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Flow cytometric measurement of labile zinc in peripheral blood mononuclear cells

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Abstract

Labile (i.e., free or loosely bound) zinc has the potential to modulate cellular function. Therefore, a flow cytometric assay for the measurement of labile zinc was developed to facilitate the investigation of the physiological roles of zinc. The zinc-sensitive fluorescent probe FluoZin-3 was used to quantify the amount of labile zinc in peripheral blood mononuclear cells isolated from human blood. Maximal fluorescence and autofluorescence of the probe were measured after the addition of zinc in the presence of the ionophore pyrithione, or the membrane-permeant chelator *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridyl-methyl)ethylenediamine, respectively. In this way, the intracellular concentrations of labile zinc in resting cells were estimated to be 0.17 nM in monocytes and 0.35 nM in lymphocytes. The method was successfully employed to monitor phorbol 12-myristate 13-acetate-induced zinc release, which occurred in monocytes but not lymphocytes, and the displacement of protein-bound zinc by the mercury-containing compounds $HgCl_2$ and thimerosal. Costaining with dyes that emit at higher wavelengths than FluoZin-3 allows multiparameter measurements. Two combinations with other dyes are shown: loading with propidium iodide to measure cellular viability and labeling with antibodies against the surface antigen CD4. This method allows measurement of the concentration of biologically active labile zinc in distinct cell populations.

Keywords: Zinc; Flow cytometry; Fluorescent probes

Zinc is an essential trace element and is required for many physiological processes, from growth to apoptosis [1,2]. It is of special importance for the immune system, where it affects both innate and adaptive immunity. Leukocyte effectiveness depends on zinc, and the functionality of monocytes, granulocytes, NK cells, T cells, and B cells is affected by zinc deprivation as well as by zinc excess [3]. Serum zinc is reduced in autoimmune diseases, such as type 1 diabetes and rheumatoid arthritis [4,5], and is associated with immunological alterations during aging [6], underscoring the importance of zinc status in the regulation of the immune response. Hence, a fast and accurate way in which to measure zinc in immune cells would be beneficial for investigations of the physiological functions of zinc in immunity.

There are several ways in which to measure cellular zinc, with the most commonly used being atomic absorption spectrometry (AAS),[1] radiolabeling with Zn65, and fluorescent probes that alter their excitation or emission properties on metal binding. These methods do not detect identical pools of cellular zinc. For example, AAS measures total cellular zinc, whereas fluorescent probes can bind only free or loosely bound zinc, that is, so-called labile zinc. The total cellular zinc is in the order of several hundred micromolar [7], whereas the concentration of labile zinc has been estimated to be picomolar up to low nanomolar [8–10]. However, cellular response is controlled by the labile portion of zinc [3].

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[1] *Abbreviations used:* AAS, atomic absorption spectrometry; PKC, protein kinase C; PBS, phosphate-buffered saline; FCS, fetal calf serum; PMA, phorbol 12-myristate 13-acetate; TPEN, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridyl-methyl)ethylenediamine; TMS, thimerosal; AM, acetoxymethyl; PE, phycoerythrin; PBMC, peripheral blood mononuclear cell.

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Zinc homeostasis is tightly controlled, and a rapidly growing number of proteins that mediate influx, intracellular distribution, and export of zinc are being identified [11].

The size of the pool of labile zinc can change due to influx [12] or oxidative intracellular release [13–15]. Such zinc fluctuations have the potential to influence physiological processes because it has been demonstrated that zinc affects several components of signaling pathways such as receptor tyrosine kinases, cyclic nucleotides, mitogen-activated protein kinases, protein kinase C (PKC), and transcription factors [16].

The aim of the current study was to develop an assay for the measurement of labile zinc in leukocytes to enable the investigation of zinc fluctuations in cells of the immune system. FluoZin-3 [17] was chosen as a zinc-specific fluorescent probe to be used for flow cytometric measurements and quantification of intracellular labile zinc. The excitation and emission wavelengths of this probe are similar to those of fluorescein, making it suitable for application with most instruments and allowing for the combination with probes that emit at higher wavelengths. Two examples of such combinations, measurement of membrane integrity (propidium iodide) and of antibodies against surface marker of leukocyte subpopulations, are presented.

Materials and methods

Materials

RPMI 1640 medium, sodium pyruvate, L-glutamine, nonessential amino acids, penicillin, streptomycin, and phosphate-buffered saline (PBS) were purchased from Cambrex (Verviers, Belgium). Ficoll was obtained from Biochrom (Berlin, Germany), and fetal calf serum (FCS) was obtained from PAA (Coelbe, Germany). Metallothionein, phorbol 12-myristate 13-acetate (PMA), bisindolylmaleimide II, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridyl-methyl)ethylenediamine (TPEN), $HgCl_2$, thimerosal (TMS), and sodium pyrithione were purchased from Sigma–Aldrich (Taufkirchen, Germany). $ZnSO_4 \times 7H_2O$ was obtained from Merck (Darmstadt, Germany), and FluoZin-3 acetoxymethyl (AM) ester and free acid were obtained from Invitrogen (Karlsruhe, Germany). Phycoerythrin (PE)-labeled anti-CD4 was obtained from ImmunoTools (Friesoythe, Germany).

Cell culture

The human acute T-cell leukemia cell line Jurkat was cultured at 37 °C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium containing 10% FCS. L-Glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), and nonessential amino acids.

Isolation and culture of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral venous blood from healthy donors by centrifugation over Ficoll–Hypaque, washed three times with PBS, and resuspended in RPMI 1640 medium containing 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) at 37 °C and 5% CO_2.

Flow cytometry

Jurkat cells or PBMCs (1×10^6 cells/ml) were loaded with FluoZin-3 AM ester (1 μM) in culture medium at 37 °C for 30 min, washed with PBS, and resuspended in PBS supplemented with 10% FCS. Aliquots of the cells were incubated with TPEN, zinc/pyrithione, PMA, TMS, or $HgCl_2$ at 37 °C as described in the figure legends. To measure cellular viability, cells subsequently were incubated with propidium iodide (10 μg/ml) for 10 min at 4 °C in the dark. For labeling of CD4, cells were loaded with FluoZin-3 and incubated with anti-CD4 PE (5 μl, 1×10^6 cells) for 20 min in PBS at room temperature in the dark. Fluorescence was recorded using a FACSCalibur (Becton–Dickinson, Heidelberg, Germany), measuring the fluorescence of FluoZin-3 in FL-1, anti-CD4 PE in FL-2, or propidium iodide in FL-3. The concentration of intracellular labile zinc was calculated from the mean fluorescence with the formula $[\mathrm{Zn}] = K_D \times [(F - F_{min})/(F_{max} - F)]$ [18]. The dissociation constant of the FluoZin-3/zinc complex is 15 nM [17]. F_{min} was determined by the addition of the zinc-specific, membrane-permeant chelator TPEN, and F_{max} was determined by the addition of $ZnSO_4$ and the ionophore pyrithione.

Cell-free fluorescence measurements

For the cell-free controls, a solution of 2 μM of the free acid of FluoZin-3 (the form that is present within cells after cleavage of the AM ester) was prepared in PBS, and 100-μl aliquots were incubated in a transparent 96-well plate for 15 min at room temperature, as indicated in the legend to Fig. 5. The resulting fluorescence was measured with a Tecan 340 fluorescence multiwell plate reader (Tecan, Crailsheim, Germany) using excitation and emission wavelengths of 485 and 535 nm, respectively.

Statistics

All experiments were performed independently at least three times. Statistical significance of experimental results was calculated by the Student's *t* test using SigmaPlot software.

Results

Measurement of PMA-induced zinc release

To measure the amount of intracellular labile zinc, PBMCs were loaded with FluoZin-3 AM ester and analyzed by flow cytometry as specified in Materials and