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Wide applicability of a flow cytometric assay to measure absolute membrane potentials on the millivolt scale

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Abstract To prove the general applicability of a recently published flow cytometric method to determine the membrane potentials of cells on the absolute (mV) scale, the validity of the premises involved were analyzed individually. Experimental evidence was given for bis-oxonol, the applied membrane potential indicator being a Nernstian dye. The results of special measurements proved that extracellular dye concentrations were not affected by cellular dye uptake under the applied experimental conditions and that the dye content of the suspending medium did not contribute directly to the measured cellular fluorescence. A direct, membrane-potential-independent contribution of the extracellular dye to cellular fluorescence was also found to be negligible, as membrane potential values of the same type of cells evaluated from measurements in the presence of different extracellular oxonol concentrations were very close to each other. The transmembrane potential of B lymphoid JY cells was measured by the method as a function of cell density in the tissue culture. Cells isolated during the log phase of growth displayed a -40 ± 4 mV membrane potential. At a high density of the culture (plateau phase), a significant increase of the membrane potential to -61 ± 3 mV was observed and a medium value of -47 ± 3.5 mV was measured at an intermediate density of the cells. Our observation indicates that nonadherent cells can also be hyperpolarized when optimal growth conditions are terminated.

Key words Fluorescence · Flow cytometry · Absolute membrane potential

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Introduction

The central role of intracellular ion concentrations in different cell physiological processes is well documented (Casabiell et al. 1991; Grinstein and Dixon 1995; Voets et al. 1995; Rader et al. 1996; Balkay et al. 1997). Transmembrane potentials may play a role in signal transduction and differentiation in a number of cells (Magni et al. 1991; Damjanovich et al. 1994; Jung et al. 1994; Loza et al. 1995). Changes in membrane potential occur in various cells within seconds after binding of ligands to appropriate receptors (Pandiella et al. 1989) and these changes may directly mediate subsequent physiological and metabolic responses in the cells involved (Szöllősi et al. 1987; Nilius et al. 1993; Riviere et al. 1995).

We recently described a fluorescence flow cytometric method which allows quantification of membrane potential measurements (Krasznai et al. 1995), but the fulfillment of the underlying suppositions was not critically investigated. In the present communication we present a thorough and detailed analysis of the fundamental assumptions on which the developed method is based. The method was also applied to monitor membrane potential changes accompanying the growth of B lymphoid JY cells from a starting state of a relatively low inoculum to a high-density plateau phase with blocked mitosis.

Materials and methods

Reagents

All chemicals were of analytical or spectroscopic grade. Bis(1,3-dibutylbarbituric acid(5)) trimethine oxonol [diBA-C_4-(3)] and the tetraacetoxymethyl ester of the dye 2′,7-bis(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF-AM) were obtained from Molecular Probes (Ore, USA) and inorganic chemicals were from Sigma. Phosphate buffered saline (PBS) contained 140 mM NaCl, 5 mM KCl, 8 mM Na_2HPO_4, and 3 mM NaH_2PO_4 at pH 7.3.

Cells

The JY cell line was maintained in RPMI-1640 medium completed with 10% fetal calf serum and antibiotics at 37 °C in a 5% CO_2 atmosphere. For experiments to study the effect of cell density, JY cultures were seeded at a concentration of 1, 0.4 and 0.18×10^6 cells/ml (samples A, B, and C, respectively) using the same cell population of plateau phase cells (having reached a density of 10^6 cells/ml) and cultured for 20 h. Before use the cells were washed with and resuspended in PBS at a concentration of 2×10^7/ml. The cell viability was routinely determined by trypan blue exclusion and it was more than 90% in each case. The cells were fixed by 2% of ice-cold formaldehyde as described previously (Krasznai et al. 1995).

The absolute membrane potential of the cells was measured by flow cytometric assay (with oxonol dye) and with the patch clamp as the reference technique (Krasznai et al. 1995; Panyi et al. 1996).

Determination of absolute membrane potential

The method has been described in detail by Krasznai et al. (1995) and can be summarized briefly as follows.

1. Free dye is assumed to distribute across the membrane according to the Nernst equation

$$\psi = RT/F \ln [D_i/D_e] \quad (1)$$

where ψ, R, T, F, D_i and D_e denote transmembrane potential, universal gas constant, absolute temperature, Faraday constant, and intracellular and extracellular concentration of the free dye, respectively. With this assumption the absolute value of ψ can be easily calculated if the free dye concentration is known on the two sides of the cytoplasmic membrane. Samples of the investigated cells were stained with diBA-C_4-(3) of different concentration (between 50 nM and 2 μM) in the suspending PBS and analyzed with the flow cytometer. A calibration curve was constructed from the observed data, displaying mean values of the measured histogram of the cellular fluorescence versus extracellular dye concentration (like curve A on Fig. 1). Owing to the interrelationship between extracellular and intracellular free dye concentrations and the membrane potential of the cells (see Eq. 1) used for the construction of the calibration curve, abscissa of this plot can be scaled either in terms of D_e or D_i (see scale on top of the figure) as

$$D_i = D_e \exp [F\psi/RT] = D_e\, C \quad (2)$$

where C is constant as long as ψ does not change, though its numerical value is not known.

2. The membrane potential of the sample of interest can be calculated from the ratio D_i^S/D_e^S using the Nernst equation (superscript S refers to the sample of interest). It is generally assumed that cell-related dye fluorescence for the same type of cells is determined entirely by the intracellular dye concentration. Based on this assumption, the intracellular dye concentration, D_i^S, in the sample of interest will be the same as that ($D_{i,c}^S$) in the very sample having the same cellular fluorescence intensity (FI^S) out of those used to construct the calibration curve. Let us denote the extracellular concentration of the dye for this sample by $D_{e,c}^S$. With the help of the calibration curve the numerical value of $D_{e,c}^S$ can be identified as the abscissa of the point of the calibration curve determined by FI^S. This in turn determines the intracellular dye concentration of the sample of interest as

$$D_i^S = D_{i,c}^S = D_{e,c}^S\, C \quad (3)$$

with the unknown constant C as detailed in paragraph 1.

3. Aiming at the determination of ψ by using Eq. (1) we still use data of a formaldehyde-fixed sample to calculate the value of the denominator of the ratio D_i^S/D_e^S in Eq. (1). This sample is made up from completely depolarized (but otherwise identical to those used in the experiment) cells. Instead of determining D_e^S one can determine D_i^0 ($D_e^S = D_i^0$, where the superscript 0 refers to the sample of fixed cells with $\psi = 0$) with fixed cells stained in the presence of exactly the same concentration of the dye as the sample of interest. This is explained by the intra- and extracellular concentrations of the membrane-permeable charged dye in the case of a zero transmembrane potential (in addition to the same extracellular dye concentration for the sample of interest, the fixed sample instrumental setting should also be kept when measuring these two samples). Here again the intracellular dye concentration, D_i^0, of the depolarized sample will be the same as that ($D_{i,c}^0$) of the very sample having the same cellular fluorescence intensity (FI^0) out of those used to construct the calibration curve ($D_i^0 = D_{i,c}^0$). The extracellular concentration of the dye for this sample will be denoted by $D_{e,c}^0$. The fluorescence intensity FI^0 identifies $D_{e,c}^0$ with the help of the calibration curve, which in turn determines again the intracellular dye concentration of this sample as well as of the fixed sample as

$$(D_e^S =)\ D_i^0 = D_{i,c}^0 = D_{e,c}^0\, C \quad (4)$$

with C as detailed in paragraph 1.

4. The transmembrane potential can be calculated according to Eq. (1). The scaling factor, C cancels in the calculation, enabling us to obtain the numerical value of ψ on an absolute scale.

Results and discussion

Assumptions required by the flow cytometric membrane potential measuring method

Transmembrane potential determinations on the absolute mV scale demand the fulfillment of the following assumptions:

1. Free diBA-C_4-(3) distributes between the two sides of the cytoplasmic membrane according to the Nernst equation.

2. The total amount of the dye taken up by the cells is negligible compared to the extracellular quantity of the fluorescent indicator throughout the whole experiment.

3. The fluorescence intensity measured from a given stained cell is a single value function of D_i in a way that any change in D_e will not alter the detected cellular dye fluorescence if the intracellular concentration of the free indicator dye remains the same.

4. A calibration curve is available, displaying the interdependence of cell-related dye fluorescence and intracellular free dye concentration. It is further assumed that the ratio $C = D_i/D_e$ is constant for all samples used for the calibration curve.

5. The cellular volume is not subject to a change in any of the samples, including also those used for the calibration.

6. A sample is available containing cells of zero transmembrane potential.

Fulfillment of the assumptions

To justify the outlined method, below we investigate the fulfillment of all the basic assumptions as defined above.

1. The membrane potential indicator diBA-C_4-(3) is a widely used dye having a broad range of applications. In our hands the cell-related fluorescence of the oxonol dye readily followed changes in the transmembrane potential and the new equilibrium was achieved within 1–2 min. The molecule is a monovalent anion with its negative charge delocalized. Based on these facts, there is no reason to doubt that the free dye is a Nernstian fluorescent indicator.

2. In order to have reliable extracellular dye concentrations for the construction of the calibration curve, one has to show that the total amount of dye accumulating in the cells under the conditions of the experiment does not significantly change the initial extracellular concentration. Negatively charged oxonol dyes offer the advantage of low intracellular accumulation compared to cyanine dyes carrying a net positive charge; thus experimental conditions may be found with negligible depletion of the dye in the suspending medium owing to cellular uptake. Dye concentration in our measurements was varied between 50 nM and 2 μM. A cell concentration of 10^6/ml was routinely used in the stained samples. To probe dye depletion, three parallel samples were prepared using 0.5×10^6/ml, 1.0×10^6/ml, and 2.0×10^6/ml concentrations of the cells and the lowest, 50 nM, dye concentration as described in Krasznai et al. (1995). In accord with the expectation based on the negative charge (and consequently low intracellular accumulation) of the oxonol, we did not detect any significant difference in the cell-related fluorescence of these samples. This observation argues for the total amount of dye taken up by the cells being negligible compared to the dye content of the suspension.

3. Our protocol to determine absolute values of the membrane potential assumes that the intensity of the fluorescence emission of a stained cell is a single-valued function of the intracellular free dye concentration. This implies that the same given intracellular free dye concentration has to determine the same cell-related oxonol fluorescence even if it is maintained under different conditions in terms of membrane potential and extracellular dye concentration (e.g. changing extracellular dye concentration relative to a reference sample may not change the cellular fluorescence if an appropriate, parallel to D_e change, alteration in the transmembrane potential will compensate for the effect of the changed extracellular dye concentration and maintain D_i identical to that in the reference sample). Owing to this reason the extracellular concentration of diBA-C_4-(3) should not exert any effect on the cellular fluorescence. To check for any eventual distortion of the detected cellular emission intensity by the direct contribution of the extracellular dye as a possible source of error, the fluorescence histogram measured from an unstained sample suspended in PBS (background or autofluorescence) was compared with that measured from a similar sample containing an extracellular nonpermeable dye. For this purpose the hydrolyzed (BCECF) form of BCECF-AM was added to the cell suspension under conditions of the experiment using a BCECF concentration giving rise to a similar fluorescence intensity reading as an oxonol solution of the highest concentration applied. The presence of extracellular BCECF changed the mean of the unstained background fluorescence less then 5%. This is a nonsignificant contribution to the fluorescence intensity of the oxonol-stained samples of 20–200 times the background. The results of these experiments clearly demonstrate that the flow cytometric method yielded true cellular fluorescence intensities for this type of cell with the maximum bias due to extracellular dye of less than 0.25%. The independence of the detected fluorescence intensity from the extracellular oxonol concentration is not evident apart from the direct contribution of extracellular dye to the emission intensity measured from the cells. Ring-like fluorescence of the oxonol-stained cells argues for a membrane-related emission intensity, which may depend on the extracellular dye concentration. Numerical values of the parameters determining the contribution of the membrane-bound oxonol emission intensity to the cellular fluorescence are not available. For this reason it was experimentally tested how much the results of the absolute membrane potential measurements are distorted by the ψ independent (and definitely D_e dependent) membrane fluorescence. Multiple samples were made up from the same cell suspension of cells of interest and zero transmembrane potential cells (obtained by formaldehyde fixation of cells from the same suspension) and fluorescence intensity data pairs (FI^S and FI^0) were measured using a number of different extracellular dye concentrations. Membrane potential values calculated from FI^S and FI^0 data pairs are displayed in Table 1. Results based on calibration curves constructed using either native or fixed (see below) cells were very similar. The displayed data belonging to different extracellular oxonol concentrations show a very low distorting effect of different D_e.

Table 1 Membrane potential (in mV) of JY cells does not change with concentration of diBA-C_4-(3) or the kind (native or formaldehyde fixed) of cells used for the construction of the calibration curve

[diBA-C_4-(3)] (nM)	Calibration curve constructed using	
	Fixed cells	Native cells
200	−61.7	−62.1
400	−60.0	−59.7
800	−59.0	−66.0

Table 2 Parameters of cell fluorescence saturation curves according to Eq. (7a)

Curve in Fig. 1	a_1	a_2	a_3	a_2/a_3
A	0.67	1154	55.9	20.6
B	1.08	584	35.2	16.6
C	8	91.3	5	18.3

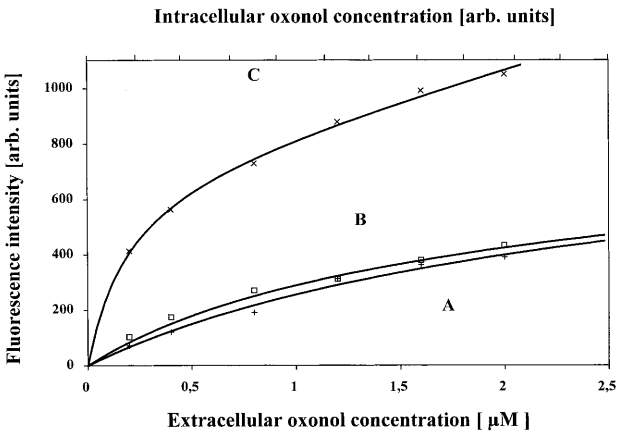

Fig. 1 Fluorescence intensity versus dye concentration plots constructed using *A* native cells in PBS (+); *B* native cells in 20 mM potassium buffer (□); *C* fixed cells in PBS (×)

4. Fluorescence intensity-extracellular dye concentration data pairs (used to construct the calibration curve) originate from samples of supposedly identical transmembrane potentials, assuring the proportionality between intracellular and extracellular concentrations of the free fluorescent label (see Eq. 2). This proportionality guarantees that the ratio of two dye concentrations belonging to two given points at random on the abscissa of the calibration curve has the same numerical value whether one uses the intracellular concentrations belonging to these calibration samples or appropriate extracellular concentrations. For this reason, ψ of the cells (defining *C*, the proportionality factor between D_i and D_e) used for the construction of the calibration curve is not a critical parameter; its numerical value is not required in the evaluation of absolute membrane potentials of specially treated cells (samples of interest).

In order to employ the calibration curve for the calculation of ψ of the samples of interest, it is required that the treatment of these samples (including formaldehyde fixation) will not change the affinity and number of intracellular dye binding sites as well as fluorescence quantum yields. These parameters are of basic importance in the determination of the total cellular emission intensity at a given intracellular free dye concentration. The interaction of oxonol with lipids and proteins is characterized by changes in the fluorescence quantum efficiency abolishing the proportional interrelationship between the amount of intracellular dye and cellular fluorescence. Unchanged binding characteristics of the investigated cells and those with $\psi = 0$ is a prerequisite to obtain undistorted membrane potential values using our method. Addressing this issue, we suggest a model to describe analytically the calibration curve using physicochemical and spectroscopic parameters of the system. Details of this model are given in the Appendix. To characterize the saturation of cell fluorescence with increasing dye concentration, three parallel titration experiments were carried out (Fig. 1). Curve A displays results of measurements made in PBS as suspending medium, curve B refers to results of similar measurements using PBS with 20 mM K^+, and data constructing curve C were obtained with paraformaldehyde-fixed cells. The gross behavior of the three titration curves is very similar; all of them can be fitted fairly well by the same analytical formula (according to Eq. 7a). Parameters of this formula vary with the conditions of the titration (with the transmembrane potential of the cells in the set of the calibration samples) but the ratio a_2/a_3 containing only parameters describing the binding characteristics remains practically unaltered (see data of Table 2). This allows one to use the same calibration curve to convert mean values of fluorescence histograms into dye concentration data, apart from a scaling constant.

5. As, under otherwise identical conditions, cellular fluorescence intensity is proportional to the cell volume, this parameter has to be constant throughout the measurements. To ensure this, all the suspending media should be accurately adjusted to isoosmolality.

6. Formaldehyde-fixed cells were used as completely depolarized cells. We detected very stable fluorescence intensity from these cells stained with diBA-C_4-(3) of the same concentration independent of the level of K^+ and Na^+ in the suspending medium, as was described by Krasznai et al. (1995).

Density of cultured cells affects membrane polarization

Absolute membrane potential values of JY cells were determined on cells isolated from cultures of different density. The same cell population of plateau phase cells was used to start culturing samples A, B, and C. The results of the membrane potential measurements carried out after 20 h culturing are displayed in Table 3, together with the results of patch clamp measurements carried out parallel to the flow cytometric absolute membrane potential determinations on the same samples. The results clearly show that the most polarized cells were those isolated from the

Table 3 Effect of cell culture density on membrane potential of JY cells

	Sample		
	A	B	C
Starting concentration of cells (10^6 cells/ml)	1	0.4	0.18
Final concentration of cells (10^6 cells/ml)	1.17±0.31	0.81±0.04	0.3±0.05
Membrane potential measured by oxonol (mV)	−61±3	−47±3.5	−40±4
Membrane potential measured by patch clamp (mV)	−63.4±5	−45.9±6.6	−40±5.5

plateau phase culture (sample A). The polarization state of these cells was very close to that of resting lymphocytes (Kiefer et al. 1980; Krasznai et al. 1995). Cells of the sample made up from the pure logarithmic phase culture (sample C) were the most depolarized. The membrane potential of these cells was very close to −36 mV, the threshold value for proliferating cells cited elsewhere (Binggeli and Weinstein 1985, 1986). Membrane potential determinations on cells isolated in a later phase of the logarithmic growth (sample B) revealed numerical values between these highly polarized and very much depolarized extremities. The close correlation of the flow cytometric and patch clamp measurements argues for a real effect of cell density. Our results indicate that a cell density-dependent change in the transmembrane potential is probably a frequent phenomenon, not limited to adherent cells. Available experimental data, however, do not allow the elucidation of the mechanism of this interrelationship. To decide whether a pronounced change in the membrane potential at a high cell density of the culture plays a direct role in inhibition of proliferation, or whether this change and the block of the cell division are simultaneous consequences of the same reason, requires further experimentation.

Appendix

Fluorescence titration is a widely used experimental procedure for the characterization of binding in an associating-dissociating fluorescent ligand-macromolecule system. The formation of the oxonol-protein complex is accompanied by an enhancement of the emission intensity of the dye. To follow quantitatively the oxonol-intracellular protein interaction, the fluorescence intensity is measured at different dye concentrations.

Emission intensity detected by the flow cytometer from a single cell is composed of the contributions of the free and bound fluorophore (I_f and I_b, respectively):

$$I = I_f + I_b = \alpha V(\phi_f c_f + \phi_b c_b) \tag{1a}$$

where α incorporates all the parameters of the excitation and detection geometry, light intensity, detector spectral sensitivity, and electronic gain; V is the volume of the cell; ϕ and c denote fluorescence quantum efficiency and intracellular concentration of the dye, respectively; and the subscripts f and b refer to the free and bound states of the oxonol, respectively.

We assume a simple association-dissociation phenomenon and homogeneous binding sites of the same kind with a total number of binding sites B_{tot}. The number of free (B_f) and occupied (B_{occ}) binding sites are related to each other as

$$K_d = \frac{B_f\ c_f}{B_{occ}} = c_f\ \frac{B_{tot} - B_{occ}}{B_{occ}} \tag{2a}$$

where K_d is the dissociation constant, and B values are numbers of the appropriate sites per unit volume.

With simple rearrangement we obtain

$$c_b = B_{occ} = \frac{c_f\ B_{tot}}{c_f + K_d} \tag{3a}$$

substituting this for c_b in Eq. 1a:

$$I = \alpha V\ \Phi_f\ c_f \left[1 + \frac{\Phi_b}{\Phi_f}\ \frac{B_{tot}}{c_f + K_d}\right] \tag{4a}$$

As for a Nernstian dye (see Eq. 2), one can write (c_0 denotes the extracellular dye concentration):

$$c_f = c_0\ \exp(-F\psi/RT) \tag{5a}$$

Equation (4a) can be rearranged:

$$I = \alpha V\ \phi_f\ c_0 \exp(-F\psi / RT) \cdot \left[1 + \frac{\phi_b}{\phi_f}\ \frac{B_{tot} \exp(F\psi / RT)}{c_0 + K_d\ \exp(F\psi / RT)}\right] \tag{6a}$$

Data points of the saturation curve (Fig. 1) can be fitted by

$$I = a_1\ c_0 \left[1 + \frac{a_2}{c_0 + a_3}\right] \tag{7a}$$

with

$$\frac{a_2}{a_3} = \frac{\phi_b\ B_{tot}}{\phi_f\ K_d} \tag{8a}$$

Thus a titration allows the calculation of the a_2/a_3 ratio from the parameters of the best fit, reporting on changes in the binding characteristics of the dye, if any.

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