# Interaction of Adrenocorticotropin-(1-24)-Tetracosapeptide with Lipid Bilayers

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**Abstract.** The interaction of the peptide hormone adrenocorticotropin with solvent-free planar lipid bilayers (BLM) and liposomes was studied by measurements of elasticity modulus perpendicular to the plane of the membrane ($E_\perp$, measured by electrostriction), surface potential difference ($\Delta\Phi_m$), electrical capacitance, capacitance relaxation following a voltage jump (yielding relaxation times for molecular dipoles or dipolar domains), and fluorescence polarization. Addition of the 6-fold positively charged peptide to one side of the membrane leads to a more positive membrane surface potential, an increase of BLM capacitance, a decrease of elasticity modulus, and faster relaxation time constants. This also caused a decrease of DPH fluorescence anisotropy of the liposome suspension modified by fluorescent dye DPH. Mixed BLM of palmitoyl-oleoyl-phosphatidylcholine (POPC) + soybean phosphatidylcholine (SBPC) (10:1 w/w), which carry a negative surface charge, exhibit considerably larger changes than electroneutral POPC membranes. Our results confirm that $ACTH_{1-24}$ binds to BLM and interacts with the hydrophobic part of the bilayer.

**Key words:** Lipid-protein interactions — Elasticity modulus — Surface potential — Capacitance relaxation — Fluorescence polarization — Bilayer lipid membranes

## Introduction

Adrenocorticotrophic hormone (adrenocorticotropin, corticotropin, ACTH) is localized in the anterior pituitary and induces the synthesis and secretion of corticosteroids from the adrenal cortex. The physiological function of these steroids secreted during stress or injury adjust the organism to the immunological and inflammatory defense mechanisms that are activated by stress (Donnerer et al. 1991).

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ACTH is a linear, flexible, amphiphilic nonatricontapeptide for which the structure – function relationship is known in some detail. Practical interest in ACTH has been stimulated due to the fact that this peptide is clinically useful as an important diagnostic tool. The field of medical application has been considerably broadened by the commercial availability of highly purified, synthetic hormone derivatives, e.g., $ACTH_{1-24}$ – tetracosapeptide. Just this derivative plays the main role in the physiological effect of ACTH (Schwyzer 1977).

The interaction of $ACTH_{1-24}$ with solvent-containing and solvent-free planar bilayer lipid membranes (BLM) has previously been studied by use of the capacitance minimization method. Schoch et al. (1979) reported that interaction of the peptide hormone with BLM led to an increase of the intrinsic electric field (difference in surface potentials) across the membrane, and that the intrinsic potential became dependent on the ionic strength on the side of the membrane opposite to that to which $ACTH_{1-24}$ was added. It was suggested that adsorption of the hormone is followed by an insertion of a part of the peptide, presumably the N-terminal region, across the bilayer (Gremlich et al. 1981). In contrast to this, analogous studies with solvent-free BLM gave no indication that $ACTH_{1-24}$ spans the lipid bilayer. The evidence for $ACTH_{1-24}$ incorporation into the membranes follows also from an infrared spectroscopic study (Gremlich et al. 1983); the binding of the hormone to negatively charged lipid was reported by Verhallen et al. (1984). Recently also Moreno and Prieto (1993) proved the existence of interaction of $ACTH_{1-24}$ with negatively charged small unilamellar liposomes by fluorescence spectroscopy. It was evident from the blue shift of Trp fluorescence and from the increase in its fluorescence quantum yield and anisotropy. These authors, however, did not find interaction of the peptide with neutral membranes at the interface membrane region.

The question arises as to the mechanism of interaction of $ACTH_{1-24}$ with solvent-free membranes. Therefore, in addition to measurements of intrinsic potential we have also studied the dielectric relaxation and membrane compressibility in direction perpendicular to the plane of the membrane, following adsorption of $ACTH_{1-24}$ to solvent-free BLM, as well as fluorescence polarization of liposomes modified by the fluorescence dye 1,6-diphenyl-1,3,5-hexatriene (DPH) upon addition of $ACTH_{1-24}$. The dielectric relaxation method allows changes in the orientation of molecular dipoles (preferentially located in the polar part of lipid bilayers) to be examined, while membrane compressibility and DPH fluorescence polarization reflect changes in the ordering of the membrane hydrophobic part.

## Materials and Methods

*Chemicals, preparation of BLM and liposomes*

$ACTH_{1-24}$ (95% purity) was a gift from Ciba-Geigy (Basel,) and was either puri-

fied by HPLC chromatography or used without purification. In both cases the effect of $ACTH_{1-24}$ on BLM was similar. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (Avanti Polar-Lipids, Inc.) and mixture of POPC + soybean phosphatidylcholine (SBPC) (Calbiochem) (10:1 w/w) were used to form BLM. BLM were prepared by the Montal-Mueller technique (Montal and Mueller 1972) in which two surface films of lipid plus *n*-hexane (Lachema) and *n*-hexadecane (Merck) (10 mg/ml, volume content of *n*-hexadecane was 20%) are apposed across a hole ($\sim$ 0.3 mm in diameter) in a thin copolymere septum (Slovnaft). After the films were spread, hexane was allowed to evaporate for approx. 10–20 min before BLM were formed. The aperture of the copolymere septum on which the bilayers were formed was pretreated with the corresponding lipid solution. Small liposomes (diameter 20–50 nm) were prepared by sonication of an aqueous dispersion of egg phosphatidylcholine (eggPC) (Sigma) or SBPC (concentration of lipids was 2 mg/ml) in a bath type sonicator UC 002 BM1 (Tesla) for 40 min.

A stock solution of DPH (Kocht-Light Co.) in acetone (0.5 mmol/l) was diluted with buffer to 2 $\mu$mol/l and then mixed 1:1 (vol/vol) with the liposome dispersion to give a final DPH concentration of 1 $\mu$mol/l and a final lipid concentration of 1mg/ml. The aqueous phase was a 9 mmol/l KCl (Lachema) solution buffered by 2 mmol/l HEPES (Sigma) (pH 7.4). All chemicals were purity grade, and were used without further purification. Experiments were done at $T = 20$°C.

The interaction of $ACTH_{1-24}$ with the membranes was monitored using *ac* voltage electrostriction, capacitance relaxation techniques and fluorescence polarization.

*Electrostriction of BLM*

The electrostriction method is based on the generation of higher harmonic components of an *ac* current flowing across the membrane. This method senses both the intrinsic electric field across the membrane (difference in surface potentials, $\Delta\Phi_m$) and the compressibility in direction perpendicular to the plane of the BLM. As shown by Carius (1976), when $\Delta\Phi_m \neq 0$ and an alternating voltage $U = U_0 \sin(2\pi ft)$ (where $U_0$ is the amplitude and $f$ is the frequency) is applied to the BLM, the membrane current contains a second current harmonic with frequency $2f$ and amplitude $I_2$ in addition to the first harmonic with frequency $f$ and amplitude $I_1$. The membrane is compressed due to electrostriction, and this is reflected in its thickness. According to Passechnik and Hianik (1977) this results in the generation of another current component with frequency $3f$ and amplitude $I_3$. Amplitudes $I_1$, $I_2$, and $I_3$ can be used to calculate electrical capacitance $C$ of a membrane, transmembrane potential $\Delta\Phi_m$, and modulus of elasticity in the direction normal to membrane surface, $E_\perp = -p/(\Delta d/d)$, where $\Delta d/d$ is the relative change in thickness, $p$ is the pressure induced by the electrostriction voltage ($p = C_s U_0^2/2d$), $C_s = C/A$ is the specific BLM capacitance per unit area, and A is the membrane

area:

$$C = I_1/2\pi f U_0 \tag{1}$$

$$E_\perp = 3C_s U_0^2 I_1/4dI_3 \tag{2}$$

$$\Delta\Phi_m = I_2 U_0/4I_3 \tag{3}$$

Parameter $E_\perp$ reflects the extent of membrane compressibility, and thus the degree of membrane ordering (Hianik et al. 1986). The measurement of the harmonic components can be performed using standard electronic equipment including resonance amplifiers (Hianik and Passechnik 1995). In the present experiments, Ag/AgCl electrodes were used to apply an alternating voltage with amplitude $U_0 = 10 - 40$ mV and frequency $f = 1$ kHz to the membranes. Values of $C_s = 7.2 \times 10^{-3}$ F/m$^2$ and $d = 2.6$ nm (Benz et al. 1975) were used for the calculations.

In measurements of $\Delta\Phi_m$ the surface charge and dipole components can be distinguished, as only the former depends on the ionic strength (Sargent et al. 1989; Sargent and Hianik 1994). In our experimental set-up the potential difference $\Delta\Phi_m$, which is a measure of the amount of substance adsorbed, is monitored continuously under computer control, and the time course is recorded following additions to the aqueous phase. The aqueous solution were stirred after each addition. Since determination of $\Delta\Phi_m$, $E_\perp$ and $C$ during stirring is accompanied by noise, the corresponding portions of the curves are indicated in the Figures by dotted lines.

*Capacitance relaxation*

The capacitance relaxation method is based on analyses of the time course of changes in the capacitance following sudden changes in the voltage applied across the bilayer (Sargent 1975a,b). Using this method one may obtain information about, e.g., reorientation of molecular dipoles and cluster formation. Capacitance relaxation was determined by measuring the time course of the displacement current following a step change of applied voltage from −50 mV to +50 mV. A detailed description of the construction and operation of the apparatus is given elsewhere (Sargent 1975a,b 1976). Capacitance relaxation curves were digitalized and recorded using a Biomation (USA) 805 waveform recorder. Calculation of relaxation times was performed using the program DISCRETE (Provencher and Vogel 1980).

*Fluorescence anisotropy*

Polarization fluorescence was recorded on a SPECORD M 40 (Carl Zeiss, Jena) spectrophotometer equipped with standard fluorescence accessories and polarizers in the excitation and emission beams. Steady state fluorescence anisotropy of DPH ($r_s = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$, where $I_\parallel$ and $I_\perp$ are the fluorescence intensities observed with analyzing polarizer parallel and perpendicular, respectively, to

the polarized excitation beam) was evaluated to assess lipid structural ordering in the hydrophobic interior of membranes (Shinitzky and Barenholz 1978). The fluorescence anisotropy values were measured during 50 min of DPH incubation in liposomes. Statistical analysis of data was performed by Student's $t$-test.

## Results

The time course of the adsorption of $ACTH_{1-24}$ to a POPC membrane is shown

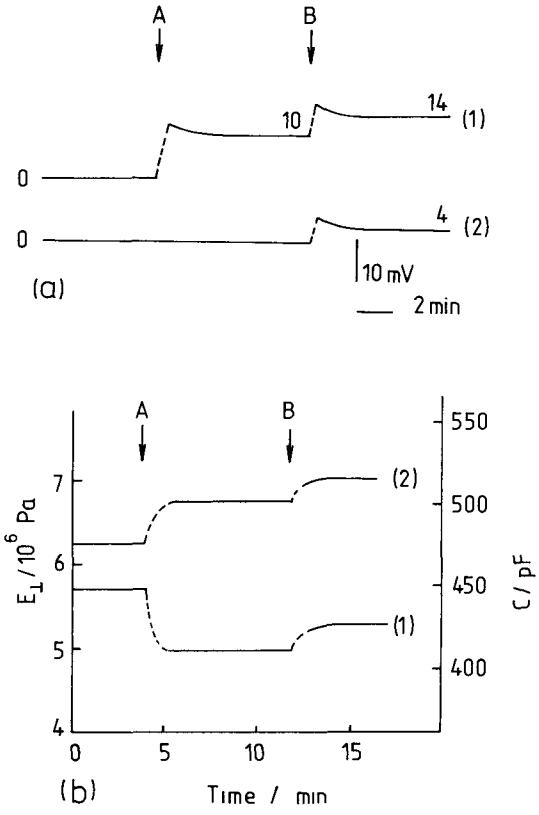


**Figure 1.** (*a*) Changes in intrinsic potential, $\Delta\Phi_m$ of solvent-free bilayers of POPC on addition of $ACTH_{1-24}$ to a final concentration of 0.11 mmol/l (time *A*), and a subsequent increase in the ionic strength on the opposite side from 10 to 110 mmol/l by addition of KCl (time *B*). The numbers above the curves indicate $\Delta\Phi_m$ in mV. Curve (2) shows the change seen after increasing the ionic strength (time *B*) with unmodified (pure) POPC, and represents the baseline for curve (1). (*b*) Changes in elasticity modulus $E_\perp$ (curve 1) and membrane capacitance $C$ (curve 2) of solvent-free bilayers of POPC upon addition of $ACTH_{1-24}$ to a final concentration of 0.11 mmol/l (time *A*), and a subsequent increase in the ionic strength on the opposite side from 10 to 110 mmol/l by addition of KCl (time *B*).

in Fig. 1*a* (curve (1)). At time *A*, upon addition of $ACTH_{1-24}$ (final concentration 0.11 mmol/l) to one side ("cis") of the bilayer, a rapid initial rise of $\Delta\Phi_m$ is followed by a slower decline of the signal to a steady state value of 10 mV. The polarity of the signal corresponds to the binding of positive charges to the cis-side of the membrane ($ACTH_{1-24}$ carries 6 positive charges under normal physiological conditions). At time *B*, the salt concentration on the opposite side ("trans") was raised from 10 to 110 mmol/l KCl. [This is done to test for the appearance of charges on the trans-side of the membrane (shielding effects), as would be expected from the incorporation reaction.] An increase in $\Delta\Phi_m$ of 4 mV is observed. (The dotted lines represent the approximative behaviour of membrane parameters during the period of the stirring of the electrolyte by a magnetic stirrer, which increased the noise of electric signals.) On curve (2) of Fig. 1*a*, the effect on the pure POPC bilayers of increasing the ionic strength on the trans-side from 10 to 110 mmol/l KCl is demonstrated. After equilibration, $\Delta\Phi_m$ also reached a value of +4 mV (this change may have been caused either by slight impurities in the lipid or may have been due to the pure lipid itself, e.g. a small head-group reorientation). A comparison of curves (1) and (2) shows that, within the experimental error, the increase in ionic strength on the trans-side of the membrane resulted in the same change of the equilibrium value of $\Delta\Phi_m$ whether or not $ACTH_{1-24}$ was added to the cis-side. Fig. 1*b* illustrates the time course of the elasticity modulus $E_\perp$ [curve (1)] and membrane capacitance $C$ [curve (2)] following the addition of $ACTH_{1-24}$ to the cis-side at time *A* and of KCl to trans-side of BLM of POPC at time *B*. Measurements of $E_\perp$ and $C$ were performed simultaneously with those of $\Delta\Phi_m$ (Fig. 1*a*). It is seen that the adsorption of $ACTH_{1-24}$ decreases the value of the modulus of membrane elasticity by about 12%, and resulted in an increase of membrane capacitance by about 8%. The increase of salt concentration on the opposite side ("trans") from 10 to 110 mmol/l KCl caused an increase in $E_\perp$ and $C$ by about 4%.

Analogous experiments were performed using solvent-free BLM of POPC+SBPC carrying approx. 10% negative surface charge mainly due to the presence of phosphatidyl glycerols. The surface charge density of these BLM was determined by a shielding experiment (see Sargent et al. 1989; Sargent and Hianik 1994) and amounted to $\sigma = 6.2 \times 10^{-2}$ elementary charges/nm$^2$. This value is about one order of magnitude higher than that seen for pure POPC BLM ($\sigma = 6.7 \times 10^{-3}$ elementary charges/nm$^2$, presumably due to charged impurities). Fig. 2*a*,*b* show the changes of $\Delta\Phi_m$, $E_\perp$ and $C$ for the charged BLM (POPC+SBPC) following the addition of 0.11 mmol/l $ACTH_{1-24}$ (time *A*), and an increase of ionic strength on the opposite side of BLM from 10 to 110 mmol/l KCl (time *B*). The equilibration times for the changes in $\Delta\Phi_m$, $E_\perp$ and $C$ were similar to those of POPC, however the magnitude of these changes was considerably higher. The results presented above were qualitatively well reproducible; they were obtained on 3–5 membranes in each series.

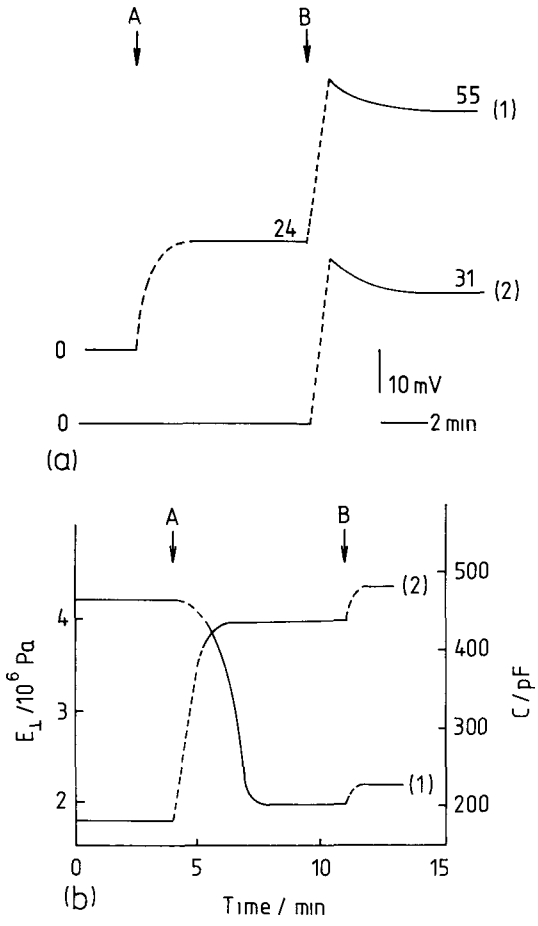


**Figure 2.** (*a*) Changes in intrinsic potential, $\Delta\Phi_m$ of solvent-free bilayers of POPC+SBPC upon addition of $ACTH_{1-24}$ to a final concentration of 0.11 mmol/l (time *A*), and a subsequent increase in the ionic strength on the opposite side from 10 to 110 mmol/l by addition of KCl (time *B*). The numbers above the curves indicate $\Delta\Phi_m$ in mV. Curve (2) shows the change seen after increasing the ionic strength (time *B*) with unmodified (pure) POPC, and represents the baseline for curve (1). (*b*) Changes in elasticity modulus $E_\perp$ (curve 1) and membrane capacitance *C* (curve 2) of solvent-free bilayers of POPC+SBPC upon addition of $ACTH_{1-24}$ to a final concentration of 0.11 mmol/l (time *A*), and a subsequent increase in the ionic strength on the opposite side from 10 to 110 mmol/l by addition of KCl (time *B*).

Capacitance relaxation results on pure, solvent-free POPC BLM before and after addition of 0.11 mmol/l $ACTH_{1-24}$ to one side of the BLM are shown in Table 1. In this experiment, current relaxation curves were averaged and the standard

**Table 1.** Capacitance relaxation components of solvent-free POPC BLM following adsorption of $ACTH_{1-24}$ (final concentration 0.11 mmol/l). Means ± S.D. were obtained by averaging of 64 current relaxation curves from one membrane

| Composition | $\tau_1$, ms | $\tau_2$, $\mu$s |
|---|---|---|
| POPC | 2.33 ± 0.63 | 111 ± 25 |
| POPC+$ACTH_{1-24}$ | 1.11 ± 0.55 | 113 ± 53 |

deviation was taken as the experimental uncertainty. Only two relaxation components were detected: for pure POPC, the slower component, $\tau_1$, had a time constant of 2.33 ms and the faster component, $\tau_2$, a value of 111 $\mu$s. Following addition of $ACTH_{1-24}$ the faster component did not change, but the slower one decreased by about a factor of two, to 1.1 ms.

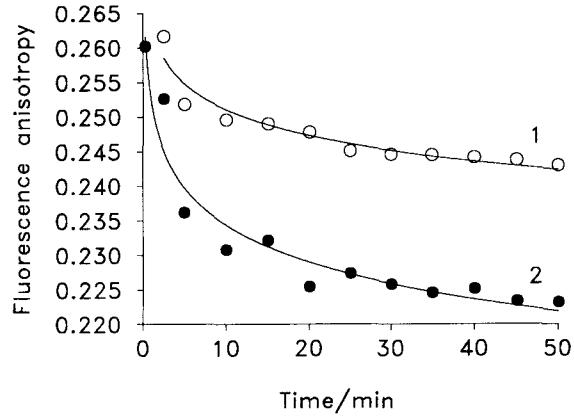


**Figure 3.** A representative time course of fluorescence anisotropy of DPH in SBPC liposomes without (1) and with (2) $ACTH_{1-24}$ (final concentration 0.153 mmol/l).

DPH incorporation into liposomes was checked by anisotropy decay within the time interval of 0–50 min (Fig. 3). No significant differences between kinetics of DPH incorporation into liposomes (both egg PC and SBPC) with and without $ACTH_{1-24}$ were observed. The DPH anisotropy values reached a plateau in 35 min. This corresponds to the steady state of DPH in the hydrophobic interior of membranes being reacted within 35–50 min. The paired Student's *t*-test revealed a significant decrease of the plateau anisotropy values in both SBPC (by 13.4%) and egg PC (by 9.5%) liposomes induced by $ACTH_{1-24}$. Anisotropy data in 45 min of

**Table 2.** DPH steady state anisotropy data for lecithin liposomes without and with $ACTH_{1-24}$ (final concentration 0.153 mmol/l). Results are as means ± S.D. of 9–11 independent experiments

| SBPC | | egg PC | |
|---|---|---|---|
| $r_s$ | $r_s^{ACTH}$ | $r_s$ | $r_s^{ACTH}$ |
| 0.233 ± 0.024 | 0.201 ± 0.035 | 0.186 ± 0.013 | 0.168 ± 0.002 |
| $P < 0.01$ | | $P < 0.05$ | |

DPH incubation are presented in Table 2. Anisotropy decay $r_s$ and/or $r_s^{ACTH}$ is directly connected with the ordering parameter $S$ of the membrane by the relation $S = \sqrt{r}_\infty/r_0$, where $r_0 = r\,(t = 0)$ and $r_\infty = \lim_{t\to\infty} r(t)$. For DPH, $r_0 = 0.39$ and $r_\infty = 4r_s/3 - 0.1$ (Shinitzky and Barenholz 1978). Our results thus reveal a decrease of the structural ordering of lipid bilayers in the presence of $ACTH_{1-24}$.

## Discussion

After addition of $ACTH_{1-24}$ the rise of $\Delta\Phi_m$ reflects a rapid adsorption of the peptide hormone. The changes of $\Delta\Phi_m$ for BLM of POPC+SBPC carrying about 10% negative charges (similar to biological membranes) are considerably higher than those of the much less electrically charged POPC BLM used. This effect has been expected owing to the Boltzmann distribution of the six-fold positively charged $ACTH_{1-24}$ between the bulk phase and the negatively charged surface. With the charge density found for the mixed POPC/SBPC BLM, the Boltzmann factor will be about 200. This explains the stronger effects seen with the charged BLM, and also implies that with naturally charged biological membranes the observed interaction could occur at $ACTH_{1-24}$ concentrations near physiological values. Our measurements of the change of surface potential upon addition of $ACTH_{1-24}$ thus confirm the results obtained on solvent-free BLM of DOPC by Gremlich et al. (1981), and indicate that $ACTH_{1-24}$ reacts less strongly with solvent-free than with solvent-containing BLM. Because the increase of the ionic strength on the trans-side causes a change in $\Delta\Phi_m$ similar to that seen with unmodified BLM, there is no evidence for a transfer of charges to the trans-side. If the incorporated to bound $ACTH_{1-24}$ ratio is the same as that determined by Schoch (1979) for solvent-containing BLM, then the expected change in $\Delta\Phi_m$ upon increasing the ionic strength on the trans-side is only of the order of 1 mV. This was showed by quantitative analysis even for the charged BLM. As this lies at the limits of the experimental accuracy, the negative result does not exclude the possibility of a translocation of $ACTH_{1-24}$ to the the trans-side of solvent-free DOPC BLM.

The increase of membrane capacitance following addition of $ACTH_{1-24}$ is presumably due to a decrease of surface tension, $\gamma$, of the cis-monolayer. Differences in surface tension will lead to a buildup of a hydrostatic pressure gradient $\Delta p \approx 2\gamma/R$, ($R$ is the radius of the bulged membrane; see, e.g. Kruglyakov and Rovin 1978), and the resulting bulging will lead to an increase of membrane area, and consequently of capacitance.

The considerable decreases of the elasticity modulus $E_\perp$ following adsorption of $ACTH_{1-24}$ indicates a disordering of the membrane, predominantly in the hydrophobic core. This strongly suggests that a part of the $ACTH_{1-24}$ molecule (presumably the N-terminus) inserts to some depth into the lipid bilayer. Very probably the disordered structure of the membrane arises around each incorporated $ACTH_{1-24}$ molecule.

The capacitance relaxation experiments support this model. As seen in Table 1, adsorption of $ACTH_{1-24}$ leads to a decrease of one of the measured relaxation time constants, suggesting increased freedom of motion of molecular dipoles in the BLM, as could result from a disturbance of the lipid structure due to the penetration of $ACTH_{1-24}$ molecules. (That the timescale of this relaxation is in the millisecond range suggests that the observed motion stems from clusters of lipid molecules.)

Results obtained by DPH fluorescence anisotropy measurements can be interpreted as a decrease of structural order in the hydrophobic part of the lecithin membrane induced by the presence of $ACTH_{1-24}$. The effect of $ACTH_{1-24}$ on disordering of charged membranes of SBPC was stronger in comparison with neutral egg PC liposomes, paralleling the results obtained on planar BLM.

Thus, in spite of the fact that the measurements of $\Delta\Phi_m$ gave no evidence for a translocation of a part of the hormone to the trans-side of BLM, the measurements of elasticity modulus $E_\perp$, relaxation time constant and fluorescence anisotropy clearly demonstrate a considerable effect of $ACTH_{1-24}$ on the ordering of the lipid environment. These findings are indicative of an interaction of $ACTH_{1-24}$ with the hydrophobic core of the BLM, and are consistent with the initial proposal by Schoch et al. (1979) of the incorporation of $ACTH_{1-24}$ into BLM.

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