Effect of Insulin on Lipid Bilayer Viscoelasticity

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Abstract. Changes in the Young elasticity modulus in perpendicular direction to the membrane surface $E_\perp$, in the coefficient of dynamic viscosity η, in the electric capacitance C, in the surface charge U_1, in the conductivity g and in the coefficient of non-linearity β of current-voltage characteristic caused by insulin were studied in bilayer lipid membranes (BLM) prepared from a mixture of egg lecithine and cholesterol (4:1, w/w) in n-heptane. Even relatively small concentrations of insulin in electrolyte ($c_i \sim 4.8 \times 10^{-11}$ mol/l) caused a diminution in parameters $E_\perp$ and η. Negative surface charge emerged on the membrane due to the insulin absorption, and U_1 gradually increased depending on the concentration of the hormone in the electrolyte. Addition of insulin was also followed by an increase in membrane conductivity and affected the value of the coefficient of non-linearity β of current-voltage characteristic. The effect of insulin on the BLM structure was discussed on the basis of the results obtained.

Key words: Insulin-bilayer lipid membranes — Viscoelastic properties — Membrane surface potential

Introduction

Insulin is well known to facilitate the transport of glucose into muscle and fat cells of the organism (Sorensen et al. 1980; Kono et al. 1982). The above process has been suggested to involve at least two different steps. During the first step, insulin binds to a specific receptor in the plasma membrane. Due to this bond, the glucose transport by a special carrier becomes activated (Step II) (Kono et al. 1982; Cushman et al. 1984). In studying membrane effects of insulin the question arises whether insulin, in addition to its interaction with the receptor, may also affect the lipid bilayer. If this is the case, physical and structural properties of the lipid bilayer may become changed, and the transport of glucose may become facilitated by active diffusion.

Membrane effects of insulin may easily be studied using artificial bilayer lipid membranes (BLM). BLM are composed of a lipid and a hydrocarbon solvent, such as *n*-heptane. BLM thickness of 5 nm as well as their other physical and structural properties are very similar to those of biomembranes (Ivkov and Berestovsky 1981). BLM are now being frequently used as a suitable model of biomembranes to study various biological and biophysical problems (Tien 1982). The study of insulin effects on lipid bilayers has so far been concentrated on the measurement of BLM conductance for glucose in the presence of insulin(Kafka 1974). Membrane conductance, while being a local characteristic of the membrane, does not allow detecting structural changes in the lipid bilayer. Due to this, and because of the problem mentioned above, our work was concentrated on the study of BLM viscoelastic properties in the presence of insulin. So far, effects of insulin on macroscopic viscoelastic properties of BLM have not been studied. However, membrane microviscosity has been known to represent an important parameter for insulin action (Amatruda and Finch 1979; Ginsberg et al. 1981; Yuli et al. 1982; Neufeld and Corbo 1982). The viscoelastic properties represent an integral physical characteristic of the membrane. They are characterized by a strong anisotropy, i.e. they are characterized by different values of the modulus of elasticity and different viscosity coefficients for different directions of deformation. E.g., in direction perpendicular to the membrane surface, deformation mainly induces changes in the hydrophobic region of BLM, i.e. in a region where active diffusion of the glucose carrier occurs. In the present paper, changes in viscoelastic properties of BLM were studied in direction perpendicular to the membrane surface, thus being characterized by the Young modulus of elasticity, $E_\perp$, and the coefficient of dynamic viscosity, η. The parameter $E_\perp$ is defined as the ratio of pressure (p) and the relative change in membrane thickness ($\Delta h/h$): $E_\perp = -p(\Delta h/h)$. The coefficient of dynamic viscosity can be expressed as the product of the Young modulus of elasticity E and the relaxation time, τ: $\eta = \tau_{E_\perp}$. The parameter η represents the time or the spectrum of relaxation times, during which the viscoelastic system changes its characteristic dimensions due to deformation induced by an external force (Passechnik and Hianik 1978).

Materials and Methods

The special method used to measured $E_\perp$ and η has been described in detail elsewhere (Passechnik and Hianik 1977; Hianik et al. 1984b). In principle, this method employs electrostriction: alternating electric voltage $U = U_0 \sin 2\pi ft$, where U_0 is the amplitude and f is the frequency, when applied to the membrane, induces membrane compression in direction perpendicular to the bilayer surface. An equivalent electric scheme of the membrane can be represented by a capacitor with a capacity C, connected in parallel with a resistor R. However, under the conditions of the experiment,

$$R \gg \frac{1}{2\pi fc}$$

and thus resistance R will in fact not affect the capacity current flowing through the circuit. The membrane thickness and thus its electrical capacitance ($C \sim 1/h$) shall change due to electrostriction. On the other hand, this change is non-linearly related to the voltage applied to the membrane:

$$C = C_0(1 + \alpha U^2)$$

where α is the coefficient of non-linearity. Consequently, in addition to the first harmonic (with amplitude A_1 and a frequency f), the third harmonic (with an amplitude A_3 and a frequency $3f$) shall also become generated in the current flowing through the membrane. The parameter $E_\perp$ is then obtained from the relation

$$E_\perp = \frac{3}{4} \frac{C_s \cdot U_0^2}{h} \frac{A_1}{A_3} \tag{1}$$

where C_s is the specific membrane capacitance ($C_s = C/S$, where S is the membrane area). The viscoelastic principle of the process of deformation shall result in a phase shift of the deformation in relation to the pressure, by an angle φ; this allows to estimate the parameter of the dynamic viscosity of the membrane. The dynamic viscosity is estimated as

$$\eta = \frac{E_\perp \sin \varphi}{2\pi f} \tag{2}$$

The method used in our experiments allows simultaneous estimation of the value of the membrane electric capacitance:

$$C = \frac{A_1}{2\pi f U_0} \tag{3}$$

If, in addition to alternating voltage also direct voltage, U_1, is applied to the membrane with the resulting voltage $U = U_1 + U_0 \sin 2\pi ft$, both the third harmonic (A_3) and the second harmonic of the current (with an amplitude A_2 and a frequency $2f$) become generated (Carius 1976).

The parameter U_1 can be obtained from the relation

$$U_1 = \frac{1}{4} U_0 \frac{A_2}{A_3} \tag{4}$$

To obtain $E_\perp$, η, C and U_1, the values of A_1, A_2, A_3 and angle φ are thus required. The latter values were recorded using a special experimental device, enabling the simultaneous measurement of all of them (see, e.g. Hianik et al. 1984b). To measure the above parameters, alternating voltage with an amplitude $U_0 = 100$ mV and a frequency $f = 995$ Hz was applied to the membrane. The parameter E was computed using values of $C_s = 0.34$ μF/cm^2 and $h = 5.5$ nm (Hianik et al. 1984b). When studying the changes of the viscoelastic properties of the membranes caused by various agonists, one should bear in mind that the third harmonic may become generated even if the agonist causes a non-linear conductivity of the membrane (see Passechnik et al. 1985). For this reason, we used a special method, described in the above mentioned work, to test the effect of insulin on the membrane conductivity.

Membranes were formed at room temperature ($T = 20$ °C), according to the method of Mueller et al. (1962) on a circular hole ($d = 0.8$ mm) in the wall of a teflon cup, dividing the vessel into two compartments of equal volumes (4 ml each). BLM were prepared from a mixture (4 : 1, w/w) of egg lecithine (Kharkov Plant of Chemical Preparations, USSR) and cholesterol in *n*-heptane (Fluka) (20 mg/ml) in Krebs-Ringer phosphate buffer prepared with re-destilled water. The solution contained (in mmol/l): NaCl 134; KCl 1.68; $MgSO_4$ 0.4; Na_2HPO_4 0.6; KH_2PO_4 0.149; pH 7.4. To this solution $CaCl_2$ was added in the final concentration of 10 mmol/l immediately before the forming of the membranes. Insulin (Calbiochem) was dissolved in Krebs-Ringer solution to give final concentrations

of 2×10^{-8} mol/l or 2×10^{-7} mol/l. It was applied by a microsyringe under constant stirring using a magnetic stirrer into one compartment of the cup to give final concentrations of insulin in the electrolyte of 4.8×10^{-11} mol/l; 6.4×10^{-10} mol/l or 8×10^{-9} mol/l respectively. Insulin-free electrolyte was simultaneously added to the other compartment to maintain hydrostatic pressure balanced and thus to prevent membrane bumping.

Results and Discussion

a) *Study of the visco-elastic parameters and the surface charge on insulin -modified BLM*

Insulin was applied into the electrolyte after the parameters A_1, A_2, A_3, had reached steady state values (~20 min following the start of the membrane formation). Insulin concentrations as low as 4.8×10^{-11} mol/l induced a gradual increase in the membrane capacitance, a decrease in $E_\perp$, and an increase in U_1 (Fig. 1). Further increasing of insulin concentration (up to 8×10^{-9} mol/l) was followed by stabilization of the values of C and $E_\perp$. The values of U_1 kept increasing at a lower rate. The increase in U_1 suggested a gradual adsorption of insulin molecules to the BLM surface. Negative value of the U_1 emerging on the side

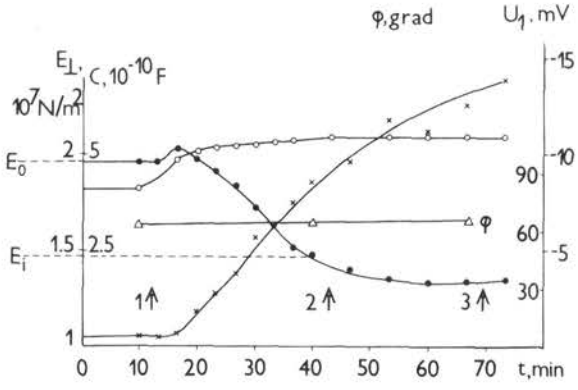

Fig. 1. An example of the kinetics of changes in $E_\perp$ (●), C(○), φ (△) and U_1 (×). Membrane prepared of egg lecithine + cholesterol (4:1, w/w) in *n*-heptane (20 mg/ml). Insulin was added to the electrolyte to one side of the membrane (the addition is indicated by arrows). Resulting concentrations of insulin in the electrolyte were: 1 — 4.8×10^{-11} mol/l; 2 — 6.4×10^{-10} mol/l; 3 — 8×10^{-9} mol/l. Electrolyte: Krebs-Ringer phosphate buffer, pH 7.4, $T = 20$ °C.

of insulin addition to the membrane, suggests an increase in the negative membrane surface charge δ. The dissociation constant of insulin (pK = 5.4) suggests that, under the conditions used in our experiment (pH = 7.4), the insulin molecule contains two elementary negative charges (Wu and Yang 1981). This suggestion concerning insulin adsorption has also been supported by a following

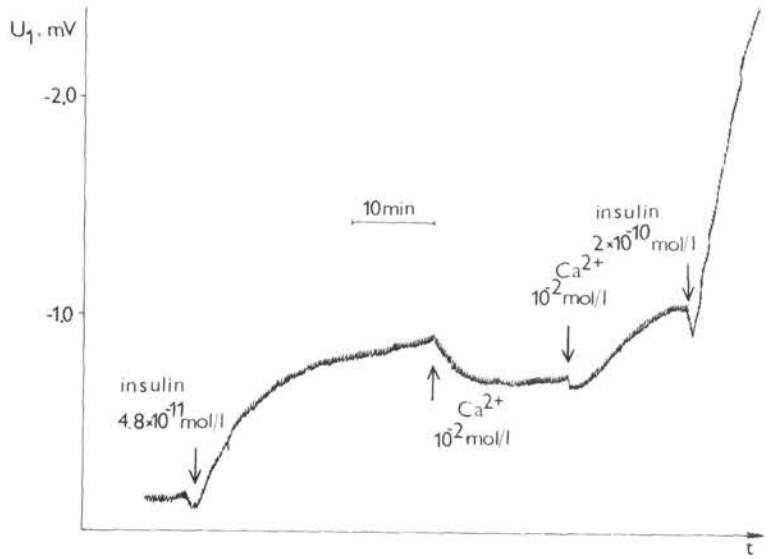

Fig. 2. Kinetics of changes in U_1. Membrane prepared of egg lecithine + cholesterol (4:1) in *n*-heptane (20 mg/ml) in the presence of 4.8×10^{-11} mol/l insulin. Ten µl of Ca^{2+} were added to the electrolyte into the insulin-containing compartment of the teflon cup (↑) or into the compartment containing no insulin (↓). Final Ca^{2+} concentration in the electrolyte was 20 mmol/l.

experiment (Fig. 2): after the addition of insulin a small amount of Ca^{2+} (final concentration of Ca^{2+} in electrolyte 20 mmol/l) was added to the same compartment of the cup as soon as the potential U_1 began rising. Calcium is known to adsorb to negatively charged membrane surface (Ohki and Papahadjopoulos 1970; Hianik et al. 1984c). It is obvious from Fig. 2 that following the addition of Ca^{2+} the value of the parameter U_1 decreased. After the addition of a similar amount of Ca^{2+} to the other side of the membrane (resulting in the compensation of the Nernst potential $\Delta\varphi\, Ca^{2+}$) the parameter U_1 kept rising. This insulin-induced change in the membrane potential U_1 agrees with findings reported by Cheng et al. (1981) and Zierler and Rogus (1981). The latter authors observed an effect of insulin on hyperpolarization of membranes of various insulin-sensitive tissues. The insulin-dependent increase in U_1 allows to estimate the size of the membrane charge. Using the theory of Guy-Chapmann (Ermakov et al. 1983), the parameter σ can be expressed as follows:

$$\sigma = (8\,\varepsilon\varepsilon_0 R\,Tc)^{1/2}\left(\mathrm{sh}\,\frac{U_1^1 F}{2RT} - \mathrm{sh}\,\frac{U_1^0 F}{2RT}\right) \tag{5}$$

where ε is the dielectric constant, ε_0 is the dielectric constant of vacuum; c is the electrolyte concentration; R is the gas constant; F is the Faraday constant; T

is the absolute temperature; U_1' is the surface potential in the presence of insulin; U_1^0 is the surface potential in the absence of insulin.

The surface potential of the membranes in the absence of insulin as measured according to the method of Sokolov et al. (1980) reached a value of $(1.8 \pm 1.1) \times 10^{-2}$ C/m^2. Fig. 3 illustrates the above relationship between both $E_\perp$

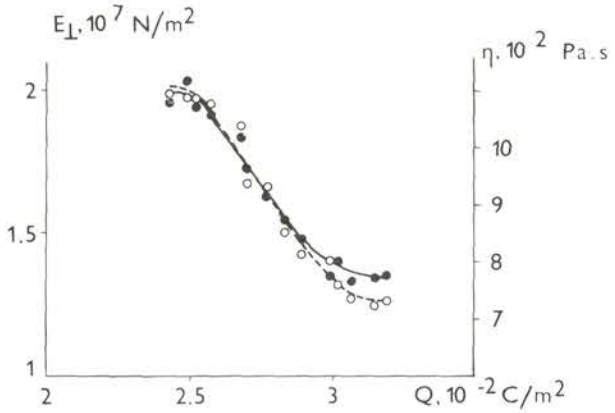

Fig. 3. Relationship between both $E_\perp$ (●) and η (○), and the membrane surface charge σ (computed for the membrane shown in Fig. 1).

and η, and the surface charge σ_i, for one of the membranes. Both $E_\perp$ and η decreased monotonously with a gradual stabilization at a practically constant level. This relationship (Fig. 3) may be interpreted as follows: Insulin adsorption is associated with the insertion of its peptidic chains into the hydrophobic region of the membrane (changes in $E_\perp$ and η) and consequently, the hydrophobic lipid chains adjacent to the insulin molecule become re-arranged. The membrane turns heterogenous, being in fact composed of two region types: one region with a changed structure characterized by the Young modulus E_m and a relative area s, and the remaining part (unchanged) of the membrane, characterized by the initial value of the Young modulus E_0 and a relative area 1-s. The resulting modulus of elasticity of the membrane, $E_\perp$ measured in our experiment, is equally dependent on both E_m and E_0 as well as on the relative areas s and 1-s, and it is determined by the relationship

$$1/E_\perp = s/E_0 + (1-s)/E_m$$

(Passechnik and Hianik (1979). A decrease in $E_\perp$ (Fig. 3) in dependence on σ_i can be explained as a gradual extension of the area of changed structure with a relatively small E_m as compared to E_0. In the region of the curve saturation ($\sigma_i = 3.1 \times 10^{-2}$ C/cm^2) the changed areas obviously overlap and further insulin

adsorption does not result in any change in $E_\perp$. This assumption makes it possible to estimate the minimum extent of membrane areas with a changed structure adjacent to one insulin molecule. As stated above, under the conditions of our experiment, the insulin molecule bears two elementary charges, thus the surface concentration of insulin in the region of saturation is

$$N=\frac{\sigma_i}{2q_e}=9.5\times 10^{16}\ \mathrm{m}^{-2}$$

(where $q_e=1.60219\times 10^{-19}$ C is the elementary charge). Taking into consideration the known BLM area ($S=5.03\times 10^{-3}$ cm^2), for the changed structure area around one molecule of insulin a diameter of 3.2 nm is obtained, a value exceeding in fact the effective diameter of the insulin molecule.

The effect of insulin on BLM depends on the initial state of the membrane. Fig. 4 shows the relationship of the relative change in modulus of elasticity, E_i/E_o, and the logarithm of the initial modulus of elasticity of the membrane E_o for 9 membranes of identical composition (egg lecithine + cholesterol, 4 : 1 w/w, in *n*-heptane, 20 mg/ml). The value of E_i was red following 30 min of insulin (4.8×10^{-11} mol/l) action on BLM (see Fig 1). The dispersion of the parameter E_o is associated with the physico-chemical characteristics of BLM formation. During the process of BLM preparation the concentration of the organic solvent in the membrane may vary, resulting in different initial values of E_o (see Passechnik and Hianik 1979; Passechnik et al. 1981). From Fig. 4 it is obvious that the higher the initial modulus of elasticity, E_o, the greater the relative change, E_i/E_o.

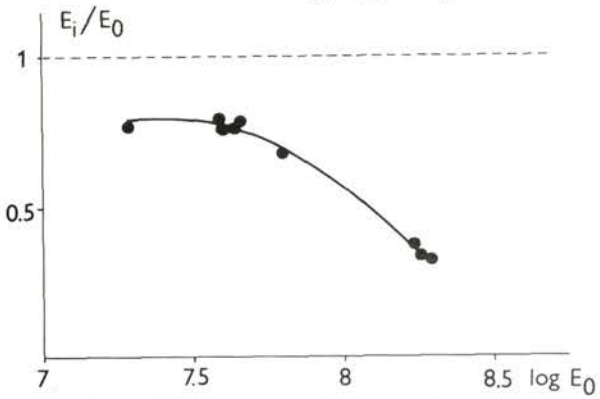

Fig. 4. Relationship between relative changes in the Young modulus of elastity E_i read after ~ 30 min of insulin action (4.8×10^{-11} mol/l) and the logarithm of the initial modulus of membrane elasticity E_o. Values for 9 different membranes. For conditions, see legend to Fig. 1.

b) *Study of insulin-induced non-linearity of BLM conductivity*

By measuring the coefficient of non-linearity β of current — voltage characteristics of the membrane the non-linearity of the BLM conductivity was determined in

dependence on insulin concentration. The meaning of the coefficient β is evident from the equation characterizing the direct current flowing through the membrane, where in the first approximation we can write: $i = Ug(1 + \beta U^2)$, U means the amplitude of direct voltage and g is the membrane conductivity. According to the value of the coefficient β, the relationship between the current and the voltage may be linear ($\beta = 0$), sublinear ($\beta < 0$), or superlinear ($\beta > 0$). Results obtained by measuring the membrane conductivity as described by Hianik et al. (1984a) showed that the addition to the electrolyte of insulin in concetrations up to 4.8×10^{-9} mol/l resulted in an increase in membrane conductivity ranging from $\sim 2 \times 10^{-11}\ \Omega^{-1}$ to $\sim 5 \times 10^{-11}\ \Omega^{-1}$ at $U = 100$ mV. The coefficient of non-linearity β dependent on the insulin concentration, is shown in Fig. 5. Experiments performed with ten membranes showed that the value of the parameter β was negative and its absolute value decreased as the insulin concentration in the membrane rised ($\beta = -2.05 \pm 0.64\ V^{-2}$) at zero insulin concentration, $\beta = (-0.68 \pm 0.26\ V^{-2})$ at insulin concentration 8×10^{-9} mol/l.

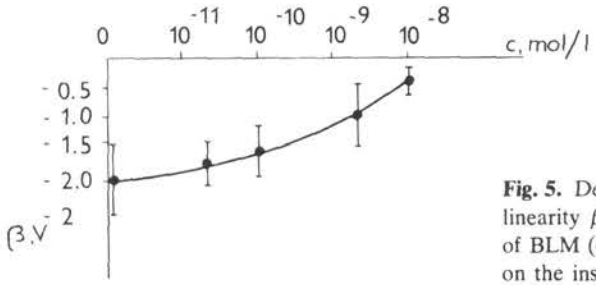

Fig. 5. Dependence of the coefficient of non-linearity β of the current-voltage characteristics of BLM (egg lecithine + cholesterol, 4:1, w/w) on the insulin concentration.

The increase in membrane conductivity and the change of the coefficient of non-linearity β of the current-voltage characteristics of BLM might be the consequence of the aggregation of insulin molecules on the membrane surface; this possibility was also discussed by Khan et al. (1978).

As mentioned above, non-linearity of the membrane conductivity may effect the value of the third harmonic, generated due to electrostriction. Let us therefore consider the influence of non-linearity of the membrane conductivity on the acquired visco-elastic parameters measured by the above electrostriction method. Using equation:

$$(A_3)_\beta/(A_3)_\alpha = \beta/\alpha(|\tilde{Z}_\beta|/|\tilde{Z}_\alpha|),$$

where $(|\tilde{Z}_\alpha|)$ is the modulus of membrane impedance for the alternating component of the current; $(|\tilde{Z}_\beta|)$ is the modulus of membrane impedance for the direct component of the current (see Hianik 1979), the amplitudes of the third harmonic is compared for electrostriction $(A_3)_\alpha$ or membrane conductivity $(A_3)_\beta$ alone. In

our case, the corresponding values for maximal insulin concentration in electrolyte ($c_i = 8 \times 10^{-9}$ mol/l, are $|\tilde{Z}_\beta| \cong 2 \times 10^{10}\ \Omega$, $|\tilde{Z}_\alpha| \cong 3 \times 10^5\ \Omega$.

$$|\beta| = 0.68\ \mathrm{V}^{-2},\ |\alpha| = \frac{3}{4} \cdot \frac{C_s}{E_\perp \cdot h} = 5 \times 10^{-2}\ \mathrm{V}^{-2}.$$

for the ratio $(A_3)_\beta/(A_3)_\alpha$ we obtained a value of 2×10^{-4}, which can be considered negligible. Moreover, while the value of the parameter (α) increased in parallel with the insulin concentration, the value of the parameter (β) diminished. The above discussed facts suggest that the interaction of insulin with BLM induced changes in the viscoelastic properties of the membrane.

All the results described were obtained in the presence of Ca^{2+} in electrolyte (10 mmol/l); this allowed the negative surface charge of the membrane to be compensated. This was necessary for the insulin interaction with BLM. The presence of Ca^{2+} in such concentration results in no changes in the value of $E_\perp$ for BLM (Passechnik et al. 1981). However, it should be noted that the question concerning the dependence of the insulin action on the presence of Ca^{2+} has not been fully elucidated as yet (Pershadsingh and McDonald 1984).

Our experiments have shown that insulin may directly affect the membrane lipid bilayer and thus change its viscoelastic properties at distances exceeding the effective diameter of the hormone molecule. The sigmoid shape of the relationship between both $E_\perp$ and η, and the surface charge (and/or insulin concentration) (Fig. 3) suggests a cooperative nature of the process of insulin-membrane interaction. In this case, it is a cooperation where incorporation of a certain amount of insulin facilitates the effect of other molecules. The cooperative nature of the insulin action and the concomitant diminution of the dynamic viscosity of the membrane may represent factors that facilitate the aggregation of insulin receptors on the membrane. This aggregation is an important step in the insulin action on target tissues (Kahn et al. 1978; Jacobs and Cuatrecasas 1981).

A very simplified model has been examined in our experiments: the interaction of insulin with an artificial bilayer membrane without any protein receptor. The results obtained may be considered as the first step towards the solution of the biophysical aspect of the insulin action on biological membranes. The discovery of the significant role of changes in the structural state of BLM characterized by the Young elasticity modulus (Fig. 4) for the interaction with insulin confirms the view that the primary step in the action of peptide hormones (binding and receptor aggregation) are probably of a simple biophysical nature and do not consume metabolic energy (Schlessinger 1980). In addition, the mechanical properties of the membrane significantly affect insulin binding to its own receptors in non-artificial membranes (Neufeld and Corbo 1982). A further step in studying these processes would be the examination of changes in biophysical properties of BLM with incorporated receptors. First experiments done in our laboratory showed that BLM

with incorporated fragments of plasma membranes from rat liver change their mechanical properties upon the addition of low concentrations of insulin; these systems can be used as a further approximation to the real biological system.

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