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The effect of transmembrane potential on the dynamic behavior of cell membranes

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The relationship between transmembrane potential and lipid dynamics in the cytoplasmic membrane of mouse thymus cells has been investigated. Changes of transmembrane potential was followed by measuring the fluorescence emission of the anionic dye, bis-(1,3-dibutylbarbiturate)trimethine oxonol (diBa-C_4-(3)). Assessment of lipid fluidity was carried out applying three fluorescent lipid probes, 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH), 12-(9-anthroyloxy)stearic acid (12-AS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) used to monitor different structural regions of the bilayer. The fluorescence anisotropy of these probes was measured as a function of temperature at two values of transmembrane potential. In the case of DPH it proved to depend on the membrane potential in the higher temperature range (above 28° C), while no such a dependence could be observed for DPH below this temperature range and for TMA-DPH and 12-AS in between 20 and 37° C. These data suggest that changes in transmembrane potential are accompanied with some local alteration in membrane lipid dynamics and/or structure.

Introduction

Physical events in cytoplasmic cell membranes such as changes in permeability to different ions, membrane potential and lipid fluidity, conformational changes of proteins, receptor capping, etc. triggered by external stimuli are of great importance, as they may play essential role in information transfer through the cell membrane. More and more information is accumulating about changes related to membrane proteins, less is known, however, about the lipid constituents. The interrelation between membrane proteins and membrane lipids can be illustrated by the concept of vertical translocation of membrane proteins [1,2]: the membrane lipid fluidity can modulate the expression (or masking) of some membrane proteins [1–5]. On the other hand, the exposure of proteins on the cell surface can be affected by changing the membrane potential [6–8]. Changes of potential gradient across the membrane of phospholipid vesicles and living cells are reported to be accompanied by appreciable modifications in membrane structure like changes in membrane rigidity [9–11] and membrane thickness [12], or conformational changes of proteins [13]. Our principal aim was to investigate whether changes of the transmembrane potential of living cells would be connected to definite changes in the structure/dynamics of certain lipid regions of the plasma membrane.

Materials and Methods

Reagents. Bis-(1,3-dibutylbarbiturate)trimethine oxonol and 1[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene were obtained from Molecular Probes. The two hydrophobic lipid probes, 1,6-diphenyl-1,3,5-hexatriene and 12-(9-anthroyloxy)stearic acid and gramicidin D ionophore were purchased from Sigma, respectively. All inorganic chemicals were Merck, analytical grade.

Cells. Balb/C mouse thymocytes were kept at room temperature and suspended to a concentration of $5 \cdot 10^7$ cell/cm^3 in normal phosphate-buffered saline (PBS) containing (in mM amounts): 150.6 NaCl, 2.7 KCl, 8.7 Na_2HPO_4, 1.5 KH_2PO_4 with pH adjusted to 7.4. PBS solutions with different K^+ content were obtained substituting NaCl isoosmotically with KCl. The viability of cells was controlled after measurements by Trypan blue exclusion and was found to be around 95%.

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; 12-AS, 12-(9-anthroyloxy)stearic acid; diBa-C_4-(3), (oxonol), bis-(1,3-dibutyl-barbiturate)trimethine oxonol; TMA-DPH, 1-[4-(trimethylammonium)-phenyl]-6-phenyl-1,3,5-hexatriene; PBS, phosphate-buffered saline.

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Fluorescence measurements. Steady-state fluorescence and anisotropy measurements were carried out in a Hitachi MPF-4 spectrofluorimeter equipped with thermostated cell holder and polarization accessories. For fluorescence emission measurements the samples were incubated in cuvettes for 10 min at 37 °C in a final concentration of $2 \cdot 10^6$ cell/cm^3 in low-Na^+, low-K^+ solution containing (in mM amounts): 248 sucrose, 1 Na_2HPO_4, 0.5 $MgSO_4$, 1 $CaCl_2$, 10 Hepes (pH 7.4).

When different extracellular [K^+] (high K^+ solutions) was required, sucrose was replaced by KCl between the concentration values of 0 and 145 mM. The concentrations of all the other inorganic compounds were the same as above. The oxonol was dissolved in dimethyl sulphoxide (60 μM) and added to the sample to a final concentration of 145 nM. The excitation and emission wavelengths were set to 485 nm and 515 nm, respectively, with 5 nm excitation and 10 nm emission slits. The gramicidin stock solution was of 45 μM in dimethyl sulphoxide and added to the sample to give the final concentration of 45 nM.

Fluorescence emission anisotropy measurements. A solution of $4.3 \cdot 10^{-3}$ M DPH in tetrahydrofuran was diluted 1000-fold by injection into a vigorously stirred normal PBS at room temperature. The obtained dispersion was stirred during 1 h in dark in order to avoid unfavourable photoisomerization of DPH and to evaporate THF. The diphenylhexatriene dispersion was mixed 10:1 (v/v) with the cell suspension of $5 \cdot 10^7$ cell/cm^3 concentration and incubated for 30 min at 37 °C in dark. The cells were then washed and resuspended in normal PBS to the same concentration.

For labelling the cells with the other hydrophobic lipid probe, $2 \cdot 10^{-3}$ M ethanol solution of 12-(-anthroyloxy)stearic acid was mixed 1:200 (v/v) with the cell suspension of $7 \cdot 10^6$ cell/cm^3 and incubated for 20 min at 37 °C. The final probe concentration was 10 μM. The dye uptake was followed in time by fluorescence intensity measurements. After incorporation of 12-AS the cells were washed and resuspended in normal PBS to a concentration of $5 \cdot 10^7$ cell/cm^3.

In contrast to DPH and 12-AS, TMA-DPH incorporates very rapidly into the cell membrane as indicated by the enhancement of the fluorescence emission of the probe [14]. Therefore, $5 \cdot 10^{-4}$ M TMA-DPH dissolved in *N,N*-dimethylformamide was injected 1:200 (v/v) at room temperature into a stirred PBS solutions containing 5 and 145 mM K^+, respectively. These solutions were added in 2:3 ratio to cell suspensions of $3 \cdot 10^6$ cell/cm^3. 5 min after the addition of the probe the steady fluorescence intensity could be measured with a negligible contribution of the free dye to the measured fluorescence.

During anisotropy measurements we have applied the final cell concentration of $2 \cdot 10^6$ cell/cm^3 in both normal and high-K^+ PBS containing 5 and 50 mM K^+, respectively. The excitation wavelength was both for DPH and TMA-DPH 360 nm and the emission was monitored at 425 nm (DPH) and 428 nm (TMA-DPH). For 12-AS, 383 nm and 440 nm were the excitation and emission wavelengths, respectively. The excitation and emission slits were always 5 and 10 nm.

Evaluation of membrane lipid dynamics. Steady-state fluorescence anisotropy values were obtained by simultaneous measurements of $I_{\parallel}$ and $I_{\perp}$, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized parallel and perpendicular to the direction of polarization of the excitation beam. The corrected fluorescence anisotropy are defined as

$$r = \frac{(I_{\parallel} - I_{\parallel}^S) - (I_{\perp} - I_{\perp}^S)}{(I_{\parallel} - I_{\parallel}^S) + 2(I_{\perp} - I_{\perp}^S)} \quad (1)$$

where the upper index S refers to the corresponding values of an unlabeled cell suspension [15]. The steady-state fluorescence anisotropy of the light emitted by a fluorophore incorporated into the lipid membrane provides information about the dynamic characteristics of its surroundings as it is expressed by the Perrin Eqn. [15]:

$$\frac{1}{r} = \frac{1}{r_0}\left[1 + \frac{C_r \tau T}{\eta}\right] \quad (2)$$

where r and r_0 are the measured and the limiting anisotropies, C_r is characteristic of the volume of the fluorophore, T is the absolute temperature, τ is the excited state lifetime and η is the average microviscosity of the lipid region around the fluorophore.

Microscopic investigations. The cells labeled with DPH were tested using a Univerśal Opton fluorescence microscope right after the labelling and 3 h later.

Determination of membrane potential. The cells were labeled in low Na^+, low K^+ medium with 145 nM oxonol and then treated with 45 nM gramicidin. This was followed by the increase of the external [K^+] by adding different [K^+] solutions to the samples. A calibration coefficient was obtained from the relationship between the relative changes in oxonol fluorescence and the calculated values of the membrane potential modified by addition of different amounts of external [K^+].

Results

When oxonol is added to suspensions of mouse thymocytes, the penetration of the dye into the cell interior is verified by an immediate and steep increase of fluorescence emission which levels off after 2 min (see Fig. 1). In the case of membrane depolarization more dye molecules can penetrate the cell membrane as

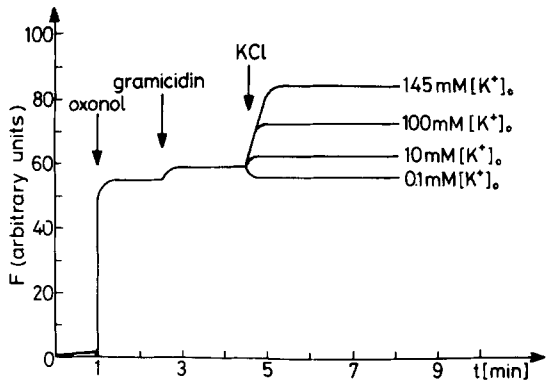

Fig. 1. Changes of oxonol fluorescence in suspension of mouse thymus cells of $2 \cdot 10^6$ cell/cm^3 concentration labeled with 145 nM diBa-C_4-(3) in low-Na^+, low-K^+ solution (containing in mM 248 sucrose, 1 Na_2HPO_4, 0.5 $MgSO_4$, 1 $CaCl_2$, 10 Hepes (pH 7.4)) after subsequent treatment with 45 nM gramicidin and different amounts of KCl. The relative increase of fluorescence signal of gramicidin-treated cells after KCl addition is related to the external K^+ concentration.

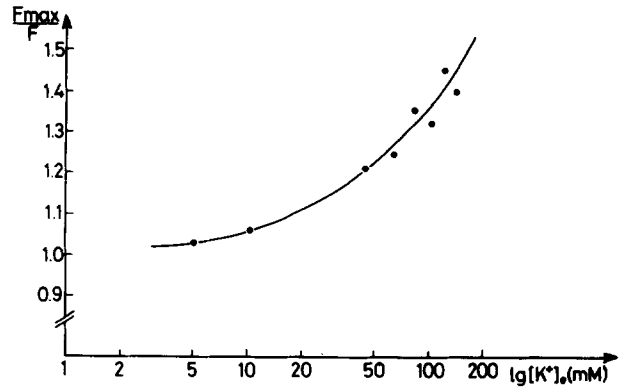

Fig. 2. The correlation between the external K^+ concentration and the relative shift in oxonol fluorescence of mouse thymus cells after treatment with 45 nM gramicidin and different amounts of KCl as well.

compared to cells under physiological conditions, resulting in a higher fluorescence signal [16]. With hyperpolarization the fluorescence intensity decreases accordingly.

After addition of gramicidin to cells in medium with low Na^+, low K^+ only a slight change of fluorescence could be observed (approx. 5% change) which indicates that the Na^+ gradient was essentially absent under the applied conditions. Addition of KCl to gramicidin-treated cells increases the fluorescence emission of the oxonol dye that reaches a steady level after 1 min. The extent of increase is related to the external $[K^+]$, as presented in Fig. 1. The maximum change is about 50% (145 mM external $[K^+]$). In low-Na^+, low-K^+ medium the K^+ depletion of gramicidin-treated cells is prevented (no external Na^+ is available to increase cellular Na^+) and the relationship between $[K^+]_o$, membrane potential and oxonol fluorescence can be investigated. Under these conditions we have used a modified form of the Goldman equation to calculate membrane potential [17]:

$$E_m = 61.5 \log_{10} \frac{[Na^+]_o + [P_K/P_{Na}][K^+]_o}{[Na^+]_i + [P_K/P_{Na}][K^+]_i} \quad (4)$$

where $[Na^+]_i = 18$ mM and $[K^+]_i = 153$ mM [17,18]. Since the permeability ratio practically does not influence the relationship, and after gramicidin-treatment the cell membrane is nearly equally permeable to Na^+ and K^+, the values of P_K/P_{Na} was taken to be 1. Depolarization induced by adding K^+ ions to the external medium resulted in a change of the membrane potential, ΔE_m of 10 to 65 mV, depending on $[K^+]_o$. The fluorescence response of the dye is about 0.5% per mV. Fig. 2 shows the experimental data of gramicidin-treated cells in low-Na^+ medium. The plot of fluorescence intensities obtained after KCl addition (F_{max}) related to the basic fluorescence (F) against the corresponding membrane potential values calculated from Eqn. 4 revealed that the relationship is sufficiently

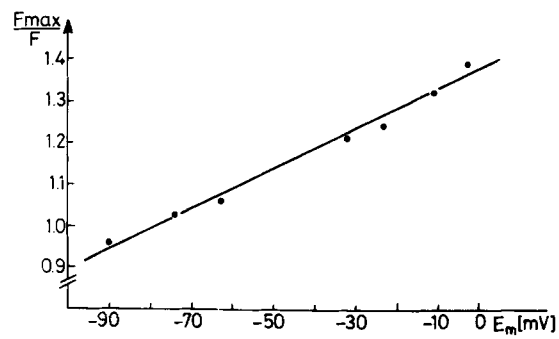

Fig. 3. The calibration curve of the fluorescence of oxonol in thymocytes at different external K^+-concentration relative to the basic fluorescence in low-Na^+, low-K^+ medium and the corresponding membrane potential values calculated from the Nerst equation (see Eqn. 3).

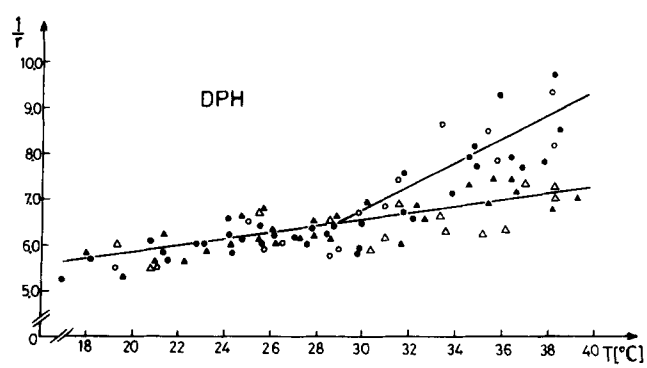

Fig. 4. The reciprocal value of the fluorescence anisotropy of DPH as a function of temperature obtained in intact (circles) and depolarized cells (triangles). Full symbols indicate increasing temperature, while empty ones refer to decreasing temperature, respectively. The experimental error of the reciprocal of the anisotropy was in the range of ±0.1–0.2 as determined by measuring the anisotropy values of six independent samples from the same cell suspension at 20 and 38° C.

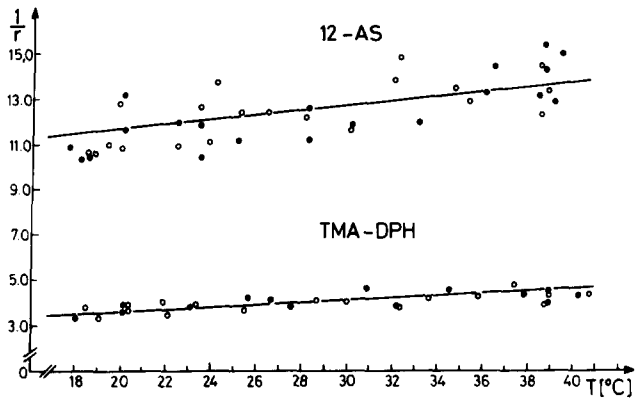

Fig. 5. The temperature dependence of the reciprocal of the fluorescence anisotropy of 12-AS and TMA-DPH in intact (full symbols) and depolarized cells (empty symbols).

linear to set up a calibration curve between the relative fluorescence intensities and membrane potential under the conditions applied (see Fig. 3).

The temperature dependence of fluorescence emission anisotropy of DPH, TMA-DPH and 12-AS was investigated by increasing/decreasing the temperature by 2–3 degrees in the range between 18° C and 38° C at two different membrane potential values set by applying 5 mM and 50 mM external [K^+]. Under the same experimental conditions DPH and TMA-DPH showed similar anisotropy values which were much higher than that of 12-AS. The temperature dependence of the reciprocal values of the emission anisotropy of DPH in intact and depolarized cell membrane are shown in Fig. 4. In the case of intact cells a break can be observed at about 28° C and above this temperature the increasing temperature results in a steeper anisotropy change as compared to depolarized cells. Essentially the same results were obtained when the temperature was decreased. The anisotropy–temperature curve for depolarized cells is nearly linear over the temperature range applied and has the same slope as the curve characteristic of intact cells in the temperature range below 28° C. Fig. 5 is a similar plot for 12-AS and TMA-DPH, as Fig. 4 is for DPH. 12-As and TMA-DPH do not seem to detect any alteration in the lipid mobility upon depolarization of the cell membrane. Accordingly, the temperature dependence of emission anisotropy of DPH seems to be sensitive for the depolarization while that of 12-AS and TMA-DPH does not.

Discussion

In our experiments an anionic potential sensitive dye, bis-(1,3-dibutylbarbiturate)trimethine oxonol was used to estimate the resting membrane potential of mouse thymocytes. The oxonol dyes undergo a potential-dependent distribution between the cytoplasm and the extracellular medium and are generally less cytotoxic than the other type of potential sensitive dyes, carbocyanines, that are experimentally proved to block mitochondrial respiration [19] and to have toxic effect on the plasma membrane of living cells as well [20]. In addition, its concentration in mitochondria is even lower than that in cytoplasm, thus the fluorescence intensities obtained are practically void from mitochondrial fluorescence and the resting membrane potential can be determined with a satisfactory accuracy [21]. Our estimate of this value is approx. −70 mV. This finding is consistent with other membrane potential values obtained for mouse [21–23], pig [22] and human lymphocytes [20,21,24]. Depolarization of cells can be induced either by altering the membrane permeability to different ions applying ionophores [17,22,23] or by varying the extracellular K^+ concentration [17,20,21,23]. According to our experiments, the 50 mM external K^+ concentration is high enough to depolarize the cells in an incubation period of 4–5 min without loosing their integrity.

In comparison of intact and depolarized cells we have studied the temperature dependence of steady-state fluorescence anisotropy of three dyes embedded in the cytoplasmic membrane. The information content of such a data set is usually related to different physical parameters discussed below.

'Lipid fluidity' is a phenomenologic term widely applied to phospholipid bilayers and natural membranes with respect to the physical state of the phospholipid matrix. By means of the fluidity concept [25] considering the bilayer (an anisotropic system) as an isotropic, homogeneous hydrocarbon fluid, the bulk lipid structure can be characterized by 'microviscosity' (η) or 'anisotropy parameter' $((r_0/r) - 1)^{-1}$, which provides a quantitative but averaged parameter of membrane fluidity. The latter is usually characterized as the extent of rotational diffusion of a fluorescent probe embedded in the analyzed region [25]. However, the steady-state anisotropy can be further resolved by means of time-dependent measurements into two major components that may vary independently [26,27]: a dynamic component which measures the rate of probe rotation, and a static component which indicates the extent of this motion (i.e., the angular range of probe rotation). The latter contribution is determined by the molecular packing (order) of the fatty acyl chains within the bilayer. Therefore changes in the value of steady-state anisotropy of a lipid probe may result from either changes in the microviscosity of the probe-environment or from alterations in the local lipid packing density. Our steady-state anisotropy data obtained with TMA-DPH and 12-AS reflect no major difference in the structural or physical state in the probed lipid membrane regions of intact and depolarized cells in the temperature range under study (see Fig. 5). By contrast, DPH reports about some alteration of the bulk lipid structure of

depolarized cells as compared to the intact ones when the temperature is above 28° C (Fig. 4). This finding can be explained by the different localization and orientation of these probes in the membrane and/or their different kinds of motion. Space filling models show that the length of the molecules of DPH and TMA-DPH is of the same order as the length of the fatty acyl chains of the lipids [28] and the dye molecules are of lipophilic nature. Therefore, it seems plausible that the long carbon chains of TMA-DPH and 12-AS is largely packed parallel to the phospholipid chains and they are anchored at the polar-apolar interphase due to their ionic charges.

The DPH molecules are likely to accommodate in the region near the centre of the bilayer [29–31] and preferentially oriented perpendicular to the surface [32]. Regarding the localization of DPH molecules in living cells, our microscopic investigations revealed that most of the fluorophores is situated in the cytoplasmic membrane even 3 h after the labeling. This is indicated by the fact that similar temperature dependence of emission anisotropy of DPH was obtained with normal and depolarized cells independently whether the given temperature range was covered by decreasing or increasing the temperature. The same indication came from the measurement of the emission anisotropy of DHP performed at 20 and 38° C, which showed no time-dependence up to 3.5 h. Although the possible translocation of DPH into the membranes of cell organelles cannot be excluded, according to the above results the contribution of such fluorophores to the total fluorescence intensity can be neglected.

Unlike 12-AS and TMA-DPH, the DPH molecules are not anchored in the bilayer, they have a 'vertical' motional freedom, as well [28–32]. This explains why DPH shows a distribution along the membrane normal inside the lipid bilayer [38]. Since the microviscosity varies along the membrane normal [33–36], the DPH molecules located at different distances from the center of the bilayer can experience different motional freedom resulted in different steady-state anisotropy values. Accordingly, the emission anisotropy of DPH is an ensamble average. This average is further altered by the possible lifetime distribution of the dye molecules. Since the lifetime decreases with the increase of the dielectric constant of the surroundings [37], the DPH molecules situated closer to the membrane surface can be characterized by shorter lifetimes due to the gradient of the dielectric constant across the membrane [38]. As it has recently been pointed out [38], the increase of the DPH mobility along the membrane normal may also result in a decrease of the average lifetime (if a dye molecule can sample all positions (characterized by different lifetimes) along the membrane normal during its excited-state lifetime). This can, again, introduce a change (increase) of the average anisotropy. Based upon the above picture we have two ways to interpret our findings with DPH:

Assuming that the DPH distribution is the same for the intact and depolarized cells over the whole temperature range we can conclude that intact cells seem to have a 'looser' membrane structure above 28° C than the depolarized cells (Fig. 4). Then, concerning the measured anisotropy, the 'looser' membrane structure of intact cells (above 28° C) could result in many effects as compared to the depolarized cells: the higher level of water prenetration may cause a decrease in the average lifetime of the excited state of DPH due to the increase of the local dielectric constant [38]; the increased level of DPH mobility, again, would decrease the average lifetime [38]. These two effects would increase the average anisotropy. Then, according to what we have found, the increased mobility (regarding both the rate and the range of motion) of the dye would have to dominate the anisotropy difference between the intact and depolarized cells (see Fig. 4). Regarding our data set obtained with TMA-DPH monitoring the membrane structure near the membrane surface, i.e., the polar-apolar interphase the application of the above picture leads to the conclusion that the increased mobility should be exactly counterbalanced by the decrease of lifetime (due to the change in the dielectric constant) over the whole temperature range. This would result in the same emission anisotropy for both intact and depolarized cells (see Fig. 5). According to our opinion this is a remote possibility which becomes even less likely in the view of our findings with 12-AS detecting no difference between the depolarized and intact cells over the whole temperature range (see Fig. 5).

Accordingly, the most reasonable interpretation of our results is that depolarization introduces (directly or indirectly) such changes in the membrane structure which are localized to the central, core region of the bilayer. We assume that above 28° C the intact cells, compared to depolarized ones, have a cytoplasmic membrane with higher mobility of the phospholipid chain ends in the core region. This allows the DPH molecules situated close to the 'membrane-core' of intact cells to experience a higher mobility as compared to those embedded in depolarized membrane. As a consequence, DPH distribution will also be more centered around the middle part of the bilayer in the intact cells. Since the DPH molecules close to the center of the bilayer have longer lifetime than those located closer to the surface [38], this difference in the distribution, in itself, can increase the average lifetime and therefore the average value of $1/r$. In this interpretation all the differences (regarding the distribution of DPH molecules, their mobility, and also the average lifetime) would result in the same type of deviations observed between the anisotropy values of depolarized and intact cells. In this case even a little change in the middle part

of the bilayer can result in a well-detectable change in anisotropy.

In conclusion we can say that a change in the potential gradient across the cytoplasmic membrane likely results in a change of the mobility of DPH molecules located in the central region of the bilayer. This change is either due to a direct effect characteristic of the bilayer structure itself (like some change in the membrane thickness) or, more likely, it is realized through conformational changes of different membrane proteins. In this latter case the changes in membrane structure probably are induced by conformational changes of membrane proteins and they are centered around and located within not too far distances from membrane proteins, i.e., in the boundary lipid regions. It is noteworthy that some authors [9,39] observed an enhanced membrane rigidity (monitored also by DPH) upon creation of transmembrane potential in lipid membrane vesicles. We observed an opposite tendency in mouse thymus cells, i.e., the depolarization of the membrane leads to an increased rigidity. We think that some potential-dependent, protein-mediated changes in the lipid structure may be responsible for the observed differences.

The relationship between the transmembrane potential and the lipid structure/dynamics observed in thymocytes seems to be interesting in relation to the transmembrane signaling processes. Further studies on different cell lines are needed, however, to see its generality. These investigations will obviously lead us towards a better understanding of rearrangements of cell surface elements associated with membrane potential changes.

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