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Estimation of membrane potentials of individual lymphocytes by flow cytometry

(cyanine dyes/ionophores/lectins/ligand–receptor interactions)

HOWARD M. SHAPIRO*, PETER J. NATALE†, AND LOUIS A. KAMENTSKY†

*283 Highland Avenue, West Newton, Massachusetts 02165; and †Ortho Instruments, Westwood, Massachusetts 02090

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ABSTRACT **The membrane potentials of individual cells can be estimated by flow cytometric quantitation of the cells' uptake of the fluorescent lipophilic cationic dye 3,3′-dihexyloxacarbocyanine iodide. Human lymphocytes separated from peripheral blood on Hypaque–Ficoll gradients are uniformly depolarized by gramicidin and hyperpolarized by valinomycin. Concanavalin A and phytohemagglutinin depolarize only a fraction of the lymphocytes. The flow cytometric technique allows precise detection of heterogeneous membrane potential responses to stimuli such as lectins; it could also provide a basis for sorting cells that respond differently to a given stimulus.**

Changes in cell membrane potential occur in various cell types within seconds to minutes after binding of ligands to transmembrane receptors (1–4), and it has been suggested (4–6) that such potential changes directly mediate subsequent physiologic and metabolic responses in the cells involved. Further elucidation of this relationship has been hampered to a considerable extent because the direct measurement of membrane potentials with implanted microelectrodes is often impractical or impossible, particularly when the cells of interest are small.

Indirect methods, employing lipid-soluble cationic indicator substances, have been developed for estimation of the potentials of cells in suspension. The partitioning of such indicators between cells and the suspending medium is a function of membrane potential, with more indicator being taken up by cells upon hyperpolarization, when the interior becomes more electrically negative with respect to the exterior. Both radiolabeled (7) lipophilic cations and fluorescent cationic dyes (8–11) have been used as indirect indicators of membrane potential.

The customary measurement procedure when cationic dyes are used as potential probes involves fluorimetric determination of residual dye in the medium after uptake by cells. This provides a rapid estimation of the average potential of the suspended cells but does not allow the detection of possibly significant heterogeneity within the cell population.

The present report describes the use of flow cytometry (12) for estimation of the membrane potentials of individual living cells stained with a lipophilic cationic dye. We have used this technique to demonstrate heterogeneous membrane potential responses to lectin stimulation in lymphocytes separated from human peripheral blood.

MATERIALS AND METHODS

Cells. Suspensions enriched in mononuclear cells were prepared by centrifugation of peripheral blood from healthy adult donors over Hypaque–Ficoll gradients (13). The cells were suspended in medium 199 (GIBCO) without added protein and were maintained at $\approx 3 \times 10^6$ cells per ml at 4°C until use.

Sample Preparation. The indicator dye used was 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] (Eastman) (8, 9). The cells to be studied were diluted to $\approx 3 \times 10^5$ cells per ml with protein-free medium 199; 10 μl of 5 μM $DiOC_6(3)$ in ethanol was then added to each milliliter of cell suspension, and the cells and dye were allowed to equilibrate for 15 min at room temperature before measurement. Lectins and ionophores, when used, were added to test cell suspensions at the same time as the dye; the incubation time between dye (and lectin or ionophore) addition to cells and flow cytometric analysis was kept constant for all samples in any given experiment. Valinomycin (Sigma; final concentration, 6 μM) and gramicidin (ICN; final concentration, 20 μM) were added from stock solutions in ethanol; phytohemagglutinin (PHA) (PHA-P, Difco; final concentration, 20 μg/ml) and concanavalin A (Con A) (Sigma; final concentration, 10 μg/ml) were added from saline stock solutions.

Measurement Procedure. After incubation, samples were analyzed in a modified Ortho Instruments FC-200 flow cytometer, using 4-mW excitation at 488 nm from an argon ion laser source. The green fluorescence emitted by intracellular $DiOC_6(3)$ was measured in the wavelength range 530–580 nm, and distributions of fluorescence from individual cells were accumulated in a multichannel pulse height analyzer (Ortho Instruments 2102). Laser power, fluorescence detector gain, and pulse height analyzer display settings were kept constant for all samples in an experiment.

Because the dye and the ionophores adhere to the plastic tubing used in the fluid handling system of the flow cytometer, we flushed the lines before and between samples with Clorox, followed by ethanol and then by a solution of 50 nM dye in cell-free medium.

RESULTS AND DISCUSSION

Fig. 1 shows distributions of total intracellular $DiOC_6(3)$ fluorescence in a control cell population and in cell populations exposed to gramicidin, valinomycin, Con A, and PHA. Gramicidin, which is known to depolarize the cell membrane, decreases the mean $DiOC_6(3)$ fluorescence per cell as well as the total amount of dye taken up by cells. Valinomycin, which hyperpolarizes the membrane, produces corresponding increases in dye uptake. When a high-potassium, low-sodium medium is used in place of medium 199, minimizing the potential change produced by valinomycin addition (8–11), we do not observe increased dye uptake in valinomycin-treated cells (data not shown). We conclude, on these bases, that intracellular $DiOC_6(3)$ fluorescence reflects the membrane potential of the cell.

The Con A-treated cell sample, when compared to the control, shows a small subpopulation of cells with decreased fluorescence, apparently resulting from depolarization. A larger

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Abbreviations: Con A, concanavalin A; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; PHA, phytohemagglutinin.

subpopulation of apparently depolarized cells can be seen in the sample treated with PHA. This result is in agreement with Taki's finding (1), based upon direct microelectrode measurements, that the mean membrane potential of lymphocyte populations is decreased within minutes after exposure to PHA.

Although lysis of the cell membrane would be expected to abolish the membrane potential, it can be excluded as a basis for the depolarization observed in our lectin- (and gramicidin-) treated cells; the nuclei of these cells do not stain when ethidium bromide is added to the isotonic medium, whereas the nuclei of lysed cells do.

When membrane potentials are estimated from bulk measurements of the fluorescence of cell suspensions, profound changes in potential in a fraction of the cells may go undetected if the affected subpopulation is not sufficiently large to shift the population mean. The flow cytometric measurement detects such changes, and thus allows a distinction to be made between agents such as the ionophores, which affect the entire cell population uniformly, and the lectins, which appear to have different effects upon the membrane populations of different cell subpopulations.

The fluorescence distributions in Fig. 1 are typically rather broad, with coefficients of variation of approximately 30%. It is not yet clear how much of the observed cell-to-cell variation in fluorescence reflects actual differences in membrane potential. In theory, it is the ratio of the concentration of free dye in the cell to that in the medium which should be directly dependent upon membrane potential. Intracellular free dye, however, is typically in equilibrium with a considerably larger amount of dye bound to various cytoplasmic constituents. Cell-to-cell variations in the amount of such dye-binding material would, therefore, be expected to broaden the observed fluorescence distribution even if the membrane potentials of all cells in a suspension were the same.

In cells of similar composition and different size, the amount, as opposed to the concentration, of intracellular dye is a function of cell size. The breadth of the $DiOC_6(3)$ fluorescence distributions in our lymphocyte populations cannot, however, be explained entirely by size differences, because the coefficient of variation of a cell size distribution obtained by forward light scattering measurements is less than 15%. In work with cultured cell lines, which have broader size distributions, we have observed that the distribution of the ratio of the fluorescence signal to the scatter signal is less broad than the fluorescence distribution (data not shown); we would expect the ratio to provide a more accurate estimate of membrane potential.

It should be possible to determine the accuracy of the fluorescence-to-scatter ratio directly by making flow cytometric measurements of liposome suspensions prepared with known constant membrane potentials. In the absence of significant sources of error in the measurement, values of the fluorescence-to-scatter ratio for individual liposomes would be expected to be narrowly distributed about the mean.

The partitioning of dyes between cells and the medium may be profoundly affected, in the absence of changes in membrane potential, by the addition of dye-binding substances to the medium. As the serum or protein concentration of the medium is increased, the amount of dye taken up by the cells decreases. Because a substantial fraction of the total amount of dye in the suspension may be taken up by cells (9), sample-to-sample variation in cell concentration can also have significant effects on the mean intracellular dye content. When protein and cell concentrations are controlled, however, the method yields reproducible results.

Potential changes after ionophore addition or lectin–receptor interaction (4) may occur more rapidly than does reequilibration of dye (or radiolabeled cation) across the membrane in response to the change. We have used flow cytometry to follow the time course of fluorescence changes in cells equilibrated with dye and then exposed to agents that alter potential; after valinomycin or gramicidin addition, the distribution typically reaches a new stable position within 3 min.

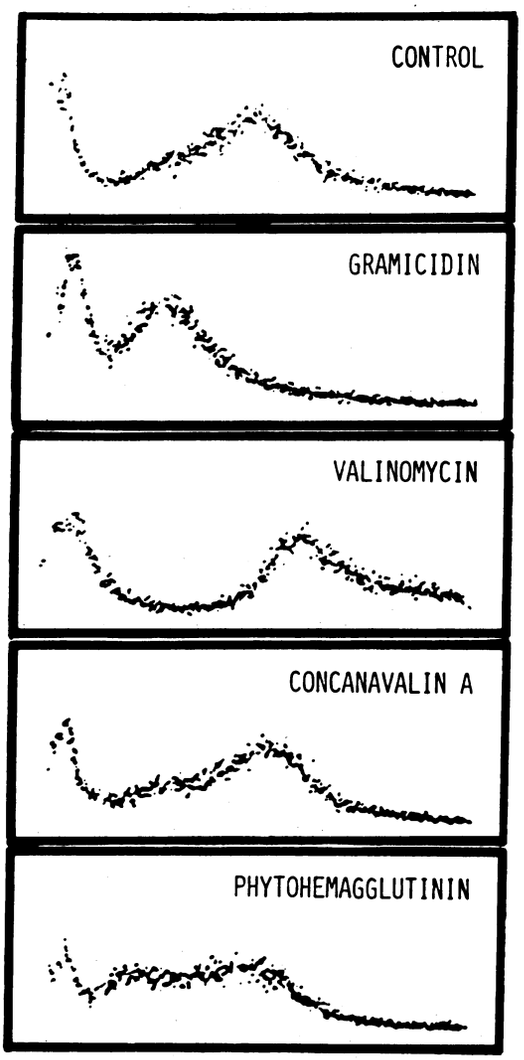

FIG. 1. Distributions of fluorescence of intracellular $DiOC_6(3)$ in a control sample of a human lymphocyte suspension and in aliquots of the same cell suspension exposed to gramicidin, valinomycin, Con A, and PHA. The horizontal coordinate represents the measured value of fluorescence intensity; the vertical coordinate indicates the number of cells with that measured fluorescence value. The relatively sharp peak at the left of each distribution results from residual erythrocytes, which remain after lymphocyte separation on Hypaque–Ficoll gradients.

Measurement of additional parameters with the flow cytometer (12) should allow us to study correlations between membrane potential and other properties of cells—e.g., cytoplasmic structure, DNA content, and the presence of various surface markers. We infer that membrane potential responses may be useful for classification of cell subpopulations; in the particular case of lymphocytes, for example, almost all peripheral blood lymphocytes bind PHA and Con A, whereas only some appear to change their membrane potentials after lectin binding. Preliminary studies with T-cell enriched and B-cell enriched samples suggest that nearly all of the T cells and few, if any, other lymphocytes are depolarized by PHA. Further

confirmation of this may be obtained by analysis of the surface antigens of cells that are and are not depolarized by the lectin.

It should be possible to use cell sorting (12) to separate individual viable cells for further study based on the magnitude and direction of their membrane potential response to a given stimulus. This would permit analyses of the relationship of potential response to later events such as growth and differentiation in culture. Although $DiOC_6(3)$ may be photosensitizing and toxic at the concentrations (1–2 μM) used for potential measurements in cell suspensions (14), we have been able to use much lower concentrations (as low as 2 nM) for flow cytometric measurements, and therefore we expect lower short-term toxicity.

We anticipate that the technique we have described will prove useful for experimental and clinical studies of cellular physiology, pharmacology, and immunology.

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