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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

methods. Because NIH 3T3 cells have been shown to release zinc after stimulation of PKC [15], samples were treated with PMA to see whether such an increase in labile zinc can be found in PBMCs with this method. After loading with FluoZin-3, corresponding fluorescence was detected in the cells (cells without FluoZin-3 did not give a significant fluorescence signal with the instrument parameters used for these measurements), but only a slight alteration was observed after stimulation of PBMCs with PMA (Fig. 1A). To distinguish the effect of PMA on the two main subpopulations of PBMCs, gates in the forward/sideward scatter density plot were set on monocytes and lymphocytes (Fig. 1B). The associated fluorescence intensities for these two types of cells are displayed separately in Figs. 1C and D, respectively. Monocytes show a clear shift to higher fluorescence intensity, indicating the release of a substantial amount of zinc, whereas fluorescence in lymphocytes is nearly unaffected. The mean fluorescence intensities of control and PMA-treated cells are displayed in Table 1, showing a significant ($P < 0.05$) effect of PMA on monocytes but not on lymphocytes.

Table 1
Mean fluorescence intensities of PBMCs loaded with FluoZin-3 and stimulated with PMA for 60 min

	Monocytes	Lymphocytes
Control	13.5 ± 1.4	8.6 ± 0.6
10 ng/ml PMA	15.8 ± 1.9	9.3 ± 1.7
100 ng/ml PMA	25.6 ± 2.8[a]	12.3 ± 2.1

Note. Cells were treated as described in Fig. 1 legend. The mean fluorescence values of three donors are shown.

[a] Significantly different from control ($P < 0.05$). Mean fluorescence intensities of monocytes and lymphocytes were significantly different ($P < 0.05$) under all three experimental conditions.

Quantification of intracellular labile zinc

The higher basal fluorescence of monocytes compared with lymphocytes (Table 1) indicates that these populations differ either in their levels of labile zinc or dye loading. Therefore, mean fluorescence intensity alone cannot be taken as a measure for the labile intracellular zinc concentration, and it is desirable to have a parameter that is independent of dye loading efficiency and that allows the expression of labile zinc as a concentration. This can be calculated from the fluorescence (F) of a nonratiometric dye if the binding constant (15 nM [17]), autofluorescence of the zinc-free probe (F_{min}), and maximum fluorescence under conditions of zinc saturation (F_{max}) are known. Here F_{min} was determined by chelation of cellular zinc with the membrane-permeant chelator TPEN that has a high affinity for zinc ($3.8 \times 10^{15}\ M^{-1}$ [7]), which exceeds that of the probe by several orders of magnitude. To determine F_{max}, the ionophore pyrithione [19] was used to load the cells with zinc, a treatment shown to cause uniform loading of all cellular compartments [20]. For correct determination of F_{max} and F_{min}, quantitative saturation and chelation are important. The data in Fig. 2A show an optimization for the concentrations of zinc (in the presence of 50 μM pyrithione) and

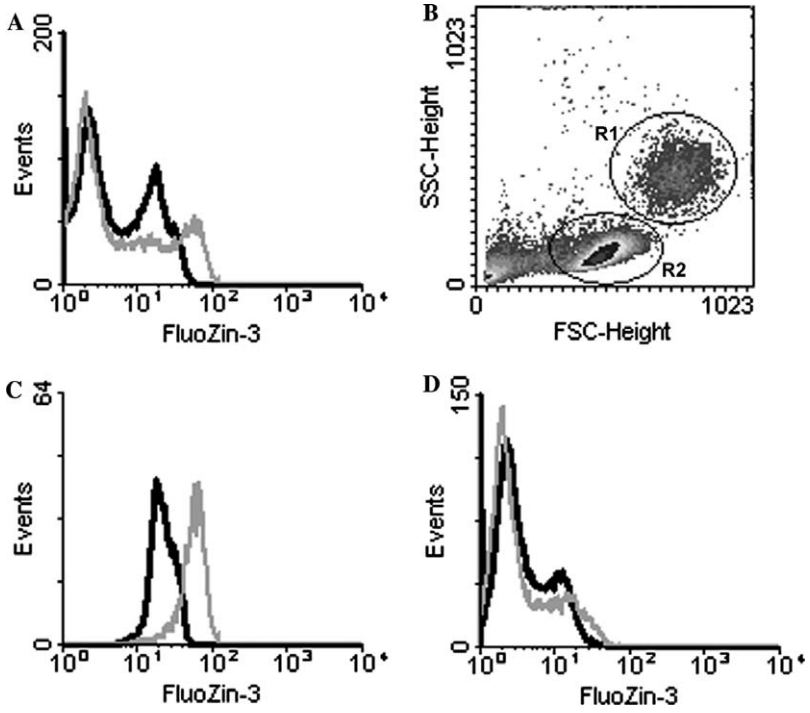

Fig. 1. PMA-induced zinc release in PBMCs. PBMCs were loaded with FluoZin-3, and the resulting fluorescence was measured by flow cytometry, either in untreated controls (black lines) or after stimulation with PMA (100 ng/ml) for 60 min (gray lines). (A) Histogram of FluoZin-3-dependent fluorescence in PBMCs. (B) Density plot, showing forward scatter (FSC) and sideward scatter (SSC) of control cells, with gates on the monocyte (R1) and lymphocyte (R2) populations. The effect of PMA stimulation on zinc-dependent FluoZin-3 fluorescence is shown for gated monocytes (C) and lymphocytes (D). Data are representative of independent experiments with blood from six donors.

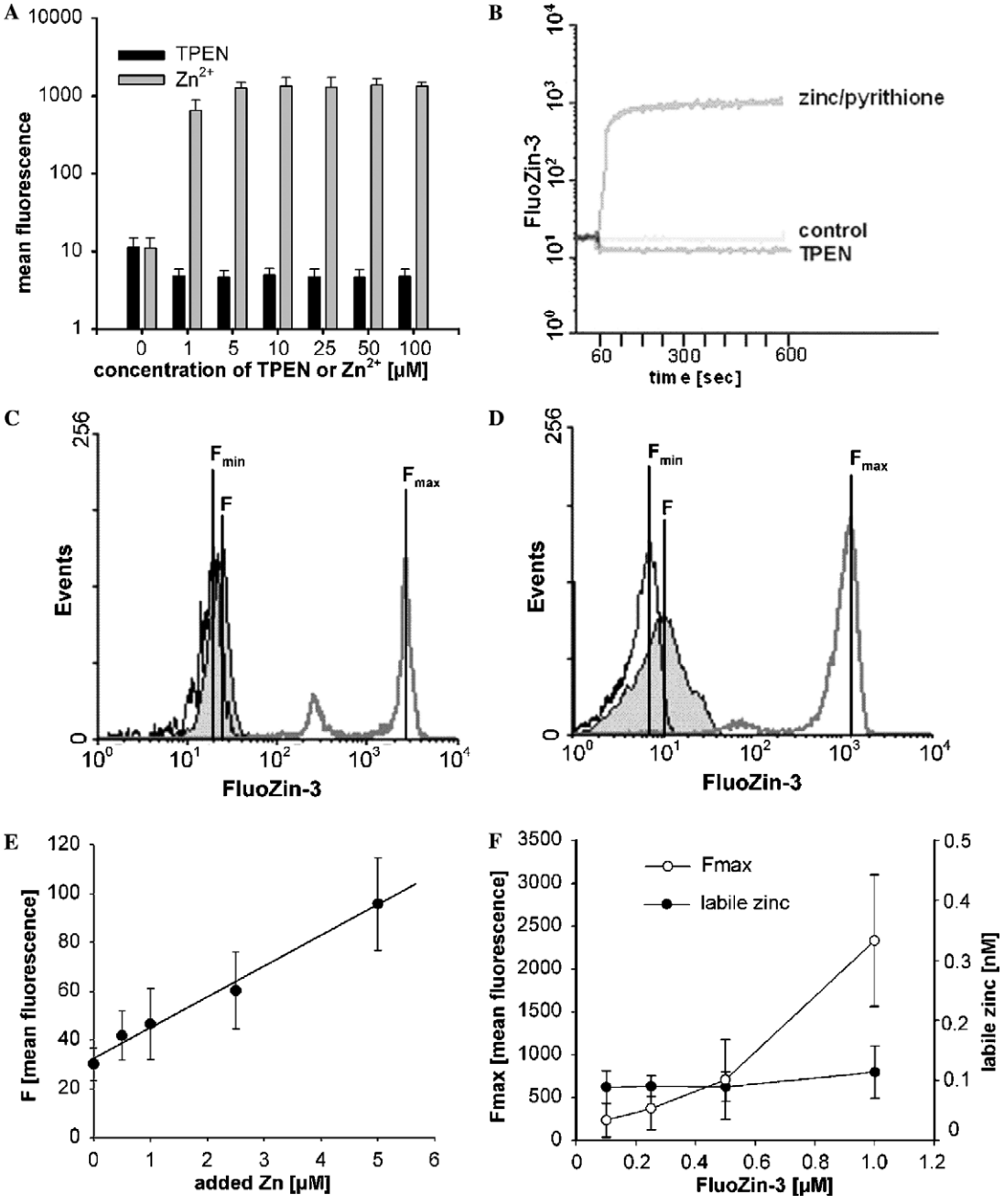

Fig. 2. Determination of F_{min} and F_{max}. Jurkat cells and PBMCs were loaded with FluoZin-3 as described above, and incubations were carried out at 37 °C. Unless indicated otherwise, in all experiments where the ionophore pyrithione was added, a concentration of 50 µM was used. (A) Fluorescence of Jurkat cells was measured after 30 min of incubation with varying concentrations of Zn^{2+} (with pyrithione) or TPEN. (B) Time-dependent plot of FluoZin-3 fluorescence of Jurkat cells treated with 50 µM TPEN or 100 µM Zn^{2+} (with pyrithione) 60 s after the beginning of the measurement. Fluorescence was observed for a total of 600 s. (C,D) PBMCs were loaded with FluoZin-3 and treated with 50 µM TPEN or 100 µM Zn^{2+}(with pyrithione) for 30 min to determine F_{min} and F_{max} in monocytes (C) and lymphocytes (D). (E) Jurkat cells were loaded with FluoZin-3 and incubated with various concentrations of zinc in the presence of 10 µM pyrithione for 10 min. Results are shown as means from four independent experiments performed in triplicate ± SEM. (F) Jurkat cells were incubated with different concentrations of FluoZin-3. F_{max}, measured in the presence of 100 µM zinc (with pyrithione, white circles), and the cellular labile zinc, calculated as described in Materials and methods (black circles), are displayed. Data are shown as means ± SD ($n = 7$).

TPEN. To ensure quick and reliable effects, 10-fold excess of the minimal effective concentrations of TPEN (50 µM) and zinc (100 µM) were used for further experiments. At these concentrations, the fluorescence for F_{max} and F_{min} can be measured within 1 min (Fig. 2B). Results of this treatment are shown for monocytes and lymphocytes in Figs. 2C and D. It is noted that the uptake of FluoZin-3 is higher in monocytes, marked by higher F_{max} and F_{min} values. This is not surprising because the dye is trapped intracellularly by cleavage of the AM ester by unspecific esterases, enzymes that are highly expressed in monocytes. To ensure a linear correlation between the fluorescence signal and labile zinc,

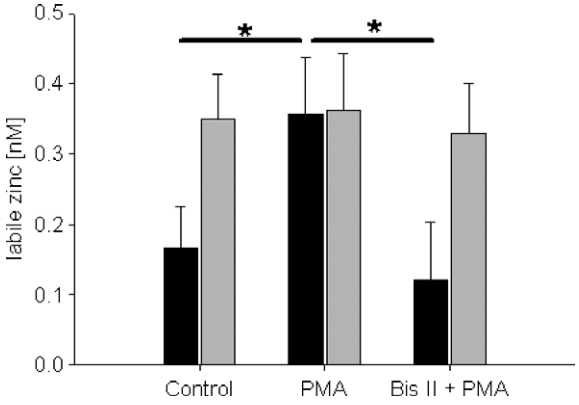

Fig. 3. Intracellular zinc concentrations and PKC stimulation. After loading with FluoZin-3, PBMCs were stimulated with PMA (100 ng/ml) for 30 min directly or after preincubation with 2 μM bisindolylmaleimide II (Bis II) for 30 min. Samples were analyzed by flow cytometry, gates were set as in Fig. 1B, and the intracellular zinc concentrations of monocytes (black bars) and lymphocytes (gray bars) were calculated as described in Materials and methods, using 100 μM Zn^{2+}/50 μM pyrithione and 50 μM TPEN to determine F_{max} and F_{min}, respectively. Statistically significant ($P < 0.05$) differences are indicated (∗).

Jurkat cells have been incubated with increasing concentrations of zinc in the presence of pyrithione (Fig. 2E). Because of the lower concentration of pyrithione (10 vs. 50 μM), less zinc is taken up compared to Fig. 2A, but fluorescence increases linearly with extracellular zinc. Because high concentrations of metal-sensing dyes potentially could deplete the labile zinc pool and thereby impair quantification [21], Jurkat cells were incubated with different amounts of FluoZin-3, from 0.1 to 1 μM, and the maximum fluorescence in the presence of zinc and pyrithione (as a measure of the total amount of intracellular dye) and labile zinc, calculated from F_{max} and F_{min} as described in Materials and methods, were compared (Fig. 2F). Although F_{max} increased proportionally to the amount of FluoZin-3 used for the incubation, the calculated concentration of labile zinc was confirmed to be independent of dye loading.

When the labile zinc concentration was calculated for PBMCs, it turned out that values of labile zinc are actually higher in lymphocytes despite the fact that higher fluorescence signals were observed in monocytes. In unstimulated cells, the concentrations were determined to be 0.17 ± 0.06 nM in monocytes and 0.35 ± 0.07 nM in lymphocytes (means ± SEM, $n = 3$). Treatment with PMA induced an increase of labile zinc in monocytes only that was completely suppressed by preincubation with 2 μM of the PKC inhibitor bisindolylmaleimide II (Fig. 3).

Parallel measurement of zinc release and cytotoxicity caused by $HgCl_2$ and TMS

Heavy metal intoxication may lead to an intracellular displacement of zinc that would be undetected when total cellular zinc is measured (because this is only shifted from one intracellular pool to another) but should be detectable when only the level of labile zinc is monitored. In addition, measurements with flow cytometry permit the combination of FluoZin-3 with other fluorophores. Costaining with propidium iodide, which is not taken up by cells with an intact plasma membrane, enables the identification of viable cells together with the measurement of labile zinc (Fig. 4A). As an example, the release of zinc by incubation with two mercury-containing compounds, $HgCl_2$ and TMS, was investigated in Jurkat cells. Mercury compounds are suspected of being involved in the development of autoimmune reactions. Also, they have a high affinity for cystein ligands, and so zinc displacement from proteins could be involved in these processes. Because these substances have a high toxicity, it is relevant to show that zinc release occurs at lower doses than those causing toxic effects. Both $HgCl_2$ and TMS did indeed increase labile zinc in Jurkat cells at concentrations below the cytotoxic levels (Fig. 4B). Although

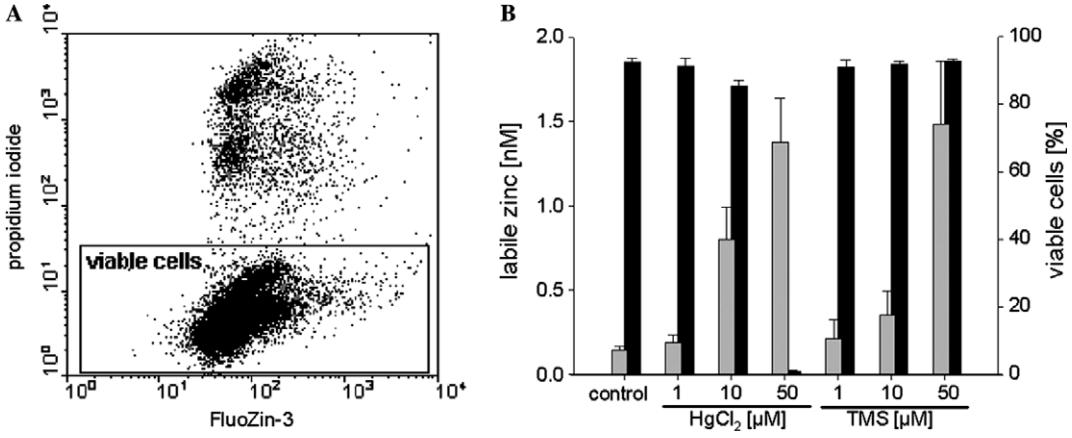

Fig. 4. Zinc release and toxicity induced by $HgCl_2$ and TMS. Jurkat cells were loaded with FluoZin-3, treated with $HgCl_2$ or TMS for 1 h, and stained for membrane integrity with propidium iodide as described in Materials and methods. (A) Dot plot of propidium iodide fluorescence versus FluoZin-3, with the region containing viable (propidium iodide-negative) cells indicated. Cells had been treated with 10 μM $HgCl_2$ prior to the measurement. A representative experiment out of four is shown. (B) Labile zinc (gray bars) and cellular viability (black bars) after incubation with different concentrations of $HgCl_2$ or TMS. Data are shown as means ± SD ($n = 4$).

the highest concentration of $HgCl_2$, 50 μM, caused more than 98% of the cells to lose membrane integrity within 1 h, TMS was not toxic under the same conditions; however, the increase in labile zinc was comparable.

A common problem with fluorescent probes for metal ions is interference of other ions. In the case of Fig. 4, it is not possible to exclude the fact that TMS or Hg^{2+} ions may bind directly to FluoZin-3 to cause the fluorescence signal instead of displacing zinc from binding sites in proteins. To address this question, the free acid of FluoZin-3, the form that is present in cells, was used for cell-free fluorescence measurements. TMS and Hg^{2+} do not affect the fluorescence of FluoZin-3 directly. However, fluorescence increases in the presence of the zinc-binding protein metallothionein, indicating a displacement of zinc ions by TMS and Hg^{2+} (Fig. 5). An interference of calcium fluctuations with the detection of zinc can be excluded because calcium (1 μM) did not increase fluorescence in vitro (1.06 ± 0.05), whereas the addition of Zn^{2+} (0.2 μM) caused a significant increase in fluorescence intensity (3.7 ± 0.11). Values are given as fold control ± S.D. ($n = 8$).

Parallel measurement of labile zinc and CD4 surface antigen

FluoZin-3 was tested in costaining experiments with fluorescently labeled antibodies against surface markers of subsets of PBMCs to further distinguish different types of lymphocytes and their labile zinc content. In Fig. 6A–I, a costaining of FluoZin-3 with PE-labeled anti-CD4 was performed to separate the signal of labile zinc in CD4-positive T-helper cells from the rest of the lymphocytes. Similar experiments with a range of different PE-labeled antibodies, including CD14 for monocytes, CD19 for B cells, and CD3 for T cells, have also been conducted in our laboratory and allowed identification of the zinc-dependent fluorescence of these cells (data not shown).

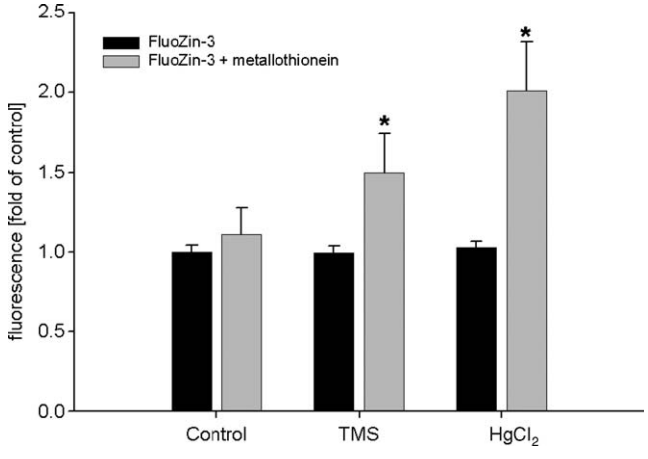

Fig. 5. Cell-free measurements with FluoZin-3. The fluorescence of the free acid of FluoZin-3 (2 μM) in PBS was measured in the absence of cells. The effects of TMS (1 μM) and $HgCl_2$ (1 μM) are compared in PBS with FluoZin-3 alone (black bars) and in the presence of the metal-binding protein metallothionein (1 μg/ml, gray bars). Data are shown as means ± SD ($n = 8$). Values that differ statistically significant ($P < 0.05$) from the controls are indicated (*).

As expected, CD4-positive cells were detected in the lymphocyte population (Fig. 6C). The zinc-dependent fluorescence intensities of total PBMCs, monocytes, and lymphocytes (gray areas) are compared with those of CD4-positive cells within these populations (black lines) in Figs. 6D, E, and F, respectively. Labile zinc in CD4-positive cells yields a single peak in the left region of lymphocyte FluoZin-3 fluorescence. A comparison of the zinc-dependent fluorescence of the cell populations investigated above with that of cells loaded with FluoZin-3 under the same conditions but without antibody treatment shows that antibody costaining has only minimal effects on the zinc status (Figs. 6G–I).

Discussion

The flow cytometric method presented here revealed different levels of labile zinc in monocytes and lymphocytes and was successfully used to monitor zinc fluctuations induced by either PKC activation or displacement of protein-bound zinc by mercury-containing compounds. For these applications, FluoZin-3 is a well-suited probe because it can be easily loaded into cells in the form of an AM ester and is retained during the measurements. Its excitation wavelength of 494 nm is a good match for excitation by an argon laser (488 nm), and the emission maximum at 515 nm allows the combination with other dyes that emit at higher wavelengths. The dissociation constant is close to the range of physiological zinc but leaves enough capacity to detect zinc release without saturation of the probe. FluoZin-3 has been reported to detect zinc with no significant interference by other metal ions, especially calcium [17,22], and our control experiments confirmed that FluoZin-3 is insensitive to calcium. FluoZin-3 and zinc form a 1:1 complex and yield a good correlation between the labile zinc concentration and fluorescence in vitro [17] and within cells (Fig. 2E). Hence, the formula introduced by Grynkiewicz and coworkers [18] allows estimation of the concentration of labile zinc when zinc–pyrithione and TPEN are used to determine F_{max} and F_{min}, respectively. This parameter facilitates comparison between different cell types and between results from different labs, relatively independent of the amount of dye loaded into the cells.

However, calculation of intracellular ion concentrations from data obtained with fluorescent probes has some difficulties. A major problem with all dyes can be high intracellular dye concentrations, described in detail in a recent publication [21]. If the dye concentration is so high that it depletes the labile zinc pool, the total amount of zinc becomes the limiting factor, so that the binding constant no longer is relevant and the concentration cannot be quantified [21]. The experiment in Fig. 2F demonstrates that increasing concentrations of FluoZin-3 yield a constant concentration of intracellular labile zinc. Because a depletion of the labile zinc pool would have led to a decrease in the calculated concentration, the amount of FluoZin-3 seems to be small enough not to severely deplete labile zinc.

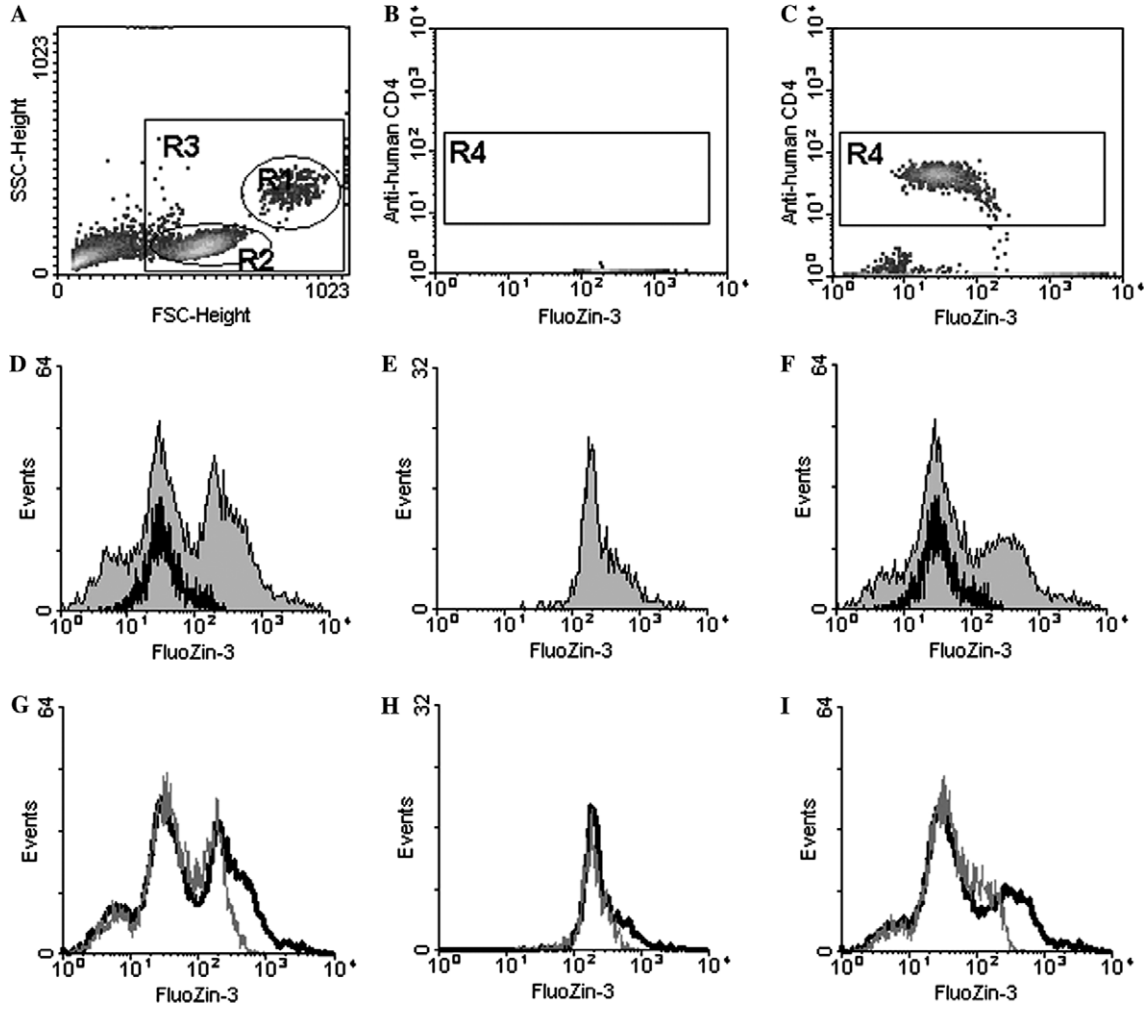

Fig. 6. Costaining of labile zinc and CD4-positive cells. Cells were loaded with FluoZin-3 and labeled with PE-coupled anti-CD4 antibodies as described in Materials and methods. (A) In a density plot of forward scatter (FSC) and sideward scatter (SSC), monocytes (R1), lymphocytes (R2), and all intact cells (R3) were marked by regions. CD4-positive cells were further selected with a gate (R4) in density plots of CD4 PE versus FluoZin-3 fluorescence of the monocyte (B) and lymphocyte (C) populations. FluoZin-3 fluorescence (gray areas) of total PBMCs (D), monocytes (E), and lymphocytes (F) are compared with that of CD4-positive cells within these populations (black lines). A potential effect of antibody labeling on zinc measurements has been analyzed by comparing zinc-dependent fluorescence of the cell populations investigated above with cells from the same donor loaded with FluoZin-3 under identical conditions but without antibody treatment in total PBMCs (G), monocytes (H), and lymphocytes (I). Data shown are representative of independent experiments with blood from three donors.

This may be due to the high quantum yield of FluoZin-3 of 0.43 [17] that allows sensitive zinc measurements at sufficiently low concentrations of this dye. Because F_{max} is saturated in the presence of only 5 μM zinc (Fig. 2A), the intracellular concentration of the probe should be significantly lower than this, whereas the effects of overload have been described for millimolar intracellular concentrations of dyes [21].

It still must be kept in mind that the dissociation constant has not been determined under intracellular conditions and that metal binding may be affected by factors not present in calibration media. Furthermore, the distribution of the fluorescent probe is an important factor, and a probe will be a reliable monitor of zinc status only in cellular compartments where it is present in sufficient quantities. When viewed by fluorescence microscopy, FluoZin-3 is evenly distributed throughout the cells [23], so the results obtained with this probe mirror the average concentration of available zinc within the entire cell, including cytoplasm, nucleus, and zinc-containing vesicles. Other dyes, such as RhodZin-3 that localizes specifically to mitochondria, can be used to monitor labile zinc specifically in distinct cellular compartments (data not shown).

A ratiometric dye would help to circumvent some, but not all, of these issues. To this point, a ratiometric dye with sufficient specificity for zinc, and a suitable affinity close to the labile zinc concentration, and with wavelengths compatible with the generally available instrumentation has not been developed, so that the method described here should represent the most reliable way of measuring labile zinc currently available.

Another issue is whether probes measure only labile zinc or also zinc that is associated with proteins. The latter effect has been demonstrated for the fluorescent probe Zinquin and the zinc-binding protein metallothionein [24]. From the results of Fig. 5, it can be inferred that FluoZin-3 does not interact with zinc bound to metallothionein because there is no increase in fluorescence when the protein is added to the dye. An increase in fluorescence is observed only when zinc is displaced from the protein.

Widely cited reports from the O'Halloran group provide evidence that the concentration of labile zinc can only be femtomolar, according to binding affinities of zinc-regulated proteins [25,26]. It must be noted that these experiments were performed in *Escherichia coli*, and the absence of free zinc in the cytosol of eukaryotic cells cannot be concluded from these data [25]. The current study and other research that used different experimental approaches, such as the use of phosphoglucomutase activity to estimate the free zinc in rabbit muscle cells to be 10^{-10} M [8] and the use of Zn65 to find 24 pM in red blood cells [9], indicate that the cellular concentration of labile zinc in mammalian cells is significantly higher than that in prokaryotes. Zinc has been discussed to regulate neuronal activity [27], growth factor signaling [23,28], and the immune system [3]. All of these functions are irrelevant for prokaryotes, and a requirement for zinc in these processes would provide an explanation for the different levels of labile zinc.

Flow cytometry with FluoZin-3 allows the measurement of labile zinc on the single cell level and the comparison of different populations of cells in one experiment. Furthermore, the combination with probes for cellular viability or surface markers enables more complex immunological investigations. Moreover, it should be possible to combine this method with cell sorting, which would allow not only the measurement of differences in cellular zinc status but also the separation of cellular populations with different labile zinc contents for functional studies.

Alterations of serum zinc have already been illustrated in a number of diseases such as type 1 diabetes and rheumatoid arthritis [4,5]. In elderly people, AAS shows that serum zinc is significantly reduced when compared with that in younger control groups, but single measurements are still well within the range of reference values [29]. Hence, AAS has no predictive value for the single patient. Interferon-α production is reduced in the elderly and returns to normal after zinc supplementation, indicating a zinc deficiency [30]. Measuring labile cellular zinc may be a parameter that allows the determination of zinc status more precisely by directly measuring leukocyte zinc status instead of zinc in the surrounding liquid. This method will allow the correlation of immunological findings with the level of labile zinc in leukocytes and, in general, will provide the possibility of monitoring the relationship between leukocyte activity and labile zinc and will help in understanding the immunoregulatory functions of this trace metal ion.

Acknowledgments

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