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Biochimica et Biophysica Acta 1278 (1996) 197–204

BBA Biochimica et Biophysica Acta

Fluorescent styryl dyes of the RH series affect a potential drop on the membrane/solution boundary

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Received 6 December 1994; revised 30 June 1995; accepted 24 July 1995

Abstract

The effects of the adsorption of the fluorescent potential-sensitive dyes RH-421, RH-237 and RH-160 on the bilayer lipid membrane were studied. It was shown that a dipole potential drop, positive in the hydrophobic part of the membrane, arose due to the dye adsorption. The dye adsorption led to a considerable increase of the rate constant of hydrophobic anion translocation through the membrane, but did not affect their partition coefficient between membrane and water. It implies that the region of the membrane where the potential drops is located deeper than the adsorption plane of hydrophobic ions. The values of the boundary potential differences were estimated by two independent methods with unilateral and bilateral application of the dyes to lipid bilayer membranes. The results suggest that RH dye molecules penetrate through the lipid bilayers. The values of ζ-potential in liposomes did not change on dye adsorption. Hence, dye molecules are adsorbed in a form that does not change the surface charge. We estimated the effects of the electric field of dye dipole layer on an individual dipole located in the same layer and on ion transport through a membrane protein Na^+/K^+-ATPase. It turned out that the local electric field of each dye dipole decayed so rapidly that a neighbouring dye molecule did not feel it. It also appeared that RH dyes could have but a minor effect on the electrogenic transport performed by the sodium pump in the examined range of dye concentrations.

Keywords: Potential sensitivity; RH dye; Membrane; Boundary potential; Adsorption; ATPase, Na^+/K^+-

1. Introduction

Potentiometric fluorescent probes are widely used for optical recordings of membrane potential changes of cells, organelles and vesicles [1] that can not be studied by electrophysiological techniques. Styryl dyes of the RH series (Fig. 1) belong to the class of 'fast' dyes, which respond in microseconds to changes in membrane potential [1–5]. They have a delocalized positive charge in the pyridinium complex and a localized negative charge on the sulfonate group at the end of the molecule (dipole moment of the order of 10 Debye [6]). Due to amphiphilic structure, RH dyes can adsorb on lipid bilayers. The sulfonate group faces water solution, and the rest of the molecule is plunged in the non-polar part of the membrane. It determines that the long axis of the dye lies perpendicular to the membrane surface [3,7–9]. Such a notion helps to explain considerable electrochromic spectral shifts observed in response to the change of transmembrane voltage [3,8–10]. However, evidence against a purely electrochromic mechanism exists [4,6]. These dyes were successfully employed for detection of local electric field changes due to Na^+/K^+-ATPase activity [7,11,12].

The considerable dipole moment of dye molecules and their orientation on the membrane make it quite possible that these dyes themselves can alter the potential drop at the membrane/water boundary. This fact creates some problems in application of RH dyes as probes. In this work we attempted to assay the location of these dyes in bilayer lipid membranes (BLM) and their influence on the electric field distribution.

Abbreviations: BLM, bilayer lipid membrane; BP, boundary potential; IFC, intramembrane field compensation; DPA, dipicrylamine; TPB, sodium tetraphenylborate.

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SSDI 0005-2736(95)00197-2

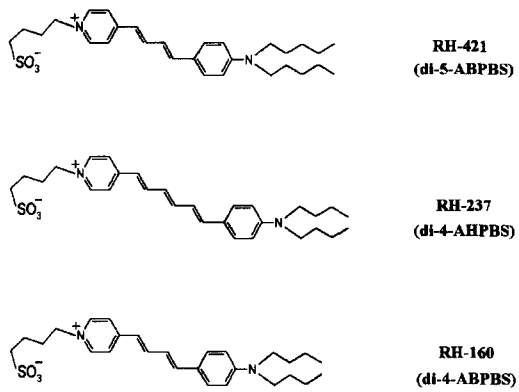

Fig. 1. Structures of potential-sensitive styryl dyes under study. The top names correspond to the classification [2], the bottom names in brackets are given according to [3,4].

The important information on the immersion depth of oriented dipole dye molecules may be gained by the investigation of the change of electric field distribution inside the membrane due to dye adsorption. The distribution of the electric potential on BLM can be determined using various experimental techniques measuring the electrostatic potential change in the planes located at different distances from the membrane surface [13,14]. In these studies three independent methods were used: (1) a current relaxation method based on monitoring the changes in the kinetic parameters of transport of hydrophobic ions across the membrane; (2) the method of intramembrane field compensation (IFC), which is sensitive to changes in the difference of boundary potentials (BP) of BLM; and (3) correlation spectroscopy measuring variation of the electrostatic mobility of liposomes (ζ-potential). These three techniques enabled us to establish the electrostatic potential change due to adsorption of dyes of RH series: RH-421, RH-237 and RH-160.

2. Materials and methods

Bilayer lipid membranes were formed from decane solution of L-α-diphytanoylphosphatidylcholine (DPhPC, 30 mg/ml; Avanti Polar Lipids, USA) on a 0.8–1.0 mm orifice in a Teflon cell, using the standard procedure [15]. The solution containing 100 mM KCl, 5 mM K_3PO_4, 5 mM sodium citrate, 5 mM Tris-HCl; pH 7.7 was used. In the case of the ζ-potential measurements this solution was diluted fivefold (pH 7.4). Solutions were prepared in distilled water with salts of analytical grade. Ag/AgCl electrodes were used to record electric parameters of BLM. All experiments were carried out at room temperature (about 22°C).

Styryl dyes RH-160 (anhydro-4-(4′-*p*-dibutylaminophenyl-1′,3′-dienyl)-1-δ-sulfobutylpyridinium hydroxide [2]), RH-237, RH-421 (Molecular Probes, USA) were dissolved in ethanol. To decrease the errors of weighing, the concentrations of RH-160 and RH-237 in these solutions were confirmed by measuring their absorption spectra with a Specord M40 (Germany) spectrophotometer; extinction coefficients were taken from [2]. All experiments were carried out in the dark to prevent decomposition of the dyes. Decomposition of the dyes during the experiment was assessed fluorimetrically with a Hitachi-850 (Japan) fluorimeter. Dipicrylamine (DPA; Koch Light, UK), sodium tetraphenylborate (TPB; Chemapol, Czechoslovakia), valinomycin (Calbiochem, USA) were also dissolved in ethanol.

The potential distribution inside BLM can be estimated from the kinetics of transmembrane transport of hydrophobic ions. The coefficient of the hydrophobic ion partition between membrane and water depends on the potential drop between the solution volume and the membrane surface. If the potential drop inside the membrane changes, this should affect the rate constant of hydrophobic ion transport across the membrane [13,16–19].

The kinetics of current relaxation in response to a voltage step on the BLM in the presence of hydrophobic ions DPA or TPB was studied using a setup similar to that described in [20]. The signal was recorded by digital oscilloscope OS 1425 (Gould, UK), then transmitted to an IBM PC. The current–time curve (Fig. 2) was approximated by an exponential function: $I = I_0 e^{-t/\tau}$. The following parameters were determined: initial membrane conductance $g = I_0/V$, where V is a voltage step applied to the membrane; characteristic time of current decline τ and time integral of current $q = I_0 \cdot \tau$ equal to the charge transferred by hydrophobic ions across the membrane during the current decay. Using these parameters, calculations of the variation of boundary potential $\Delta\varphi_b$ and its components on the inner ($\Delta\varphi_\tau$) and outer ($\Delta\varphi_q$) sides of the

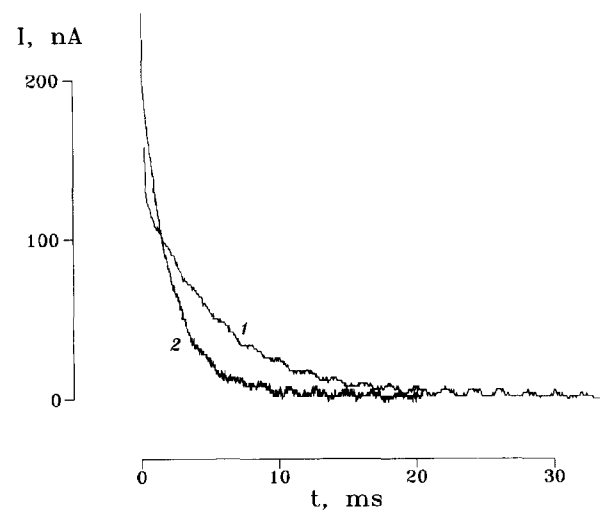

Fig. 2. A typical example of the current relaxation curve after a voltage step applied to the BLM in the presence of 1.25 mM TPB: 1, before dye adding; 2, after RH-421 adding (0.56 μM) on both sides of the membrane. The voltage was dropped from 0 mV to 5 mV at the time $t = 0$.

hydrophobic ions adsorption plane were performed as described in [13]:

$$\Delta\varphi_b = \frac{RT}{zF}\ln\left(\frac{g}{g_o}\right) \quad (1)$$

$$\Delta\varphi_q = \frac{RT}{zF}\ln\left(\frac{q}{q_o}\right) \quad (2)$$

$$\Delta\varphi_\tau = \frac{RT}{zF}\ln\left(\frac{\tau}{\tau_o}\right) \quad (3)$$

where F is the Faraday constant, R is the universal gas constant, T is the absolute temperature, z is the valence of the transported ion, g_o, q_o and τ_o are respectively the initial values of conductance, charge and time constant measured prior to dye injection into the cell. To follow the changes of these parameters during the process of dye adsorption, the measurements were repeated at intervals of about a minute.

To assess the effect of RH dye adsorption on valinomycin-induced conductivity of the BLM, the total change of BP $\Delta\varphi_b$ was estimated using Eq. (1) where g_o and g stayed for DC-conductivity values measured in the presence of valinomycin respectively before and after the dye addition [14].

The difference of boundary potentials on BLM was measured by the method of intramembrane field compensation using the second harmonic of capacitive current [21]. The boundary potential difference established after unilateral application of the dye was recorded. In case when all changes of potential occur only on one side of the membrane, this potential difference coincides with the variation of the BP induced by the adsorption of a compound under study on the corresponding membrane/water interface [14,22].

The electrophoretic technique allows to measure the potential drop from the solution volume to the slip plane localized near the membrane (ζ-potential). Comparison of the values of ζ-potential and the total drop of BP measured by other techniques allows evaluation of the contribution of the diffusion layer to the total potential drop.

Electrophoretic mobility was measured on multilayer liposomes using the technique based on recording the autocorrelation function of a scattered laser beam by a Zetasizer-2 instrument (Malvern Instr., UK). Multilayer liposomes were prepared from DPhPC in the following way: the lipid solution in chloroform was dried using a vacuum pump, then it was shaken with an appropriate electrolyte solution. The concentration of lipids was 1 mg/ml electrolyte. Concentration of compounds under study was varied by successive addition of their concentrated solutions to the liposome solution. The procedure of ζ-potential determination was similar to [23].

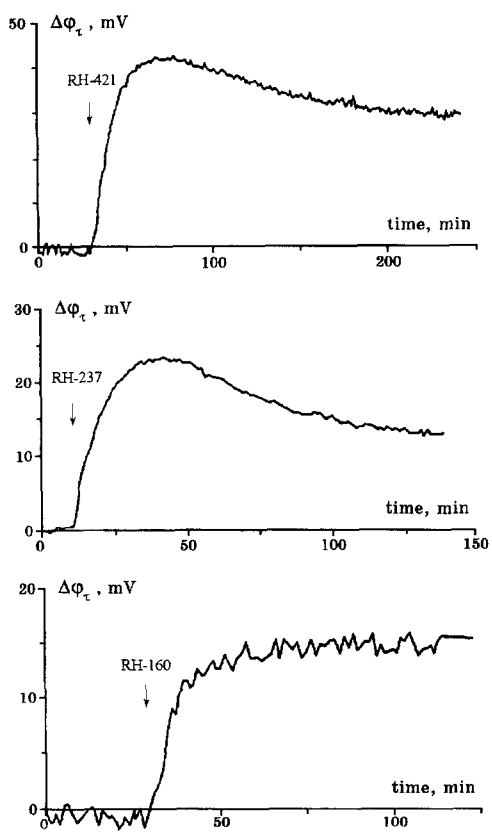

Fig. 3. The characteristic kinetics of variation of $\Delta\varphi_\tau$ calculated from the measurements of current relaxation (Eq. (3)) in the presence of 1.25 μM TPB under addition of RH dyes on both sides of the membrane. The arrows indicate the moments of dyes addition. Variations of dyes concentration in the solution as a result of additions: RH-421, from 0 to 0.56 μM; RH-237, from 0 to 0.38 μM; RH-160, from 0 to 2.38 μM.

3. Results

The adsorption of RH dyes on the BLM led to significant acceleration of the transport of hydrophobic anions DPA and TPB through the membrane. Charge q carried through the membrane by the ions remained practically constant, whereas the time constant τ of current decay decreased in a dose-dependent manner (Fig. 2). Due to this effect, current relaxation recordings were limited by time response of the amplifier, and in case of DPA were not possible. Therefore, for quantitative estimates in these experiments, only TPB was used. The variation of relative change in τ with time after addition of dyes into the cell calculated as $\Delta\varphi_\tau$ using Eq. (3) is presented in Fig. 3. One can see that after addition of RH-421 or RH-237 into the cell $\Delta\varphi_\tau$ increases, passes through the maximum, then decreases and reaches the steady-state value. Fig. 4 (points 2) illustrates dose-dependence of the steady-state values of $\Delta\varphi_\tau$. Fig. 5 compares the dependencies of the steady-state values of $\Delta\varphi_\tau$ on the concentration of the three dyes under investigation (RH-421, RH-237 and RH-160). The potentials created by RH-421 and RH-237 are approximately equal, but they significantly exceed $\Delta\varphi_\tau$ for RH-160 at the same concentrations.

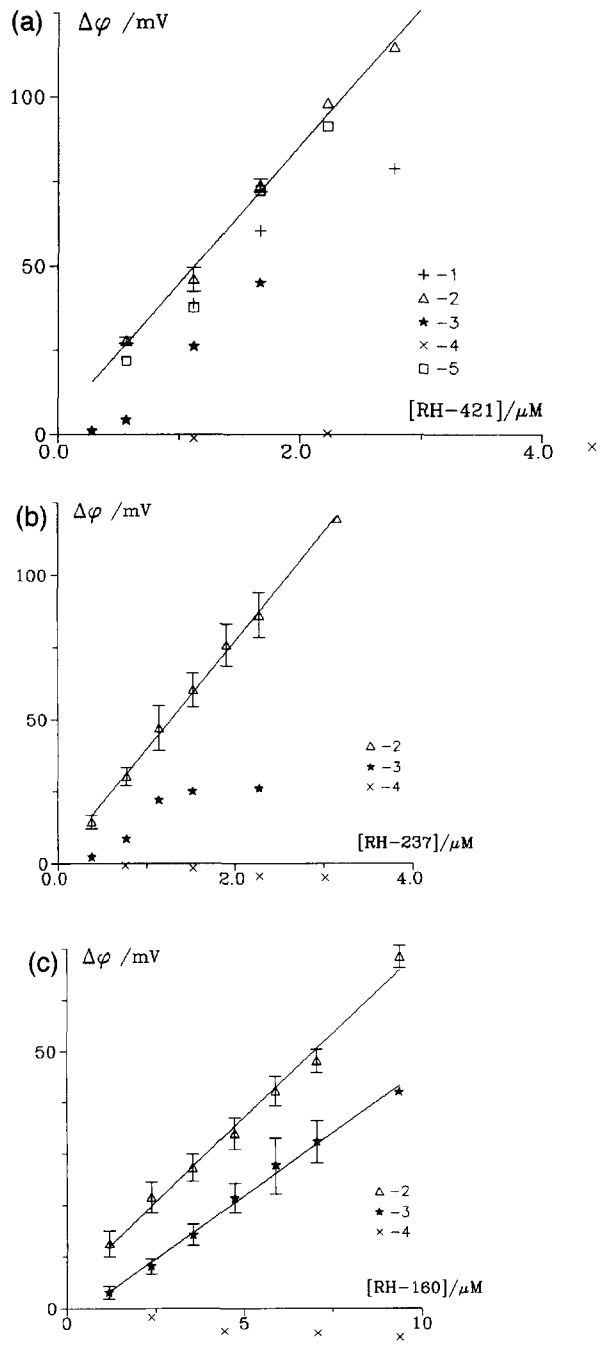

Fig. 4. Adsorption of styryl dyes on the BLM: (a) RH-421, (b) RH-237, (c) RH-160. The stationary values of the following parameters as a function of dyes concentration in the solution are represented here: 1, $\Delta\varphi_\tau$ calculated from the measurements of current relaxation (Eq. (3)) in the presence of 1.25 μM DPA; 2, $\Delta\varphi_\tau$ in the presence of 1.25 μM TPB; 3, the drop of BP $\Delta\varphi_b$ measured by the IFC method; 4, ζ-potential of liposomes from DPhPC; 5, $\Delta\varphi_b$ calculated from the conductivity change in the presence of 0.5 μM valinomycin for RH-421 using Eq. (1).

The injection of RH-421 leads to reduction of the valinomycin-induced conductivity of the BLM. Fig. 4a (points 5) shows the change of boundary potentials calculated from the relative change in the conductivity using Eq. (1). One can see that the values of the potential obtained

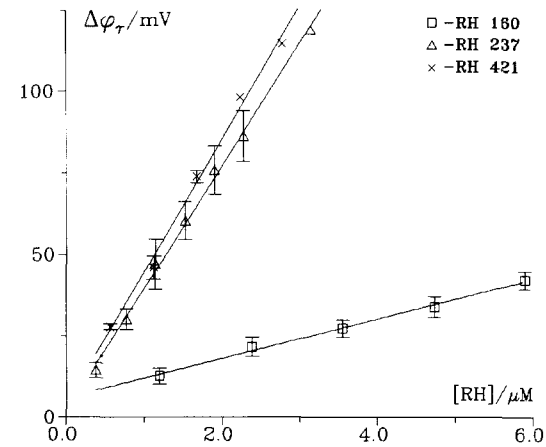

Fig. 5. Comparison of the potentials changes in the hydrophobic part of the membrane $\Delta\varphi_\tau$ for RH-160, RH-237 and RH-421 from Fig. 4a,b,c as a function of dyes concentration.

from the measurements with valinomycin are very close to those from the hydrophobic ion experiments.

Fig. 6 represents time course of the difference of boundary potentials of the BLM measured by IFC method under unilateral application of RH dyes. After dye addition the potentials pass through maximum and then slowly decrease to steady-state values like in Fig. 3. The dose-dependencies of these values are shown in Fig. 4 (points 3). The stationary values of $\Delta\varphi_b$ measured by this method are lower than the potentials obtained by current relaxation method, where the addition of dyes was symmetric with respect to the membrane (Fig. 4, points 2).

The change of ζ-potential on liposomes (Fig. 4, points 4) after addition of RH dyes into solution was negligible. It

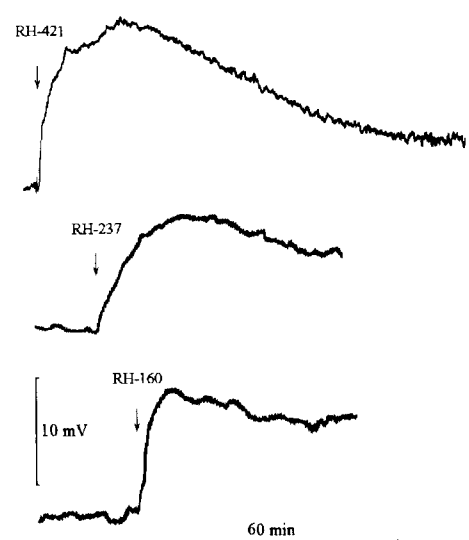

Fig. 6. The characteristic kinetics of variation of the drop of BP $\Delta\varphi_b$ measured by the IFC method under addition of RH dyes on one side of the BLM. The arrows indicate the moments of dyes addition. Variations of dyes concentration in the solution as a result of additions: RH-421, from 0 to 0.56 μM; RH-237, from 1.14 to 1.52 μM; RH-160, from 0 to 2.38 μM.

means that the dyes are adsorbed on membrane/water interface in a form such that the total surface charge of the boundary is not changed.

4. Discussion

4.1. Electrostatic potential created by the dyes inside the membrane

In principle, the dye molecules adsorbed on the membrane can influence the transmembrane transport of hydrophobic ions by different mechanisms: (i) structural reorganization of the membrane, (ii) direct interaction between dyes and hydrophobic ions and (iii) change of electrostatic potential at the membrane/solution boundary. We suggest that the last mechanism is the most important. The following observations support this suggestion:

The adsorption of the dyes suppresses membrane conductance induced by valinomycin that carries positive charges through the membrane whereas in the presence of negatively charged ions (DPA or TPB) the conductance increases, moreover, the values of potential calculated from the data obtained with valinomycin and TPB coincide (Fig. 4a);

The relative change in time constant τ of current decay does not depend on the sort of hydrophobic ions (DPA or TPB);

Finally, variation of the boundary potential difference produced by unilateral application of the dyes was revealed by the IFC method based on another principle, different from conductance measurements.

Thus, we assume that dye adsorption changes the potential drop at the membrane/water boundary so that the interior of the membrane acquires more positive potential with respect to the water solution.

The values of the boundary potential difference measured by the IFC method were lower than those based on the kinetics of the hydrophobic ions transport and measurements of valinomycin-induced conductivity. This discrepancy is most probably related to the fact that in the case of IFC-measurements the BLM was unilaterally exposed to the dye tested. The potential drop could change on both sides of the membrane due to the penetration of the dye molecules to the other side of the membrane. This effect becomes significant if steady state flow of the dye through the BLM is comparable with the dye flow through the membrane interfaces. The difference between the potentials measured by the methods reflects the membrane permeability to the dyes. As can be seen from Fig. 4, this difference is more pronounced in case of RH-237 and it is the least in the case of RH-160. From this we can infer that RH-237 passes through the membrane better than the other dyes, and RH-160 does it worse than the others. This can be ascribed to the difference in the structure of the dye molecules: RH-237 has the most delocalized positive charge on the chromophore, and RH-421 has the longest hydrophobic tail.

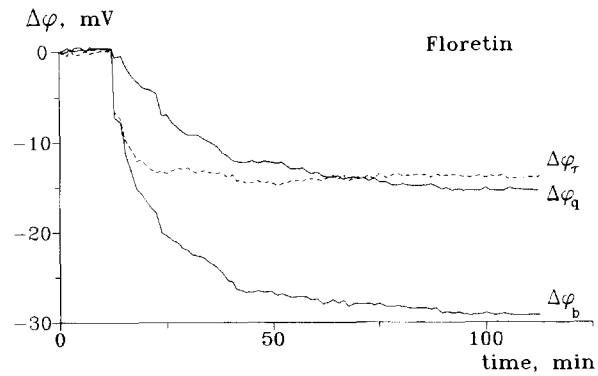

Fig. 7. Variation of boundary potential $\Delta\varphi_b$ and its components $\Delta\varphi_q$ and $\Delta\varphi_\tau$, calculated from the measurements of current relaxation (Eqs. (1)–(3)) in the presence of 1.25 μM TPB under addition of 0.62 μM floretin on both sides of the BLM. The arrow indicates the moment of floretin addition.

As ζ-potential does not change on adsorption of RH dyes, the dyes are adsorbed on the membrane in a form that does not change the surface charge. This implies that the potential drop induced by dye adsorption can only be assigned to ordered dipoles of adsorbed molecules.

Two important features of RH dye-induced potential drop should be noted. First, unlike the majority of known compounds influencing a dipole potential drop, RH dye adsorption induces interior positive potential. Second, the dye adsorption affects only the time constant of the hydrophobic ion transport through the membrane; this means that the potential drop is located deeper than the adsorption plane of hydrophobic ions. Thereby the dyes in hand are also different from well studied compounds, such as 2,4-D [24] or floretin [18,19,25], where both parameters (as τ as q) changed. As experimental results obtained with floretin vary in a wide range in different works, we repeated the measurements and compared the effects of floretin and RH dye adsorption under the same conditions. Our results in Fig. 7 show evidence that both $\Delta\varphi_q$ and $\Delta\varphi_\tau$ change after floretin addition.

The potentials induced by RH-421 and RH-237 are approximately identical, but significantly exceed $\Delta\varphi_\tau$ for RH-160 at the same concentrations (Fig. 6). This can be attributed to the differences in their molecule structure (Fig. 1). The chromophore of RH-237 molecule is longer in comparison with RH-160. Therefore its dipole moment may be higher and the ordering of RH-237 molecules may have a more pronounced effect on $\Delta\varphi_\tau$. RH-421 molecules have the longest hydrophobic tail, and probably this explains a comparatively high binding constant of RH-421 and the membrane. Besides, RH-421 molecules may better conserve their orientation in the membrane (normal to the membrane surface).

One of the unexpected results is the time-dependence of the dye-induced potential drop. This is likely related to the

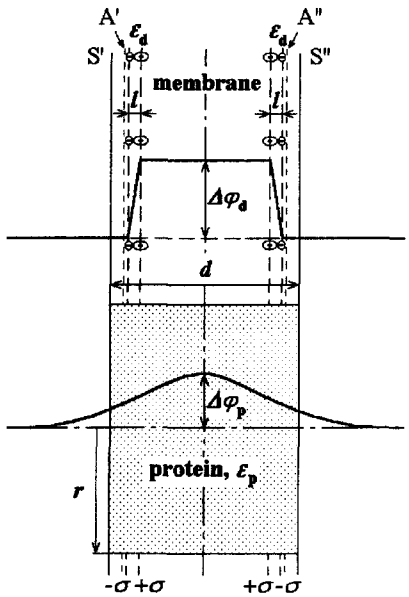

Fig. 8. Electrostatic model for the estimation of local electric potential distributions due to adsorption of dipole dye molecules on both sides of the membrane with a transmembrane protein molecule. $\Delta\varphi_d$, $\Delta\varphi_p$ are the electric potentials; ε_l, ε_p the relative dielectric constants; r the radius of the protein molecule; d the thickness of the membrane; l the thickness of the layer where the potential drops; σ the surface charge density; A',A" adsorption planes of hydrophobic ions; S',S" membrane surfaces (see explanations in the text).

change of a dye molecule state in the BLM, for example, as a result of their partial dimerization.

Thus, we showed that adsorption of RH dyes induced the potential drop at the membrane boundary. It was attributed to oriented dipoles of dye molecules. The above data also enabled us to infer that dye molecules penetrated through the membrane, and the potential drops appeared at both of its boundaries even when the dye was injected unilaterally.

4.2. Applicability of RH-dyes as probes

As these dyes are employed as probes for recording electric fields in the membrane, it is necessary to assess to what extent the electric field induced by oriented dye molecules themselves can disturb the measured parameter.

First of all it should be noted that the potentials measured represent the average values in a plane which is parallel to the membrane and located deeper than the dye dipoles. If potential drops are equal on both of the membrane boundaries, the average potential $\Delta\varphi_d$ is a variation of the height of potential barrier in the interior of the BLM, and the averaging can be carried out in the plane located in the middle of the membrane (Fig. 8). This average potential markedly affects the transport of hydrophobic ions through the membrane. For example, if dye adsorption increases potential by 100 mV, the rate constant of the hydrophobic anion translocation through the BLM will increase by almost two orders of magnitude.

We will estimate two possible disturbances of intramembrane electric field distribution due to adsorbed dipoles of dye molecules.

4.2.1. Lateral changes in electric field

In the first case we will consider the influence of the electric field of dye dipole layer on a dipole situated in the membrane. Obviously the strongest electric field arises inside the dipole layer, so we will estimate the possible disturbance for a dipole in the same layer. The dipole–dipole interaction was discussed in detail in [26]. It was shown that in case of floretin adsorption this interaction was negligible. We will make the same estimations for RH-421. The extent of the influence of dye dipole on another will depend on how fast the dipole field of each dye molecule decays in the lateral direction. If this field decays slowly, this must lead to the electric interactions between neighbouring dye molecules and finally to deviation from linearity of the average potential measured experimentally [26]. The fact that dose-dependence of the potential appears to be linear (Fig. 5) suggests that in this case all the interactions including the electric ones are most likely minor. It means that the local electric field of each dye dipole decays so abruptly that a neighbouring dye molecule does not feel it. This conclusion can be also confirmed by quantitative estimations using the results reported in [26].

To do this we first estimate the surface area s_0 per one adsorbed dye molecule. We will assume that the dipole moment vectors p of adsorbed dye molecules are perpendicular to the membrane surface. The average potential drop inside the membrane can be calculated by the formula of a flat capacitor

$$\Delta\varphi_d = \frac{pn}{\varepsilon_d \varepsilon_0} \quad (4)$$

were $n = 1/s_0$ is the dye surface concentration in the adsorption plane, ε_d the relative dielectric constant of the medium where the layers of dye dipoles situated, ε_0 the electric constant. Clarke et al. [6] have determined the ground-state dipole moment of RH-421 in chloroform solution (12 Debye). They note that the dipole moment of the molecule may depend on its surroundings: it may have a lower value in a less polar solvent. The value of p may be even less due to the deflection of the long axis of the dye from the normal to the membrane surface. So we take the value of the dipole moment $p \approx 10$ D $= 3.34 \cdot 10^{-29}$ C m for the molecule in the membrane. Now, if we take the average potential $\Delta\varphi_d = 100$ mV and $\varepsilon_d \approx 4$, then from Eq. (4) we can define the area per one dye molecule $s_0 = 9.4$ nm^2. It means that if the average potential equals 100 mV, the dye molecules are spaced at intervals of about 3 nm. Noteworthy is that from experimental estimates of dye concentration corresponding to the potential 100 mV one can calculate the value of the dye partition coefficient. This value obtained in our experiments for RH-421 is of the same order of magnitude as reported in [7] ($2.5 \cdot 10^5$).

Now, similarly to [26], we consider a 'central' dipole surrounded by other dipoles distributed homogeneously in the adsorption plane, excluding an area with radius $(\pi n)^{-1/2}$ that represents the area occupied by the central dipole. The energy of the central dipole U_o in the field of all surrounding dipoles will modify the adsorption constant by a factor of

$$\beta = \exp\left[-\frac{U_o}{k_B T}\right] = \exp\left[\frac{\sqrt{\pi \varepsilon_d \varepsilon_o p(\Delta\varphi_d)^3}}{2k_B T}\right] \approx 0.8 \quad (5)$$

where k_B is Boltzmann's constant and T absolute temperature. Consequently, if $\Delta\varphi_d$ ranges from 0 to 100 mV, β ranges from 1 to about 0.8. It means that the local electric field of adsorbed dipole dye molecules decays in lateral direction so fast that at their surface density corresponding to the average potential 100 mV the dipole–dipole interaction between adsorbed molecules modifies the value of the adsorption constant by only about 20%. This minor effect is not observed in the experiment at all (Fig. 4a, points 2): the dose-dependence of the potential is linear over all the measured range of dye concentrations.

4.2.2. Dipole potential in the interior of a protein molecule

The above estimation accounts for the dipole–dipole interaction. Now let us consider the ion–dipole interaction. In this case it is necessary to evaluate how dye dipoles adsorbed on both sides of the membrane affect the height of potential barrier inside the membrane. As already discussed, dye dipoles dramatically affect the kinetics of the transport of hydrophobic ions that sense the 'average' potential. However, if ions are transferred in the interior of a large protein molecule, in a medium which is more polar than the hydrophobic part of the membrane, then the influence of the dipoles situated in the lipid part of the bilayer can be considerably reduced. This fact has been already noted in studies of single gramicidin channels [27] or single potassium channels in myelinated nerve [28]. It was shown there that dipole potential changes practically have no effect on single channel conductance. The theoretical estimations of the reduction of dipole potential inside the ion channels were made by Jordan [29].

As the dyes in question are used as potential-sensitive probes for the recording of charge transport due to the Na^+/K^+-ATPase activity [7,11,12], it would be interesting to estimate to what extent the field of adsorbed dye molecules is reduced inside the protein, provided that the dipoles do not penetrate into the protein. On the other hand it would be also appropriate to use RH dyes not as probes but as a tool to modify Na^+/K^+-ATPase activity by changing the electric field distribution in its interior. The structure of any ion conduction pathway inside the Na^+/K^+-ATPase is not clear yet, but many investigators assume it is similar to that of an ion channel [7,11,30]. We will consider the protein as a uniform cylinder with dielectric permeability higher then that of lipid bilayer.

Let us assume that the dye is adsorbed on both sides of the membrane, and the protein presents a cylinder of radius r (Fig. 8). We now imagine the layer of adsorbed dye molecules on both of membrane boundaries as two planes of opposite charge with surface charge density $\sigma = \pm en$, where $e = 1.6 \cdot 10^{-19}$ C is the electron charge. These two planes are separated by a distance $l = p/e$. Using electrostatic relations we can find the potential $\Delta\varphi_p$ created by four charged planes in the centre of the protein which cuts a disk of radius r out from each of them:

$$\Delta\varphi_p = \frac{\sigma}{\varepsilon_o \varepsilon_p}\left[\sqrt{r^2 + d^2/4} - \sqrt{r^2 + (d/2 - l)^2}\right] \quad (6)$$

here the potential at infinity is zero, d is the thickness of the membrane and ε_p the relative dielectric constant of the protein. Now, if we compare $\Delta\varphi_p$ with the potential in regions of the membrane far away from the protein $\Delta\varphi_d = \sigma l/\varepsilon_o \varepsilon_d$, then taking $l \approx 0.2$ nm, $\varepsilon_p \approx 10$ and $r \approx d \approx 5$ nm, we obtain: $\Delta\varphi_d/\Delta\varphi_p \approx 6$. It means that the change of potential induced by the dye dipoles inside the protein will be six times less than the change of potential inside lipid bilayer. Perhaps, this difference would be more pronounced if we take into account the image effect, i.e., the effect of shielding of the membrane dipole potential by the image field due to polarization charges induced at the membrane/protein boundary. Such an effect was considered by Jordan [29] in the model with the narrow water pore. Our estimates are in a good agreement with Jordan's results.

Hence, we can infer that RH dyes have rather minor effect on the electrogenic transport performed by the sodium pump in the examined range of dye concentrations. This influence was not found in direct experiments [31]. To induce the potentials comparable to the measured ones in the protein, the dye concentration in the solution should be raised by at least one order of magnitude, assuming that the contribution of the dipole–dipole interaction to the adsorption will be small, and the dye concentration dependence of the dipole potential will be linear.

Acknowledgements

This work was financially supported by Russian Foundation of Fundamental Research (grant #93-04-7652) and the Deutsche Forschungsgemeinschaft (Sonderforschungbereich 156). We would like to express our gratitude to H.-J. Apell for providing us with styryl dyes and all his helpful support. We are also thankful to Yu.A. Chizmadzhev for fruitful discussions and to A.Ya. Dunina-Barkovskaya for the help in preparing this manuscript for publication.

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