Determination of Intracellular Calcium in Vital Cells by Flow-Cytometry

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

Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} concentration. Third, the Ca\textsuperscript{2+} ionophore A23187 [5] which was used for Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} levels and Ca\textsuperscript{2+} influx into vital cells were measurable by flow cytometry with the INDO-1 dye.

Five \muL of a stock solution containing 1 mg ml\textsuperscript{-1} (1 mM) of INDO-1 ester (Molecular Probes, Junction City, Oregon, USA) as Ca\textsuperscript{2+} indicator and 2 mg ml\textsuperscript{-1} (3 mM) 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 \muL Ehrlich mouse ascites cells suspended at a concentration of 1 x 10\textsuperscript{6} cells ml\textsuperscript{-1} in HBS buffer (0.15 M NaCl, 3 mM HEPES, pH 7.35). The solvent in the stock solution was dimethylformamide (DMF). Five \muL non-fluorescent A23187-Br Ca\textsuperscript{2+} ionophore at a concentration of 5 mg ml\textsuperscript{-1} (8.3 mM) in DMF and 5 \muL of CaCl\textsubscript{2} solutions were added to obtain extracellular Ca\textsuperscript{2+} concentrations between 0.1 \muM and 20 mM. Similarly 5 \muL of ethylenediamine-tetraacetate acid (EDTA) stock solution were added to quantitatively chelate the Ca\textsuperscript{2+} ions.

The simultaneous flow-cytometric measurements of the electrical cell volume and two fluorences of each cell were performed with a Fluvo-Metricell flow-cytometer [10] equipped with a hydrodynamically focused cylindrical sizing orifice of 80 \mum diameter and 40 \mum 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 nm 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\textsuperscript{2+} concentration (Fig. 1).

The spectral response of the flow cytometer was determined by running
1 mM solutions of INDO-1 dye through the measuring chamber in the presence of 2 μM to 20 mM 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: found 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 mM 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 μM was reached. The intracellular INDO-1 concentration was independently determined 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 mM 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 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 mM EDTA and 166 μM 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 μM. The dissociation constant of protein-bound Ca$^{2+}$ is in the mM range [12]. INDO-1 binds Ca$^{2+}$ with a dissociation constant of 2 × 10$^{-5}$ M [6] and accumulates in cells to concentrations in the mM 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.