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Assessment of Membrane Potential Using Confocal Microspectrofluorimetry

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Some slow potentiometric dyes, e.g., 3,3′-dipropylthiacarbocyanine and tetramethylrhodamine methyl ester, exhibit fluorescence spectral changes on redistributing from aqueous medium to cells. This effect has been used for spectroscopic discrimination of the emissions from free and bound dyes in cells. Such a discrimination can, in principle, allow for the assessment of cell membrane potential in individual cells, using the Nernst equation applied to the ratio of free dye fluorescence intensities inside and outside of the cell.

KEY WORDS: Membrane potential; redistribution dyes; microspectrofluorimetry; confocal microscopy.

INTRODUCTION

Slow (redistribution) dyes are important among various groups of potential sensitive fluorescent probes, since they offer a possibility to assess membrane potential in individual cells on an absolute scale [1,2]. The cell membrane potential can be determined according to the Nernst equation. The Nernst equation is applied to the ratio of probe concentrations inside and outside of the cell, which is revealed by the ratio of respective fluorescence intensities [3,4]. As a rule, this approach needs a correction for a non-Nernstian binding of some dye fraction to intracellular structures and macromolecules, such as proteins. Unfortunately, it is a difficult task since the dye binding rate can vary as a function of intracellular pH and/or ionic strength.

We found that some slow potentionmetric dyes, e.g., 3,3′-dipropylthiacarbocyanine[diS-C_3(3)] and tetramethylrhodamine methylester (TMRM), exhibit fluorescence spectral changes on redistributing from aqueous media to cells. This effect has been used for spectroscopic discrimination of the emissions from free and bound dyes in cells. Microspectrofluorimetric measurements on single cells have been performed in confocal mode with laser excitation, to discriminate the respective intracellular and extracellular signals [5].

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METHODS

Chemicals. The fluorescent probes diS-C_3(3) and TMRM were purchased from Molecular Probes (USA). Hepes, bovine albumin, and valinomycin were from Sigma Chemical Company (USA).

Cells. Human T47D mammary tumour cells, isolated from the pleural effusion fluids of a patient with breast carcinoma [6], were obtained from Dr. H. Magdelenat (Institut Curie, Paris, France), and maintained at 37°C in a humidified atmosphere of air–CO_2 (95/5%, v/v). Confluent cultures prepared in 50-mm petri dishes were used for membrane potential measurements.

Cell Labeling and Membrane Potential Calibration. The culture medium was replaced by 20 m*M* Hepes before cell labeling and membrane potential calibration. The membrane potential in T47D cells was changed by

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a valinomycin clamping of cell membranes at different extracellular KCl concentrations. Five KCl concentrations in Hepes buffer were used—3.5, 20, 50, 100, and 132 m*M*. NaCl was added together with KCl in such a way that the concentration of Cl^- ions in the medium was set to a constant value of 135.5 m*M*. Cells were labeled in either a 5×10^{-8} *M* solution of diS-C_3(3) or a 1×10^{-6} *M* solution of TMRM or in Hepes. Spectra were measured after a 5-min equilibriation period of incubation of cells with the dye and valinomycin (1×10^{-6} *M*) at room temperature.

Confocal Microspectrofluorimeter. The UV-VIS confocal laser microspectrofluorimeter was built around a Zeiss UMSP 80 UV epifluorescence microscope (Carl Zeiss, Oberkochen, Germany), a Spectra Physics 2025 argon ion laser (Spectra-Physics, USA) as the excitation source, a Jobin-Yvon HR640 spectrograph (ISA, France), and a 1024 diode-intensified optical multichannel analyzer (Model IRY-1024G, Princeton Instruments, Inc., USA), [7]. The spatial resolution was 0.5 μm in all three directions using a Plan-Neofluar 63/1.2 water-immersion objective (Carl Zeiss, Oberkochen, Germany).

Fluorescence Measurements. The cellular fluorescence emission spectra of the probe (F_c) were analyzed in terms of a linear combination of two model components: (i) the fluorescence emission spectrum of a free dye in aqueous solution (F_w) and (ii) that of a protein-bound form of the dye (F_b). Thus;

$$F_c = aF_w + bF_b \quad (1)$$

The linear decomposition was based on the least-squares fitting method according to Salmon *et al.* [8]. The corresponding calculated aF_w values were taken as the contribution of the fluorescence intensity of free (Nernstian) dye in cytosol.

The normalized fluorescence spectra of dyes in Hepes were substituted for F_w. The F_b models were obtained in the following way: Several probe fluorescence spectra were measured, both in hyperpolarized cells in petri dishes and in 2% albumin solutions in water. These spectra should obviously comprise both F_w and F_b. Their aF_w fractions were estimated empirically. The F_w spectrum was multiplied with a suitable coefficient and then subtracted from the experimental fluorescence spectrum of the dye in hyperpolarized cells or 2% albumin solution. The procedure was repeated several times, increasing the coefficient value until part of the resultant differential spectrum became zero. A weighted average of the empirical differential spectra was determined for the set mentioned above and taken as the model component F_b.

RESULTS AND DISCUSSION

Emission Spectra of diS-C_3(3) and TMRM in Cells and Aqueous Solutions

The model fluorescence spectra (normalized to account for different fluorescence quantum yields) of free diS-C_3(3) in Hepes buffer and bound diS-C_3(3) in cells are presented in Fig. 1. The model spectrum of the bound form of the dye was obtained as described above. It exhibits a spectral red shift of 10 nm compared to that of the free dye in Hepes. The experimental spectra measured in T47D-labeled cells and diS-C_3(3)-stained albumin solution [1×10^{-6} *M* diS-C_3(3); 2% albumin] were practically identical. Thus the spectral change observed after redistribution of the dye from the aqueous medium to cells can be interpreted in terms of dye binding to cytosolic proteins (Plasek *et al.*, in press). With TMRM, the dye accumulation in cells and its binding to proteins have been monitored by the change of the emission spectrum half-width rather than by the simple spectral shift (data not shown here).

Probe Fluorescence Response to Changes in Extracellular Potassium Concentration

At the various extracellular potassium concentrations the fluorescence probe response was analyzed for three cells, at three points in each. We tried to avoid measurements at very bright, isolated spots that occur in

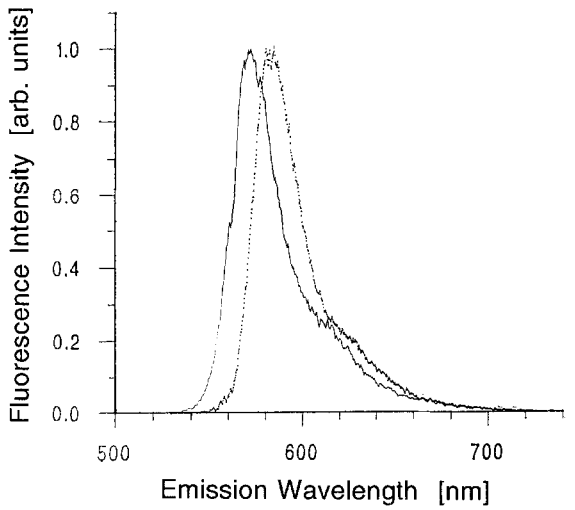

Fig. 1. Fluorescence emission spectra of diS-C_3(3) in Hepes (solid line) and bound to cytosolic proteins (dotted line); λ_{exc} = 514.1 nm.

cell cytoplasm. For each set of nine spectra a mean value of free dye fluorescence intensity, $(aF_w)_{mean}$, was determined $[(F_w)_{in}]$. In all investigated cell preparations the intensity of dye fluorescence in the extracellular medium, $(F_w)_{out}$, was also measured.

As long as we can assume that the respective fluorescence quantum yields of the dye fluorescence in cytosolic water and in Hepes are equal, the fluorescence intensity ratio $(F_w)_{out}/(F_w)_{in}$ reflects the ratio of dye concentrations C_{out}/C_{in}. The membrane potential in cells can therefore be estimated according to the equation.

$$\psi_m = (RT/F) \ln[(F_w)_{out}/(F_w)_{in}] \quad (2)$$

In Fig. 2 the results of the membrane potential assessment in cells at different extracellular potassium concentrations are plotted against the theoretical increment of the potassium diffusion potential, $\Delta\psi_K$, defined with the potassium diffusion potential at $[K^+]_{out} = 3.5$ m*M* taken as a reference:

$$\Delta\psi_K = \psi_K - \psi_{3.5} = (RT/F) \ln([K^+]_{out}/3.5) \quad (3)$$

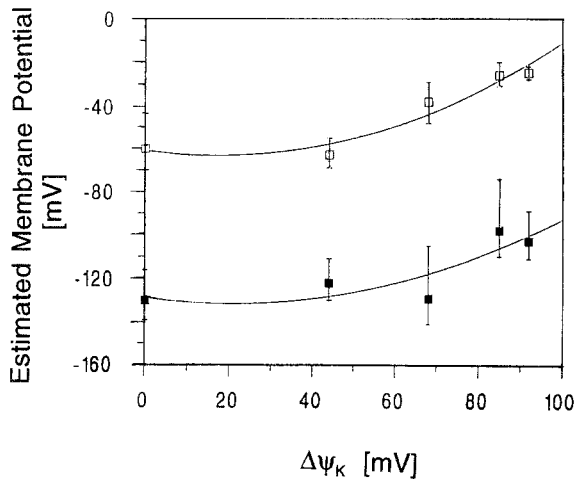

Fig. 2. TMRE and diS-C_3(3) fluorescence response to membrane potential established in T47D cells by valinomycin clamping at different extracellular potassium concentrations: TMRE, open squares; diS-C_3(3), filled squares.

Both potentiometric probes exhibited similar responses to variations in the extracellular potassium concentration. With TMRM, realistic values of ψ_m were assessed. Using diS-C_3(3), absolute values of membrane potential were obviously overestimated. Both the low reliability of the spectroscopic determination of the aF_w component, which is very weak in diS-C_3(3) spectra in cells, and the moderate difference between the respective quantum yields of dye fluorescence in cytosolic water and in Hepes could account for this effect. In agreement with the results of Ehrenberg *et al.* [3], a considerable variability was found between individual ψ_m values in different cells, as well as in different locations within a single cell. The relationship between the measured membrane potential and the theoretical potassium diffusion potential is not linear, which suggests that the potassium-specific valinomycin clamping of cell membranes did not surpass other ionic conductivities.

In conclusion, the new spectroscopic technique of measuring membrane potential in individual cells is capable of providing realistic estimations. However, further investigations are necessary to obtain Nernstian dyes exhibiting more pronounced spectral changes in fluorescence after binding to cytosolic proteins, preferably with few accompanying changes in their fluorescence quantum yield after binding.

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