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CALCIUM-INDUCED HETEROGENEOUS CHANGES IN MEMBRANE POTENTIAL DETECTED BY FLOW CYTOFLUORIMETRY

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Ionophore-induced changes in the cell-associated fluorescence of samples of approx. 50 000 individual murine L1210 leukemia cells which had been incubated with the voltage-sensitive dye 3,3′-dihexyloctacarbocyanine iodide (DiOC6(3)) were monitored by flow cytometry. The K^+ ionophore valinomycin (1 μM) produced homogeneous changes in the fluorescence of the entire population, the magnitude of which was dependent upon the concentration of extracellular K^+. These changes allowed the estimation of the potassium equilibrium potential of the cells, by the null-point method, to be -11.9 mV. The Ca^{2+} ionophore A23187 (500 nM) produced heterogeneous changes in fluorescence, with populations of both hyperpolarised and depolarised cells. In addition, the depolarised population underwent an apparent size change, with a reduction in cell volume. This heterogeneity of response resulted in a minimal change in the median fluorescence value for the whole population, which suggests that it would not have been detectable by methods dependent upon net population-averaged changes in fluorescence. Removal of extracellular Na^+ or preincubation of cells with amiloride (500 μM) effectively eliminated the depolarised population. Removal of extracellular K^+ increased the hyperpolarised population. These findings provide evidence for the presence of Ca^{2+}-induced Na^+ exchange and Ca^{2+}-induced K^+ efflux mechanisms in these cells which may be expressed simultaneously in the cell population.

Introduction

Changes in the membrane potential of cells reflect some of the complex alterations of ionic homeostasis which are associated with cell activation, such as the response to hormonal stimuli, and studies of these changes are widespread. In small cells, these studies have been facilitated by the development of indirect fluorescent or radiochemical probes for the measurement of membrane potential, and their employment has circumvented some of the technical difficulties associated with the use of classical electrophysiological methods [1–6]. There are, however, a number of problems associated with the use of these probes, particularly with the fluorescent dyes [7–10], not the least of which is that the measurement of the accumulation of the probe in a large population of cells has to be averaged, either in terms of fluorescence or the accumulation of a radiolabel, over the entire population. This renders such methods incapable of the detection of either inherent or induced heterogeneity of response. The technique of flow cytometry allows the accumulation of data on the measurement of individual cell-associated fluorescence in a large population of cells within a short

time period, and so circumvents this problem when a fluorescent probe is used.

Few studies of membrane potential have utilised this technique [11–13]. In one such investigation, where an inherently heterogeneous cell population was used in a mixed lymphocyte reaction, quantitation of their different responses in membrane potential was achieved by the construction of dual-parameter isoamplitude plots [13]. A similar approach has been employed in this report which utilises the murine leukemia cell line L1210. The results show that the technique allows the detection of A23187-induced, heterogeneous changes in membrane potential which would not have been detected by population-averaged methods.

Materials and Methods

Exponentially growing L1210 cells were harvested from Fischer's medium containing 10% horse serum (Gibco Laboratories) and were equilibrated at $5 \cdot 10^5$ cells/ml for 1 h at 37°C in Fischer's medium alone, gassed with 5% CO_2/95% air. Aliquots were removed and allowed to reach room temperature (21°C) and then 300 nM of DiOC6(3) (Kodak Ltd.) was added in ethanol (0.5%, v/v, final concentration). Ionophores and other compounds were added simultaneously with the dye and the mixtures were incubated in the dark for 10 min. Samples, filtered through 37 nylon mesh (Small Parts Inc., Miami, FL), were then assayed for fluorescence and light scattering on a Becton Dickinson FACS IV flow cytometer (Sunnyvale, CA.) mounted with a Spectra Physics 164-05 laser (Mountview, CA.) operating at 488 nm, with 520 nm- and 535 nm-long pass barrier filters (Ditric Optics Inc., Hudson, MA.), which were used to distinguish fluorescence from light scatter. Data on the fluorescence and light scatter of individual cells were accumulated on approx. 50 000 cells in the 'DPC dual parameter mode' and isoamplitude plots were constructed by use of a computer program similar to that described previously [13]. Experiments were reproduced a minimum of three times. For the 'Null point' assay of the potassium equilibrium potential, L1210 cells were resuspended in a medium containing a total of 156 mM of combinations of KCl and NaCl, together with 2 mM $MgCl_2$ and 5 mM glucose in 0.1 mM Tris-HCl (pH 7.4). Data were accumulated as above, with a precise 10 min incubation with dye and either 0.5% ethanol (v/v) (control) or 1 μM valinomycin (0.5% ethanol (v/v) final concentration) with varying K^+ concentrations. When Na^+-, K^+-, or Ca^{2+}-free media were used, choline chloride was substituted for either NaCl or KCl, and Ca^{2+} was omitted from a Krebs-Ringer solution consisting of 118 mM NaCl, 4.7 mM KCl, 25 mM $Na(K)HCO_3$, 1.18 mM $MgCl_2$, 1.17 mM $Na(K)H_2PO_4$, 600 μM $CaCl_2$, (equivalent to the Ca^{2+} concentration in Fischer's medium), and 5.6 mM glucose.

Results and Discussion

Fig. 1a shows an isoamplitude plot of the distribution of the voltage-sensitive dye DiOC6(3) in a sample of 52 829 cells. In recording the data in the dual parameter mode, the ordinate is an arbitrary scale of 1 log unit of fluorescence and the abscissa a linear, arbitary scale of light scatter. Both scales, as shown in Fig. 1c, are divided into a grid of 64 channels. To construct the isoamplitude plots, corresponding channels of fluorescence and light scatter with the maximum number of cells were recorded and all other corresponding channels were represented as a decile fraction of this number. Thus, in Fig. 1a, the cell number in the peak corresponding channels was 254; this was designated as being equal to 10 (= *), together with those channels lying within 10% of this value. For each experiment, the cell number in the peak channels and the medians of fluorescence and light scatter were calculated.

Comparisons of the distribution of cell volume, as measured by a Coulter Counter Channelyser (inset Fig. 1a, ν, frequency), and forward light scatter, show them to correspond closely, as has been reported by others [14]. In a large series (greater than 200) of such isoamplitude plots of L1210 leukaemia cells, a homogeneous population was observed, with the dye being distributed normally according to cell size. Such a distribution is interesting, given the evidence that profound changes in E_m have been reported to occur during the cell cycle, particularly a large depolarization at the G_1-S interface of the cell cycle [15]. It might, therefore, have been expected that cells in the

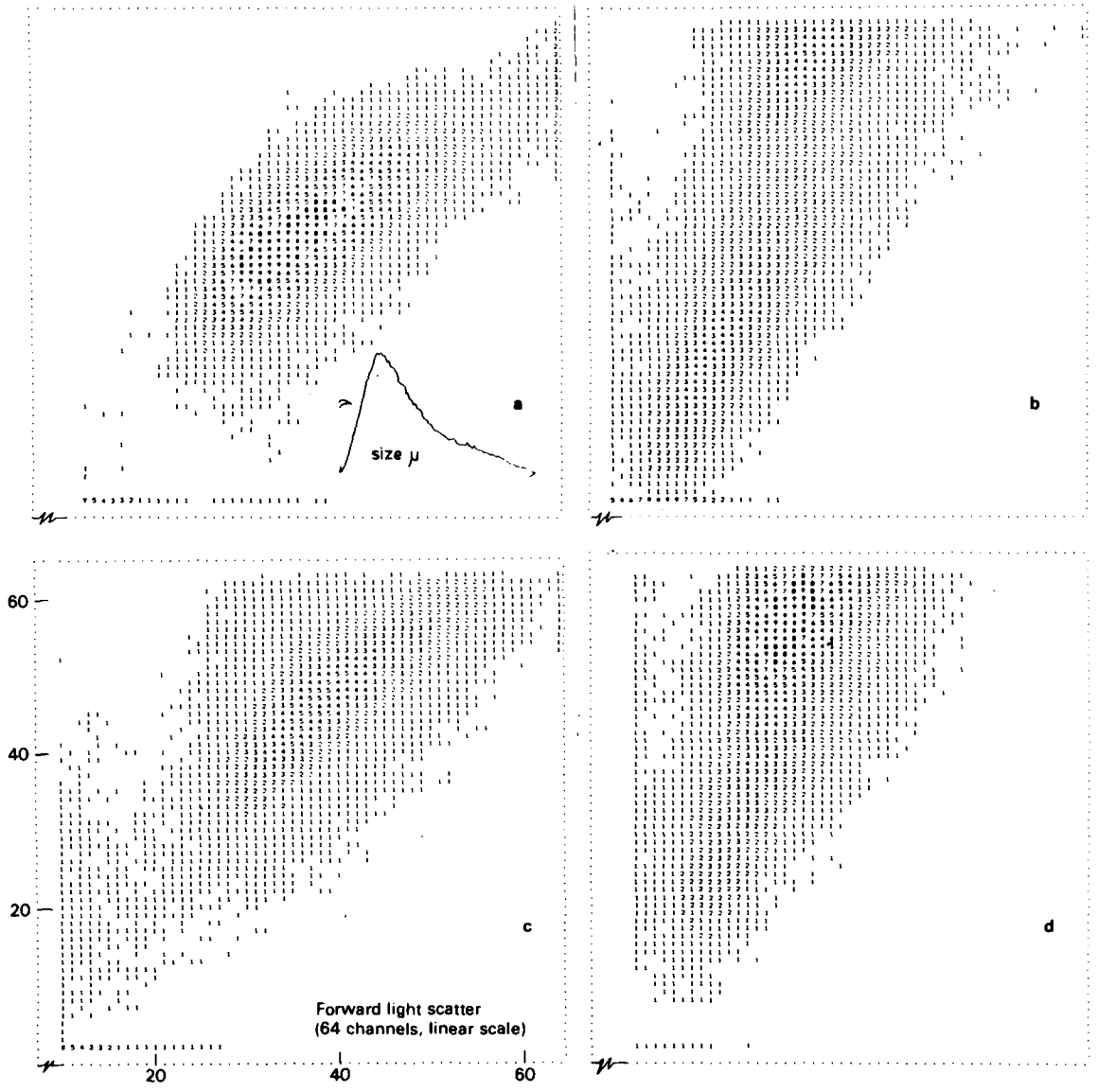

Fig. 1. Isoamplitude plots of fluorescence versus forward light scatter. Approx. 50000 L1210 cells which had been incubated for 10 min with 300 nM DiOC6(3) in Fischer's medium were monitored for fluorescence at 488 nm with a FACS IV flow cytometer. On a 64×64 channel grid, an isoamplitude plot was constructed with the peak channel(s), which contained the maximum number of cells of a particular, corresponding fluorescence and light scatter which is denoted as *(= 10). The remaining population was then denoted as decile fractions of this value. a, controls; b, 500 nM A23187; c, 500 nM A23187 with 500 μM amiloride; d, K^+-free potassium Ringer plus 500 nM A23187.

lower size channels, which centrifugal elutriation and cytometry have shown to be in the G_1-S phase (data not shown), would contain a high proportion of depolarized cells. The nature of these profound changes in E_m, which were induced experimentally in mouse lymphocytes by a variety of mitogens, has been questioned recently [16] and it remains to be determined, using synchronized cells from elutriation experiments, to clarify whether cell cycle-dependent changes in E_m occur with L1210 cells which are progressing normally through the cell cycle.

It was first established by a null point assay of the K^+ equilibrium potential that changes in fluorescence of L1210 cells incubated with DiOC6(3) were E_m-related [17]. In common use, the voltage-sensitive changes in the fluorescence of cyanine dyes, such as used here, are recorded as a function of changes in the extracellular dye fluorescence, that accumulated by the cell according to its potential being quenched by an undefined mechanism. Thus, there is normally an inverse relationship between E_m and fluorescence [17]. In the present experiments, changes in what must be assumed to be the cell-associated, unquenched fluorescence of the dye were recorded, and a direct relationship between this cell-associated fluorescence and E_m needs to be established.

There are a number of problems associated with the 'null point' assay [7,8], not the least of which are the effects that both valinomycin and the dye may have on cellular energetics. Some controversy also exists regarding the valinomycin concentrations required to dissipate the plasma membrane K^+ gradient [18–19]. We found that valinomycin concentrations of 5 μM caused a consistent hyperpolarization (i.e., a relative increase in fluorescence) of the entire population of cells in a homogeneous manner, even when potassium concentrations as high as 150 mM were used. With 1 μM valinomycin, variable extracellular potassium concentrations and a dye concentration of 300 nM, however, changes in the fluorescence median (ordinate in Fig. 1a) were consistent with the cells having a K^+ equilibrium potential. Fig. 2 shows the changes in the median fluorescence which occur under such varying conditions of K^+ concentration. An extrapolated null point of 73 mM K^+ at 21°C and an internal K^+ concentration of 117 mM, determined by the methodology of Gargus and Slayman [20], gave a value for the K^+ equilibrium potential, calculated as described by Laris et al. [17], of -11.9 mV. This null point value is similar to that obtained by the use of a dye in Ehrlich ascites cells [17].

Equilibration of the potassium gradient across the plasma membrane by valinomycin and of other ions by other ionophores may lead to concomitant changes in the concentration of associated ions, including H^+, in the cell. It is therefore possible that the observed changes in cell-associated fluorescence reported here, and by others [11–13], may represent changes in the pattern of intracellular dye distribution, binding and/or quenching of fluorescence brought about by a combination of factors, in addition to changes in the potassium equilibrium potential. This problem, barely addressed in other studies which utilise direct measurements of cell-associated fluorescent probes of membrane potential [11–13] or pH [21], demands detailed analysis of the behaviour of the fluorescent probe DiOC6(3) under each particular condition of changing intracellular ion concentration. Model studies, using different but related dyes, suggest that there may be heterogeneity in the fluorescence signal according to whether they are bound to different cellular environments [22,23]. However, limited studies comparing cell-associated fluorescence of DiOC5(3) in human neutrophils with the radiochemical probe of potential, trimethylphenylphosphonium bromide, showed identical results for the two probes of potential [11]. With the above-mentioned reservations in mind, we have, like others, assumed that increases in fluorescence represent hyperpolarisation and that decreases in fluorescence represent

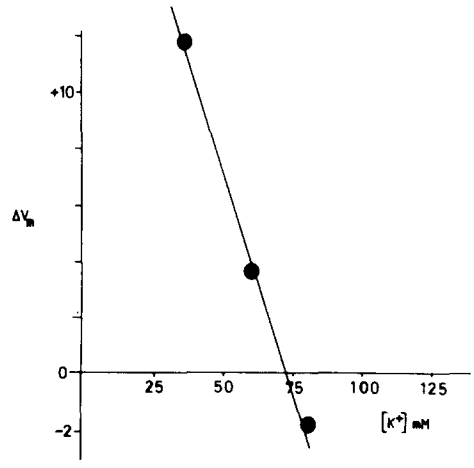

Fig. 2. The potassium equilibrium potential of L1210 cells. Approx. 50000 L1210 cells incubated with 300 nM DiOC6(3) in a medium with varied K^+ concentration with or without 1 μM valinomycin for 10 min, were monitored for fluorescence with a FACS IV flow cytometer. The change in median fluorescence (ΔV_m) between controls and ionophore-treated cells was measured. The extrapolated 'null point' at which $V_m = 0$ was 73 mM K^+. Results are the means of three experiments.

the depolarisation of L1210 cells under all the conditions reported here but, clearly, future studies must address the problems of fluorescence heterogeneity, which may be generated by particular conditions of changed intracellular ionic concentration.

The generally homogeneous responses of L1210 cells to the K^+ ionophore valinomycin, in which the majority of the cell population moved to a higher or lower median fluorescence according to external K^+ concentration, contrasts markedly with that obtained when cells were incubated with 500 nM of the calcium ionophore A23187 (Fig. 1b). With this agent, the isoamplitude plot demonstrated the disadvantages of population-averaged determinations of membrane potential, since it shows that despite an insignificant change in the population-averaged median fluorescence (V_m) (control $V_m = 34.79$; 500 nM A23187 $V_m = 35.76$), distinct and reproducible (18 experiments) heterogeneity of response occurs. This pattern of response was completely lost when Ca^{2+} was removed from the external medium, suggesting that the changes induced by this low concentration of A23187 were due to an increase in cytoplasmic Ca^{2+}. As the concentration of A23187 was progressively increased from 500 nM to 10 μM, a concomitant loss of the fraction of hyperpolarised cells and a parallel increase in the depolarised fraction occurred.

In addition to the observation of the distinctly heterogeneous populations of cells measured by fluorescence, changes in cell size by forward-light scattering were also produced. The depolarized population in Fig. 1b, with a median fluorescence channel of 21, consisted of cells which were smaller than those of the control population and which constituted approx. 28% of the cell population. The relatively hyperpolarized fraction in Fig. 1b, at median fluorescence channel 59 on the ordinate, had a light scatter median of 36, which was similar to the control median (Fig. 1a). We can only speculate on this apparent size change in the depolarized cells, and suggest that as intracellular Ca^{2+} levels rose to a certain threshold value, contractile elements of the cytoskeleton were activated or that the cell volume was changed osmotically. This action would also have the effect of producing a relatively higher intracellular Ca^{2+} concentration, which appears to coincide with, or is responsible for, the observed depolarization. Experiments to clarify these alternatives are in progress.

Coincubation of L1210 cells with 500 nM A23187 and 500 μM of amiloride effectively prevented the appearance of the depolarized population (Fig. 1c); it is presumed that this is evidence for a Na^+-induced depolarization mediated by the electrogenic Na^+-Ca^{2+} exchanger which amiloride has been reported to block [24]. This is a ubiquitous transport mechanism for maintaining low intracellular Ca^{2+} [25]. Amiloride itself had no effect on the potential of L1210 cells, which suggests that the Na^+-Ca^{2+} exchanger is normally electrically silent. Experiments conducted in Na^+-free Krebs solution with 500 nM A23187 gave a similar result, supporting the hypothesis that the depolarisation was brought about by sodium influx, but direct proof of the involvement of the Na^+-Ca^{2+} exchanger awaits determinations of ion flux and these experiments are currently being undertaken.

The hyperpolarized fraction shown in Fig. 1b, which represents approx. 22% of the entire population in Fischer's medium ($[K^+] = 5.3$ mM), increased to 46% when 500 nM A23187 were added to L1210 cells suspended in K^+-free Krebs medium (Fig. 1d). This finding is indirect evidence for the presence of a Ca^{2+}-activated K^+ efflux channel in these cells, which resembles that of normal mouse lymphocytes [26]. Further studies to characterize the mechanism of Ca^{2+}-induced hyperpolarization of these cells are underway. One possible alternative mechanism is that if Na^+-influx mediated by Na^+-Ca^{2+}-exchange occurs, this may stimulate the $(Na^+ + K^+)$-ATPase. However, 1 mM ouabain which is a concentration of ouabain which effectively blocks ^{86}rubidium transport into these cells and, therefore, affects $(Na^+ + K^+)$-ATPase activity (Chahwala and Hickman, unpublished data) did not block the A23187-induced hyperpolarization.

In conclusion, it has been demonstrated that in L1210 murine leukemia cells, changes in intracellular Ca^{2+} induced by the ionophore A23187 cause heterogeneous changes in the fluorescence signal from L1210 cell-associated DiOC6(3) as indicated by flow cytofluorimetry. A null point assay of the potassium equilibrium potential supports the hy-

pothesis that these changes in fluorescence are related to membrane potential and, at this stage, it is presumed that this relationship extends to the changes in fluorescence brought about by alterations of calcium homeostasis. From these experiments it appears that these cells may have a Ca^{2+}-activated K^+ efflux channel and a Na^+-Ca^{2+} exchange system which are possibly activated sequentially as the internal concentration of Ca^{2+} increases, a hypothesis which is currently under investigation. Such heterogeneity of response is not assessable by population-averaged methods using radiochemical probes or fluorescent dyes. These methods may lead to misinterpretation of the nature of the ionic events triggered at the plasma membrane in response to various stimuli, many of which may involve changes in Ca^{2+} concentration, not only when heterogeneity of cell type exists, such as in mixed lymphocyte reactions, but also when cell lines are used.

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References

1 Cohen, L.B., Salzberg, B.M. and Grinvald, A. (1978) Annu. Rev. Neurosci. 1, 171–182
2 Waggoner, A.S. (1979) Annu. Rev. Biophys. Bioeng. 8, 47–63
3 Waggoner, A.S. (1979) Methods Enzymol. 55, 689–695
4 Grinvald, A., Hildesheim, R., Farber, I.C. and Anglister, L. (1982) Biophys. J. 39, 301–308
5 Lichtstein, D., Kaback, H.R. and Blume, A.J. (1979) Proc. Natl. Acad. Sci. USA 76, 650–654
6 Cheng, K., Haspel, H.C., Vallano, M.L., Osotimehin, B. and Sonenberg, M. (1980) J. Membrane Biol. 56, 191–201
7 Johnstone, R.M., Laris, P.C. and Eddy, A.A. (1982) J. Cell. Physiol. 112, 298–301
8 Smith, T.C. (1982) J. Cell. Physiol. 112, 302–305
9 Rink, T.J., Tsien, R.Y. and Pozzan, T. (1982) J. Cell. Biol. 95, 189–196
10 Rogers, J., Hesketh, T.R., Smith, G.A., Beaven, M.A., Metcalfe, J.C., Johnson, P. and Garland, P.B. (1983) FEBS Lett. 161, 21–27
11 Seligmann, B.E. and Gallin, J.I. (1983) J. Cell. Physiol. 115, 105–115
12 Levinson, R., Macara, I.G., Smith, R.L., Cantley, L. and Housman, D. (1982) Cell 28, 855–863
13 Nerl, C., Valet, G., Schendel, D.J. and Wank, R. (1982) Naturwissenschaften 69, 292–294
14 Mullaney, P.F. and Dean, P.N. (1970) Biophys. J. 19, 764–772
15 Keifer, J., Blume, A.J. and Kaback, H.R. (1980) Proc. Natl. Acad. Sci., USA 77, 2200–2204
16 Deutsch, C. and Price, M. (1982) J. Cell. Physiol. 113, 73–79
17 Laris, P.C., Pershadsingh, H.A. and Johnstone, R.M. (1976) Biochim. Biophys. Acta 436, 475–488
18 Felber, S.M. and Brand, M.D. (1982) FEBS Lett. 150, 122–124
19 Smith, T.C. and Robinson, S.R. (1981) J. Cell. Physiol. 106, 399–406
20 Gargus, J.J. and Slayman, C.W. (1980) J. Membrane Biol. 52, 245–256
21 Murphy, R., Powers, S. and Cantor, C.R. (1984) J. Cell. Biol. 98, 1752–1762
22 Ivkova, M.N., Pechatnikov, V.A. and Ivkov, V.G. (1983) Gen. Physiol. Biophys. 2, 473–486
23 Packard, B.S., Karukstis, K.K. and Klein, M.P. (1984) Biochim. Biophys. Acta 769, 201–208
24 Smith, R.L., Macara, I.G., Levinson, R., Housman, D. and Cantley, L. (1982) J. Biol. Chem. 257, 773–780
25 Blaustein, M.P. and Nelson, M.T. (1982) in Membrane Transport of Calcium (Carafoli, E., ed.), pp. 217–236, Academic Press, New York.
26 Rink, T.J., Montecucco, C., Hesketh, T.R. and Tsien, R.Y. (1980) Biochim. Biophys. Acta 595, 15–30