Naturwissenschaften 72, S. 600 (1985)

Determination of Intracellular Calcium in Vital Cells by Flow-Cytometry

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The presence of Ca^{2+} ions is of great importance for normal cell growth and cell function. The intracellular Ca^{2+} concentration is high in nucleus, mitochondria, endoplasmic reticulum, and cell membrane (0.1 to 1 m*M*), while it is low in the cytoplasm (1 to 10 μ*M*). Most of the Ca^{2+} is protein-bound. Approximately 10% of cytoplasmic Ca^{2+} is, however, present in free form (0.1 μ*M*) (review in [1]).

Ca^{2+} can be determined by a number of different methods amongst which the use of fluorescent dyes is of particular interest for studies with vital cells. Quin-2 esters [2, 3] penetrate the cell membrane, the ester is cleaved within the cell by cytoplasmic esterases and free Quin-2 accumulates in vital cells. The fluorescence of the Quin-2 molecules increases between five- to eightfold in the presence of Ca^{2+} ions [2].

The Quin-2 fluorescence in single vital cells is measurable with a flow cytometer [4]. The following problems with the Quin-2 dye were, however, encountered when the influx of Ca^{2+} ions into rat bone marrow cells was measured. First, Quin-2 has a low fluorescence yield. Second, cellular fluorescence intensity varied with cell size or esterase activity and was therefore not always proportional to the intracellular Ca^{2+} concentration. Third, the Ca^{2+} ionophore A23187 [5] which was used for Ca^{2+} calibration is highly fluorescent, which limits the sensitivity of the assay.

The recently synthesized INDO-1 [6, 7] dye, in contrast, fluoresces approximately 30 times more brightly than Quin-2. In addition, the color of the emitted fluorescent light shifts in the presence of Ca^{2+} from a peak emission at 480 nm to 405 nm. Flow cytometric measurements are, therefore, largely independent of fluorescence intensity, similar to the case of the pH-sensitive dye DCH (2,3-dicyanohydroquinone) [8] for intracellular pH measurements in vital cells. It is also favorable that the Ca^{2+} ionophore A23187 has become available in a nonfluorescent form A23187-Br [9] (HSC Research Development Corp., Toronto, Ont., Canada).

The purpose of the present study was to investigate whether Ca^{2+} levels and Ca^{2+} influx into vital cell were measurable by flow cytometry with the INDO-1 dye.

Five μl of a stock solution containing 1 mg ml^{-1} (1 m*M*) of INDO-1 ester (Molecular Probes, Junction City, Oregon, USA) as Ca^{2+} indicator and 2 mg ml^{-1} (3 m*M*) propidium iodide (Sigma Chemicals, Munich, FRG) as DNA stain to distinguish and separate dead cells from vital cells were incubated for 15 min at room temperature with 250 μl Ehrlich mouse ascites cells suspended at a concentration of 1×10^6 cells ml^{-1} in HBS buffer (0.15 *M* NaCl, 3 m*M* HEPES, pH 7.35). The solvent in the stock solution was dimethylformamide (DMF). Five μl non-fluorescent A23187-Br Ca^{2+} ionophore at a concentration of 5 mg ml^{-1} (8.3 m*M*) in DMF and 5 μl of $CaCl_2$ solutions were added to obtain extracellular Ca^{2+} concentrations between 0.1 μ*M* and 20 m*M*. Similarly 5 μl of ethylenediamino-tetraacetic acid (EDTA) stock solution were added to quantitatively chelate the Ca^{2+} ions.

The simultaneous flow-cytometric measurements of the electrical cell volume and two fluorescences of each cell were performed with a Fluvo-Metricell flow-cytometer [10] equipped with a hydrodynamically focused cylindrical sizing orifice of 80 μm diameter and 40 μm length operated at an electrical current of 0.23 mA in HBS buffer. Fluorescence was excited by a HBO-100 high-pressure mercury arc lamp (Osram, Munich, FRG) between 300 and 400 nm. Fluorescent light pulses from single cells between 418 and 440 nm for the first light channel (F1) and fluorescence pulses between 500 to 700 for the second light channel (F2) were collected, together with the electrical volume signal of each cell in List-Mode on magnetic tape, followed by evaluation and display of the data using FORTRAN IV computer programs developed earlier [11]. The F1/F2 ratio of each cell was calculated and the mean F1/F2 ratio of all cells was plotted against the extracellular Ca^{2+} concentration (Fig. 1).

The spectral response of the flow cytometer was determined by running

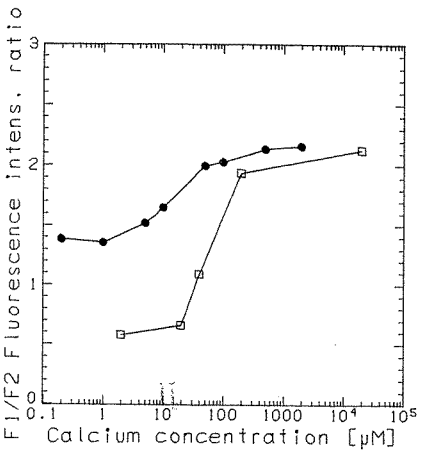

Fig. 1. Titration of free INDO-1 with Ca^{2+} in the sample beam of the flow cytometer (□) and within vital Ehrlich ascites cells (●). The F1/F2 ratios were determined from the fluorescences measured in the first (F1) and second (F2) fluorescence channel of the flow cytometer

1 m*M* solutions of INDO-1 dye through the measuring chamber in the presence of 2 μ*M* to 20 m*M* Ca^{2+}. The electrical voltage at the photomultiplier tubes of the F1 and F2 light channels was read with a voltmeter for each Ca^{2+} concentration. The resulting curve of F1/F2 ratios is also plotted in Fig. 1.

The free INDO-1 molecules enriched within the cells and a signal-to-noise ratio of 10:1 was obtained with Ehrlich ascites cells, indicating that the cells were brightly fluorescent. The addition of known extracellular Ca^{2+} concentrations and A23187-Br ionophore increased the F1/F2 ratio of the intracellular INDO-1 dye until saturation occurred around 1 m*M* Ca^{2+} (Fig. 1). INDO-1 binds Ca^{2+} in a 1:1 molar ratio. Therefore, a 50-fold enrichment of intracellular free INDO-1 over the extracellular INDO-1 ester concentration of 20 μ*M* was reached. The intracellular INDO-1 concentration was independently redetermined in the following way: First, INDO-1-stained Ehrlich ascites cells were washed five times by centrifugation (2 min, 6000 *g*) with an excess of HBS buffer and resuspended in HBS. A23187-Br ionophore, together with 2 m*M* Ca^{2+}, were added to open the cell membrane for Ca^{2+} and to saturate all intracellular INDO-1 with Ca^{2+}. Second, an aliquot of the cells was measured in the flow cytometer in the presence of 5 μm monosized fluorescent latex particles of known volume and particle concentration. The mean volume of the cells was calculated by comparison with the volume of the latex particles. The absolute cell concentration in the suspension was determined from the ratio of the number of cells to the number of fluorescent latex particles. Third, another aliquot of the cells was centrifuged and the cell pellet was lyzed with a known amount of distilled water. The fluorescence of the supernatant was determined in a Perkin Elmer LS-5 microprocessor-controlled fluorimeter (Perkin Elmer, Überlingen, FRG) in the presence of 2 m*M* Ca^{2+} after recentrifugation. The INDO-1 concentration in the lysate was calculated by comparison with measurements of solutions of known INDO-1 concentration. From these determinations, a value of 1.25 m*M* for the intracellular concentration of INDO-1 was calculated for Ehrlich ascites cells. Both values for the intracellular concentration of INDO-1 coincided reasonably.

The F1/F2 ratio of intracellular INDO-1 at low Ca^{2+} concentrations remained at a value of 1.45, which is substantially higher than the F1/F2 value of 0.60 obtained from the spectral calibration of the instrument (Fig. 1). The value could not be further decreased even in the presence of 2 m*M* EDTA and 166 μ*M* A23187-Br ionophore. This indicates that either a small amount of INDO-1 dye was in an inaccessible compartment, or that some INDO-1 had bound to cellular structures which changed its spectral properties or that part of the INDO-1 ester remained uncleaved within the cell, which is unlikely. In case the INDO-1-containing compartment is inaccessible to the ionophore, the lowest F1/F2 ratio of 1.45 for cells corresponds to a Ca^{2+} concentration of 60 μmol in such a compartment according to the calibration curve of the flow cytometer (Fig. 1).

From the ionophore experiments (Fig. 1), it is evident that Ca^{2+} influx into vital Ehrlich ascites cells can be readily monitored by a flow-cytometric measurement in the range 1 μ*M* to 1 m*M*. It was of interest whether the F1/F2 ratio of intracellular INDO-1 of cells stained in the absence of ionophore could be decreased by addition of A23187-Br ionophore and EDTA because this would indicate that not only Ca^{2+} influx but also Ca^{2+} concentrations in vital cells were measurable by this technique. This was indeed the case. The F1/F2 ratio of fresh Ehrlich ascites cells decreased from originally 1.53 to 1.25 in the presence of 2 m*M* EDTA and 166 μ*M* A23187-Br ionophore. A similar decrease was obtained for rat bone marrow cells. Extrapolation of these values on the cellular calibration curve of Fig. 1 indicates an intracellular Ca^{2+} concentration of approximately 10 μ*M*. The dissociation constant of protein-bound Ca^{2+} is in the m*M* range [12]. INDO-1 binds Ca^{2+} with a dissociation constant of 2×10^{-7} *M* [6] and accumulates in cells to concentrations in the m*M* range, as shown above. INDO-1, therefore, is capable of binding free and protein-bound intracellular Ca^{2+} in accessible compartments. The easiest explanation for the results is that the total cytoplasmic Ca^{2+} concentration of Ehrlich ascites cells is determined with INDO-1. In this case a uniform cytoplasmic stain with a decrease of fluorescence in the area of the cell nucleus is expected. This was indeed observed on microscopic examination of INDO-1-stained cells.

Altogether the flow-cytometric method seems of value for the measurement of cellular Ca^{2+} concentrations in heterogeneous cell populations of tumors, metastases, lymph nodes, thymus, and bone marrow. The advantage of the flow-cytometric method are twofold: first, the cells remain intact during staining and measurement and second, heterogeneities amongst cell populations which are lost in cuvette assays of intact cells or cell lysates can be fully appreciated. A variety of experimental possibilities exist now to either investigate the exchange of intercellular signals in complex cell culture systems or study heterogeneous cell populations within a short time after removal from the organism.

Received August 9, 1985

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