Cytosolic calcium determination: a fluorometric technique

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Overview

The activation of many mammalian cells, including human blood cells, by physiological stimuli evokes a rapid change in cytosolic Ca^{++} levels. The role of calcium as a possible messenger in signal transduction is presently under investigation due to the advent of several new highly fluorescent indicators. The Ca^{++} fluxes observed in human blood cells—platelets, neutrophils, and monocytes—are rapid, are initiated in milliseconds in some cells, are maximal by 30 seconds, and can be detected with probes such as Quin 2, Fura-2, and Indo-1. Suspension studies using spectrofluorometry, which reflect a value which is the average of all cells, are commonly used; however, recently the adaption of flow cytometry to the measurement of Ca^{++} changes in individual cells has been extremely successful. Flow cytometric measurements using a fluorescence activated cell sorter allow analysis of several activation parameters simultaneously, including changes in cytosolic Ca^{++} and pH, as well as in membrane potential, and also permit correlation of these changes with ligand and antibody binding. Since Ca^{++} release into the cytoplasma is one of the earliest signs of cell activation, the adaptation of the fluorescent Indo-1 loading technique to other cell types will further the advance of studies seeking to elucidate the role of cytosolic Ca^{++} in cell activation.

Materials and methods

Blood cell isolation

Blood was drawn from normal human volunteers and anticoagulated with 0.38% sodium citrate.

Platelet isolation

Platelets were isolated by gel filtration using a Sepharose 2B column equilibrated with HEPES buffer (137 mM NaCl, 3.3 mM NaH_2PO_4, 5.5 mM D-glucose, 2.7 mM KCl, 3.8 mM HEPES, 0.98 mM $MgCl_2$, and 0.15 Units/ml apyrase), pH 7.4 as previously described.[1]

Leukocyte isolation

Leukocytes were isolated from citrated blood by dextran sedimentation followed by Ficoll-Hypaque gradient centrifugation.[2] Polymorphonuclear leukocytes (neutrophils) were separated from contaminating erythrocytes by hypotonic lysis. The resulting cells (95% polymorphonuclear leuko-

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Supported by grants HL15335, HL07501, HL19717, and AM31056.

cytes) were resuspended in phosphate buffered saline (PBS) (125 mM NaCl, 8 mM NaH_2PO_4, 5 mM KCl, 5 mM glucose), pH 7.4, and stored on ice in a Ca^{++} and Mg^{++}-free medium to minimize aggregation.

Mononuclear leukocytes were obtained from the Ficoll-Hypaque plasma interface and subsequently washed twice in PBS, pH 7.4. Monocytes were resuspended in Medium 199 (Microbiological Associates, Inc., Bethesda, MD) supplemented with 100 U/ml penicillin-G, 100 g/ml streptomycin sulfate, and 10% fetal bovine serum (FBS), at a concentration of 2×10^7 cells/ml. The monocyte suspension was plated on plastic culture dishes and the cells were then cultured at 37°C (5% CO_2, 100% humidity) until needed (0, 24, 48, and 96 hours), as previously reported. After harvesting, the cells were resuspended in PBS + glucose and were kept on ice until needed.

Indo-1 acetoxymethyl ester loading

Stock Indo-1 acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR) was diluted to 1 mM in DMSO, aliquoted, and stored dessicated at −20°C.

Cells at a concentration of 10^7/ml (PBS + glucose) were loaded for 7 minutes with 5 μM (neutrophils)[4], or for 30 minutes with 1 μM (monocytes)[3] Indo-1 acetoxymethyl ester at 37°C. Excess probe was removed by diluting the cells five-fold with cold PBS + glucose, which was then followed by centrifugation at 150*g* for 10 minutes at 4°C. Cells were resuspended in PBS + glucose (10^7/ml monocytes, 5×10^7/ml, neutrophils) and kept on ice until needed.

Washed platelets (2×10^8 per ml) were incubated with 2 μM Indo-1 acetoxymethyl ester at 37°C for 15 minutes. Excess probe was removed by gel filtration over a second Sepharose 2B column equilibrated with HEPES buffer, pH 7.4 as described.[5-7] Platelet concentrations were determined by turbidity measurements using a Perkin-Elmer 576 spectrophotometer calibrated against a Coulter Counter. A routine wash, as described for leukocytes, was not performed since platelets are extremely fragile cells and require EDTA during centrifugation to prevent aggregation. The amount of dye remaining in the cell suspension after loading was calibrated according to the fluorescence titration curve of the cell-permeant (hydrolyzed) form of Indo-1 in a solution of digitonin lysed cells. No significant leakage of dye was detectable in leukocytes stored on ice for up to 2 hours after loading. Platelet suspensions did leak Indo-1 significantly when kept at 37°C. Storage of loaded platelets at room temperature did decrease the amount of leakage; however, a correction is still necessary and will be discussed below.

Measurement of cytosolic Ca^{++} in cell suspensions

The Indo-1 fluorescence of platelets (3×10^7/ml HEPES) or leukocytes (2×10^6/ml PBS + glucose with 1.0 mM CaCl and 1.5 mM $MgCl_2$/ml [KRP]) was monitored using a Perkin-Elmer 650/10 spectrofluorometer in a thermostated 1 cm cuvette with continuous stirring at 37°C. Excitation of Indo-1 occurs at 355 nm, with emission detected at 2 wavelengths, 405 and 485 nm, the maxima for fully Ca^{++}-liganded and for Ca^{++}-free Indo fluorescence, respectively. Since the binding of calcium alters the fluorescences at these two emission wavelengths, relative changes in the ratio F_{405}/F_{485} can be used to quantitate cytosolic Ca^{++} concentrations without regard to the quantity of probe localized in each cell or to the size of the cell.[8] Fluorescence was continuously monitored before and after stimulation at both emission wavelengths, and the ratio of intensities 405 nm/485 nm determined at 10-second intervals.

A correction for probe leakage in platelets was necessary. A sample was removed before and after stimulation for all platelet runs and centrifuged at 10,000*g*, using an Eppendorf microcentrifuge. The fluorescence of the supernatant at 405 and 485 nm (405/485 nm) was subtracted from the suspension fluorescence before calculating the ratios at the appropriate time

point. This procedure was required for platelets only, especially at low thrombin concentrations, to correct for probe leakage.

The relationship $[Ca^{++}] = K_D S(R - R_0/R_s - R)$ was adapted from the method of Grynkiewitz et al.,[8] for use in cell suspensions where R is equal to the ratio of the fluorescences emitted at 405/485 nm. The minimal Ca^{++} ratio, R_0 (Ca^{++}-free Indo), maximal Ca^{++} ratio, R_s (Ca^{++}-bound Indo-1) were determined in digitonin-lysed cell suspensions using EGTA (10 mM) or Ca^{++} (1 mM $CaCl_2$) buffers, respectively. The constant S is equal to the fluorescence of the Ca^{++}-free Indo-1 at 485 nm, determined in a 10 mM EGTA solution of digitonin-lysed cells, divided by the fluorescence of the Ca^{++}-saturated Indo-1 at 485 nM, determined in digitonin-lysed cell suspensions with 1 mM $CaCl_2$. The published K_D for Indo-1 is 250 nM at physiological pH.[8]

Measurement of cytosolic Ca^{++} in individual cells

Cells loaded with Indo-1 were diluted to the appropriate concentration (10^7 per ml HEPES buffer for platelets, 10^6 per ml KRP for leukocytes). Fluorescence measurements were obtained using a FACS 440 dual laser system (Becton Dickinson) equipped with a Consort 40/PDP-11/23 microcomputer (Digital Corp.). A primary 4 watt argon laser tuned to 357 nM was to excite Indo-1. Two fluorescence emission peaks at 405 and 485 nm were monitored, using 405/20 and 485/22 band-pass filters in front of the respective photomultipliers. Data were collected for 2-4000 resting cells at a flow rate of 1000/sec to determine the baseline distribution of probe. Stimuli were then added directly to the sample and data collected at the rate of 2-4000 cells per time point after stimulation, at approximately 5-second intervals, with the first time point at 10 seconds. All data were collected in the linear mode to allow calculation of mean channel fluorescence ratios. Changes in cytoplasmic Ca^{++} levels were calculated by subtracting the ratio of the mean channel fluorescences before stimulation from the respective ratio after stimulation at each time point.

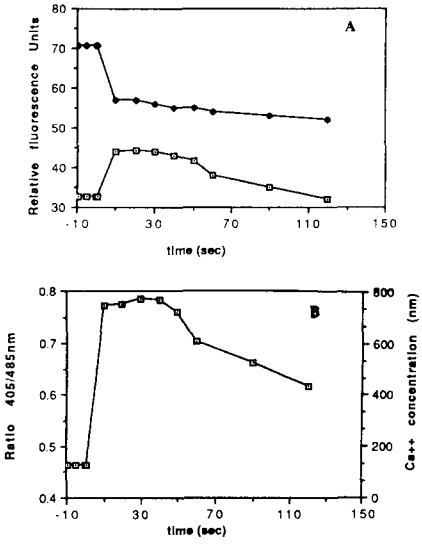

Figure 1 Tracing of thrombin-induced platelet stimulation using Indo-1. The fluorescence of human platelets (3×10^7/ml) loaded with 2 μM Indo-1 were monitored at excitation 355 nm, emission at 405 and 485 nm. An initial baseline fluorescence was determined prior to stimulation with 0.05 U/ml thrombin at time = 0 sec. The fluorescence at each wavelength was monitored versus time (*1A*). The ratio of the fluorescences at 405 and 485 nm was calculated at each time point and plotted versus time (*1B*).

Results

Cell activation studies performed by monitoring relative changes in intracellular Ca^{++} concentrations have been extremely successful in our laboratory as well as others.[1,3-7,9-16] The results for a typical platelet experiment are shown in *Figure 1*, and resemble those found with neutrophils and monocytes[3,4,13,17] *Figure 1* depicts the fluorescence of platelets loaded with 2 μM Indo-1, monitored at 405 and 485 nm (excitation at 355 nm). A baseline fluorescence level was determined at each wavelength prior to stimulation with thrombin. Indo-1 fluorescence at 405 nm increases with Ca^{++} binding and the fluorescence at 485 nm decreases with Ca^{++} binding (*Figure 1A*). The relative ratio of these fluorescences can then be calculated and plotted (*Figure 1B*). The ratio of 405/485 nm can be used to quantitate $[Ca^{++}]_{in}$, as described in *Methods*.

The flow cytometer has been used successfully to monitor thrombin-induced platelet Ca^{++} changes with Indo-1. *Figure 2* illustrates representative histograms of the baseline Indo-1 fluorescence and the subsequent histogram 15 seconds after thrombin stimulation at three thrombin concentrations. At a saturating thrombin concentration (lower panel), the histogram shifts to the right indicating an increase in the Indo-1 ratio (405/485 nm) in the entire population. The increase in the 405/485 ratio is indicative of an increase in $[Ca^{++}]_{in}$. In the upper and middle panel, one observes a partial platelet response, (i.e., some of the cells exhibit an increase in the Indo-1 ratio while others remain at the baseline fluorescence). Again the relative change in this ratio can be used to quantitate intracellular Ca^{++} fluxes.

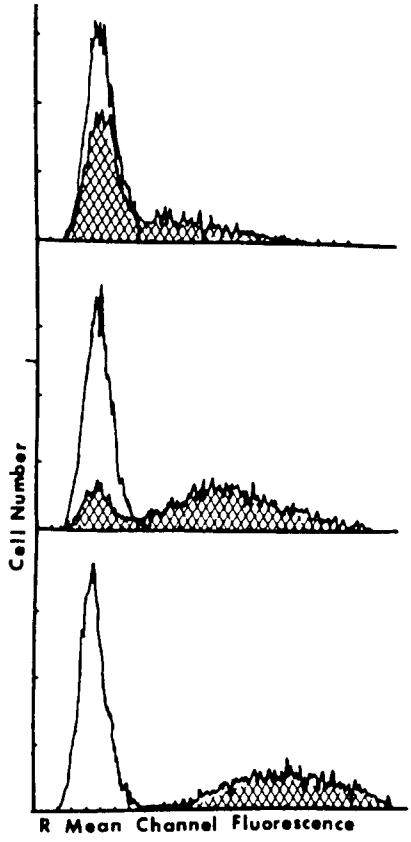

Figure 2 Flow cytometric histograms of Indo-1 loaded platelets before and after thrombin-induced stimulation of human platelets. The emissions at 405 and 485 nm of platelets (10^7/ml) loaded with Indo-1 were monitored on a fluorescence activated cell sorter. Histograms illustrate the baseline Indo-1 fluorescence (nonshaded) and the change in fluorescence 15 seconds after stimulation with 0.0025 U/ml (top panel), 0.005 U/ml (middle panel), and 0.05 U/ml (bottom panel) (shaded). Plotted is the ratio (405 nm/485 nm) of mean channel fluorescences versus platelet number.

Discussion

Many mammalian cells, including human blood cells, exhibit rapid changes in cytosolic calcium concentrations in response to physiological stimuli. With the recent advances in probe development, it has become possible to measure and study nanomolar levels of Ca^{++} within a viable cell, without significant perturbation of the working system, through the development of cytoplasmic fluorescent indicators, such as Indo-1, Fura-1, and Quin-2.[8] Quin-2 has been a useful tool for detection of basic differences in mechanisms of calcium influx and release from internal stores. However, Quin suffers from three disadvantages: a low K_D for Ca^{++} (115 nM), a significant buffering capacity which minimizes probe sensitivity at physiologic calcium ranges, and a single excitation, single emission signal so that calibration curves must depend on uniform probe concentration and cell size.[8-10]

In comparison to Quin-2, Indo-1 has a 30-fold greater fluorescence, a weaker affinity for calcium, a better selectivity against Mg^{++} and other heavy metals,[8] and a wavelength shift from 485 to 405 upon calcium binding. As a result of this shift, one can use the ratio of fluorescences at the two emission wavelengths, elicited at a single excitation wavelength, which is then independent of probe concentration and cell size, but is a function of intracellular $[Ca^{++}]$ concentrations. These characteristics allow Indo-1 to be used at very low (1 μM) concentrations, leading to an even greater reduction in the buffering of calcium and damping of its concentration transients.[8]

Our laboratory has, until recently, used changes in membrane depolarization, initial resting potentials, and degranulation, as a means to assess the stimulus response of our cell preparations. Cytosolic Ca^{++} fluxes, as well as the initial Indo-1 ratio (indicative of the resting $[Ca^{++}]_{in}$), have replaced membrane potential measurements as an accurate and reproducible control technique to evaluate our cell preparations. Indo-1 loading of cells thus indicates whether or not our cells, after preparation, are unperturbed and functionally responsive.

The fluorescence activated cell sorter enables individual cellular responses to be monitored continually after cell stimulation. Our laboratory has successfully measured intracellular Ca^{++}, pH, membrane potential changes, and ligand and antibody binding in platelets, monocytes, and leukocytes.[3-7,15,16] Due to the limitation of the lasers, Fura-2 cannot be used. The flow cytometer has several major advantages over suspension fluorometric studies: 1) a smaller number of cells is required per run; 2) continuous monitoring of individual cellular responses, including differently responding populations, is possible; 3) simultaneous binding of ligand or antibody to cell surface binding sites can be evaluated; and 4) simultaneous measurements of an additional activation parameter (e.g., pH_i, with Ca^{++}) can be accomplished with the appropriate probe and flow cytometric set-up.

Although the role of the calcium transient is still undefined, it may act as a second messenger (for review, see references 11 and 12) and is thought to play a role in the regulation of cytoskeletal protein polymerization. Calcium fluxes can occur extremely rapidly, and, in these blood cells (monocytes, platelets, neutrophils) are temporarily associated with a concomitant cytoplasmic pH change,[1,17] membrane depolarization,[1,3,6,13,14,18] and oxidative burst (in monocytes and leukocytes),[3,13,14] events whose interdependence remains unelucidated.

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