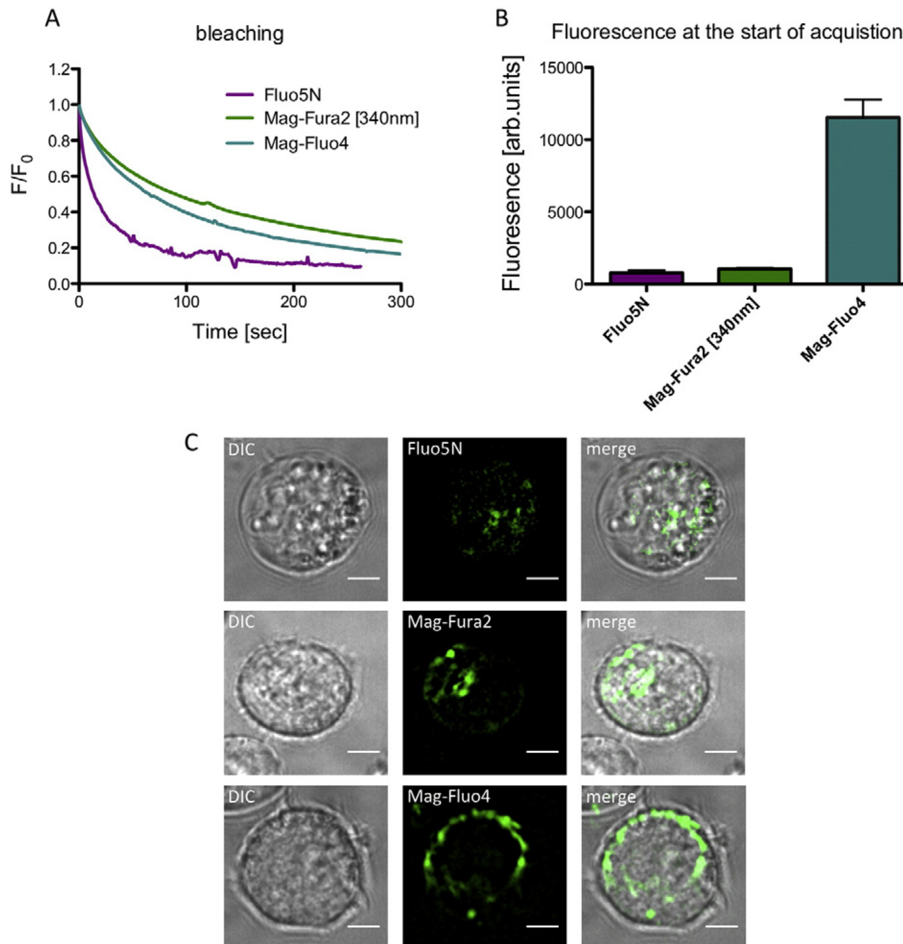

Fig. 1. Properties and cellular distribution of Fluo5N, Mag-Fluo4 and Mag-Fura2 in Jurkat T cells. (A to C) Jurkat T cells were loaded with the acetoxymethylesters of Ca^{2+} indicators Fluo5N (10 μM), Mag-Fluo4 (5 μM) and Mag-Fura2 (10 μM). Acquisition rate was 1 frame/s. Exposure time varied from 1 s (Fluo5N) to 200 ms (Mag-Fluo4) and 25 ms (Mag-Fura2). Bleaching (A) and initial fluorescence intensity (B) were measured. Curves indicate the mean of global fluorescence signals measured in single cells and normalized to the initial frame, based on 8 to 25 individual Jurkat T cells loaded with the respective dyes. (C) Cellular distributions of Fluo5N, Mag-Fluo4 and Mag-Fura2 (380 nm wavelength was used) after loading in Jurkat T cells. Deconvolution was applied to obtain digital confocal images.

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