Insulin-Induced Changes in Mechanical Characteristics of Lipid Bilayers Modified by Liver Plasma Membrane Fragments

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Abstract. Insulin interaction with BLM with incorporated fragments of rat liver plasma membranes, containing hormone receptors, was studied by determining Young modulus of elasticity of bilayer lipid membranes in direction perpendicular to the surface, $E_\perp$. The presence of membrane proteins in a concentration of 60 $\mu g.ml^{-1}$ induced a significant decrease in parameter $E_\perp$ (to approx. 50 %) as compared with values obtained in non-modified membranes during insulin action (concentration interval $10^{-11} - 10^{-9}$ $mol.l^{-1}$). The extent of the effect was dependent on the initial phase state of the membrane, on cholesterol content in BLM as well as on membrane proteins concentration in lipid bilayer.

Key words: Mechanical properties — Lipid bilayers — Membrane receptors — Insulin action

Introduction

In a previous work (Hianik et al. 1987), insulin effects on viscoelastic properties of bilayer lipid membranes (BLM) were studied. The Young modulus of elasticity in direction perpendicular to membrane surface, $E_\perp$, and the coefficient of dynamic viscosity, η, showed considerable changes in relation to the initial physical state of BLM. The above work can be considered as the first approximation of the hormone-membrane interaction process. Aimed at further approaching the model to the real biological system, insulin interactions with BLM were studied, also using BLM with incorporated fragments of rat liver membranes with insulin receptors. The advantage of this approach is in that it enables studying one of the possible stages in the mechanism of hormone reception. During this stage involving hormone-receptor interaction, conforma-

tion changes of the macromolecular system in the membrane can occur (for review see Jacobs and Cuatrecasas 1981), which may spread to the lipid environment of the membrane. The method of measurement of $E_\perp$ proved to be particularly suitable for recording processes induced by protein conformation changes in BLM (Hianik and Vozár 1985).

Materials and Methods

Membranes were formed according to Mueller et al. (1962) on a circular hole ($d = 0.8$ mm) in a teflon cup wall, dividing the vessel into two identical volume ($\sim$ 4 ml) compartments. BLM were prepared from a 4 : 1 mixture of egg lecithin (Kharkov Plant of Chemical Preparations, USSR) and cholesterol (Fluka) in n-heptane (Kodak) (20 mg ml^{-1}). The lipid solution also contained rat liver membrane fragments in concentrations of 40—160 μg membrane proteins per 1 ml lipid solution isolated according to Emmelot et al. 1974). Protein concentration was detemined by the method of Lowry et al. (1951). BLM were formed in Krebs-Ringer phosphate buffer prepared with bidistilled water supplemented with 10 $mmol.l^{-1}$ $CaCl_2$; $CaCl_2$ was added immediately before starting membrane formation. Owing to the membranes being formed from a lipid solution containing biomembrane fragments, the receptor orientation in BLM was virtually symmetrical (see Hianik and Vozár 1985). Electrolyte temperature was kept constant (T = 20 °C) using a thermostat. Insulin (Calbiochem) dissolved in Krebs-Ringer solution with 0.05 % bovine serum albumin (Sevac, Prague) was added to one compartment to final sequential concentrations of 10^{-11}; 10^{-10}; and 10^{-9} $mol.l^{-1}$. Identical volumes of electrolyte were simultaneously added to the other compartment to balance hydrostatic pressure. Insulin was added approx. 30 min following membrane formation. This interval was sufficient to allow the parameters measured to reach steady-state values. Subsequent hormone administrations were performed in 30 min intervals. Membrane $E_\perp$ values were also read from continuous records in 30 min intervals.

A special analysis was carried out to check the specificity of the insulin-receptor binding. For this purpose, mechanical properties of liver membrane fragments-modified BLM with the hormone receptors treated by trypsin were determined. Trypsin (Serva) with a specific activity of 4 $U.mg^{-1}$ was used. Three ml of 1 $mmol.l^{-1}$ $NaHCO_3$ (pH 7.4) solution containing isolated membranes (1.5 mg membrane proteins per 1 ml) were added to 1 ml of 1 $mmol.l^{-1}$ $NaHCO_3$ (pH 7.4) solution containing 4 $mg.ml^{-1}$ trypsin. The mixture was incubated at 4 °C for 20 min. Then, 1 ml of Lima bean trypsin inhibitor (Sigma, 3.52 $mg.ml^{-1}$) was added and the mixture was subsequently centrifuged at 15,000 g for 10 min in a Janetzki K-24 centrifuge. The sediment was resuspended in 4 ml of 1 $mmol.l^{-1}$ $NaHCO_3$ solution (pH 7.4). A repeated centrifugation at 15,000 g for 10 min yielded sediment which was resuspended in 3 ml of 1 $mmol.l^{-1}$ $NaHCO_3$ and lyophilized. The test for specific insulin binding according to Schilling et al. (1979) showed no specific insulin binding to trypsin-treated membranes.

In each experimental series measurements were performed on 5—8 membrane samples. Student's t-test for nonpaired data was used and P-values of 0.05 or less were considered significant. Mean values and mean quadratic deviations were calculated. Linear regression was calculated using the least squares method.

The variable $E_\perp$ was measured using the electrostriction method (Passechnik and Hianik 1977). The Young modulus of elasticity $E_\perp = -\frac{p}{\Delta h/h}$ characterizes the degree of membrane compressibility (changes in its thickenss, $\Delta h/h$) upon the application of external pressure p, induced by alternat-

ing electrostriction voltage $U = U_0 \sin 2\pi ft$, with an amplitude $U_0 = 140\,\text{mV}$ and a frequency $f = 1\,\text{kHz}$.

Results

The parameter $E_\perp$ of BLM with receptors containing membrane fragments was dependent on fragment concentration in the lipid solution (Fig. 1). It can be seen (Fig. 1, curve 1) that $E_\perp$ decreased conspicuously around low membrane fragment concentrations (60 μg membrane proteins per 1 ml lipid solution). One possible explanation for these changes may be a substantial effect of hormone receptor proteins on the structural state of the lipid bilayer (see Passechnik et al. 1981). A region of altered membrane structure obviously occurs around the receptor. The growth of $E_\perp$ at higher membrane fragments concentration ($c > 60\,\mu$g membrane protein per 1 ml lipid solution) may result from stepwise overlapping of altered regions. It should be noted that an analogous effect had been observed in studying photoinduced changes in mechanical properties of BLM with membrane fragments containing bacteriorhodopsin (Hianik and Vozár 1985).

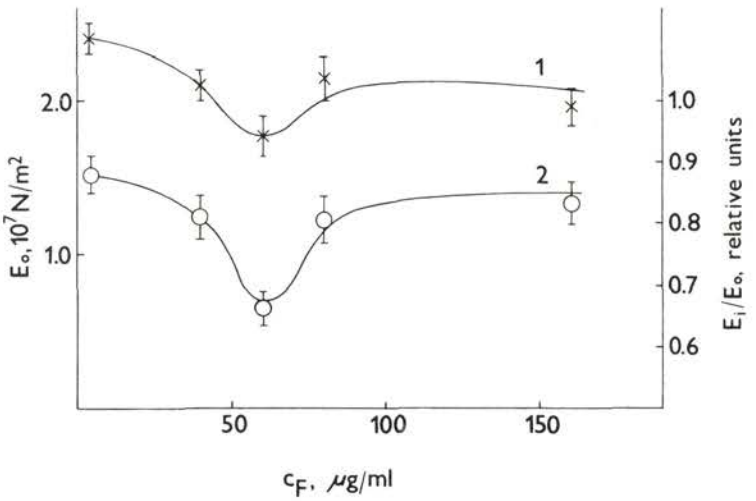

Fig. 1. Dependence of modulus of elasticity of BLM, E_0, on the concentration of rat liver membrane fragments in the lipid solution (1). Relative change in modulus of elasticity E_i/E_0 of BLM in dependence on the concentration of membrane fragments following ~30 min of insulin action (10^{-10} mol.l^{-1}) (2).

Insulin interaction with modified membranes generally resulted in $E_\perp$ decrease. The resulting effect was dependent on the concentration of membrane

fragments in the lipid solution. For quantitative expression of changes in BLM modulus of elasticity during insulin (10^{-10} mol.l^{-1}) action, the relationship E_i/E_o (E_o — initial modulus of elasticity; E_i — modulus of elasticity measured after 30 min of action of the corresponding insulin concentration), and concentration of membrane fragments in the lipid solution was constructed. The ratio E_i/E_o decreased with increasing concentrations of membrane protein, reaching a minimum at 60 μg.ml^{-1} and increasing thereafter (Fig. 1, curve 2). Also, changes in modulus of elasticity E_i/E_o were determined in relation to insulin concentration in electrolyte (Fig. 2). Fig. 2 clearly illustrates that increasing insulin concentrations in electrolyte are associated with a stepwise decrease of E_i/E_o, as corresponding to a growth of membrane deformability (deformability is defined as $M = 1/E_\perp$). Fig. 2 also shows that maximum decrease of E_i/E_o at the initial insulin concentration (10^{-10} mol.l^{-1}) occurs at membrane protein concentrations of 0; 40; 80; and 160 μg.ml^{-1}. A further decrease of $E_\perp$ with increasing insulin concentrations was virtually prevented by „ageing" of the membrane associated with a stepwise increase of modulus of elasticity.

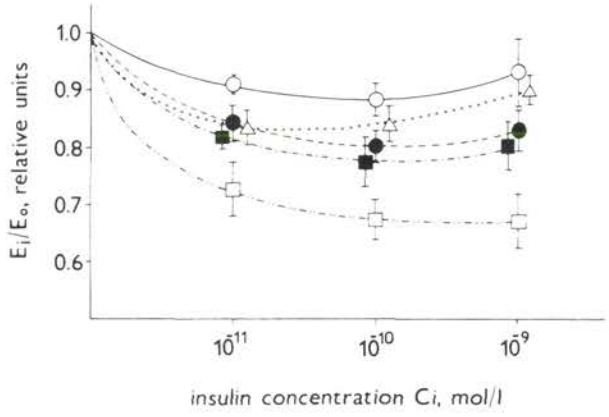

Fig. 2. Effect of insulin concentration on relative changes in modulus of elasticity E_i/E_o of BLM with various membrane fragments concentrations. ○ — 0; ● — 40; □ — 60; ■ — 80; △ — 160 μg membrane proteins per 1 ml lipid solution.

Fig. 3 illustrates the influence of trypsin on insulin binding. The effect of insulin on trypsin-treated membrane fragments modified BLM (in a concentration of 60 μg membrane proteins per 1 ml lipid solution) leads to practically equal relative changes of $E_\perp$ as in the case of interaction of insulin with unmodified BLM (curves 1 and 3). Curve 2 (Fig. 3) illustrates the effect of insulin on BLM modified by untreated membrane fragments at the same concentration of 60 μg membrane proteins per 1 ml lipid solution.

Fig. 4 (curve 1) shows kinetics of the ageing process of BLM modified by liver membrane fragments (electrolyte without insulin was added to BLM in the same 30 min intervals). Correction of relationship E_i/E_o for membrane ageing, e. g. for a BLM with a protein concentration of 80 μg.ml^{-1} (curve 2) yields relationship E_i/E_o as represented by curve 3. It is obvious that, due to the correction, the segment of E_i/E_o growth seen on the non-corrected curve, becomes lost. The relationship E_i/E_o reaches steady state at 10^{-8} mol.l^{-1} insulin. Nevertheless, the E_i/E_o curve may reach steady state values as soon as at 10^{-10} mol.l^{-1} insulin, with 10^{-8} mol.l^{-1} insulin leaving the value of E_i/E_o unchanged. Our experimental results do not allow distinguishing between the above alternatives.

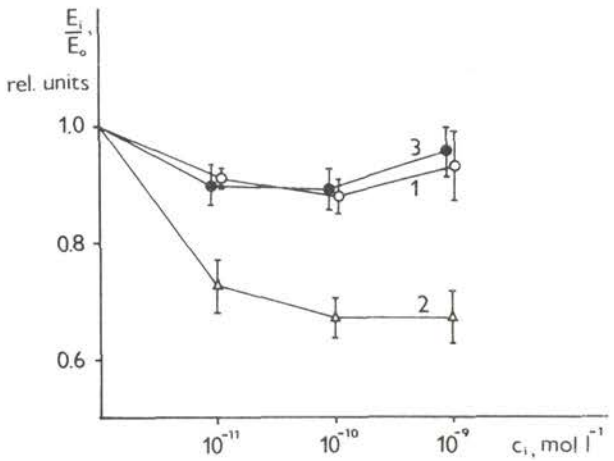

Fig. 3. Effect of insulin concentration on relative changes in modulus of elasticity E_i/E_o of BLM non-modified (1), modified with membrane proteins (60 μg. ml^{-1}) (2), modified with membrane fragments treated by trypsin (60 μg.membrane proteins per 1 ml lipid solution) (3).

Cholesterol has a strong effect on insulin action on membranes (Yuli et al. 1982). The presence of a considerable proportion of cholesterol in the lipid bilayer (33 mol %), inducing phase transition as well as changes in microscopic and macroscopic membrane characteristics (Hianik et al. 1986) considerably affects the action of insulin on both modified and non-modified membranes. Fig. 5 shows the dependence of the relative change in membrane modulus of elasticity E_i/E_o on insulin concentration in the electrolyte for various cholesterol concentration in the lipid solution used to form membranes. Two different membrane systems were studied: non-modified membranes (curves 1, 2) and liver membrane fragments-modified BLM (curves 3, 4). At large cholesterol

concentrations (33 mol %) the value of E_i/E_o of non-modified membranes increases with the increasing insulin concentration (curve 1). The nature of the changes in the relationship is close to that expressing the process of membrane ageing (Fig. 4, curve 1). Hence, at large cholesterol concentrations in BLM, insulin does not effectively influence mechanical properties of the membrane.

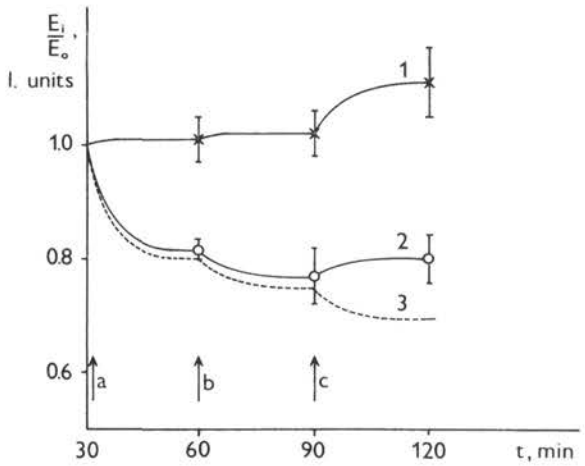

Fig. 4. Kinetics of changes in relative modulus of elasticity E_i/E_o of liver membrane fragments-modified BLM (80 μg membrane proteins per 1 ml lipid solution). 1 — kinetics of BLM „ageing" 2 — following insulin addition to the electrolyte: *a* — 10^{-11}, *b* — 10^{-10}, *c* — 10^{-9} mol.l^{-1}. 3 — relationship 2 corrected for membrane „ageing". Arrows indicate insulin addition.

Fig. 5 also shows E_i/E_o (c_i) for a relatively low cholesterol concentration (11 mol %) (curve 2); a similar cholesterol concentration is typical of many biomembranes (Ivkov and Berestovsky 1981). This latter cholesterol concentration induces no significant changes in physical characteristics of bilayers (Hianik et al. 1986). It is clear from Fig. 5 that at 11 mol % cholesterol, 10^{-10} mol.l^{-1} insulin induces an approximately 12 % decrease (statistically significant; Student's *t*-test; $p < 0.005$) of E_i/E_o of non-modified membrane. As shown earlier, 10^{-10} mol.l^{-1} insulin induces a strong decrease of E_i/E_o (by 32 % in average, curve 4) (statistically significant; $p < 0.001$) when acting on BLM containing both hormone receptors and 11 mol % cholesterol. The large cholesterol concentration (33 mol %) affects also insulin action on modified membranes. At 10^{-10} mol.l^{-1} insulin in the electrolyte, the change in BLM modulus of elasticity E_i/E_o will be approximately 22 % (curve 3).

It can be concluded that large cholesterol concentrations in lipid bilayers will entirely suppress insulin-induced conformational changes of non-modified

BLM, and decrease by 10 % in average the effectivity of insulin action on hormone receptor-modified membranes (statistically significant; $p < 0.05$).

The model approach employed in membrane reconstitution raises the question concerning the possible effects of the organic slovent, *n*-heptane, used

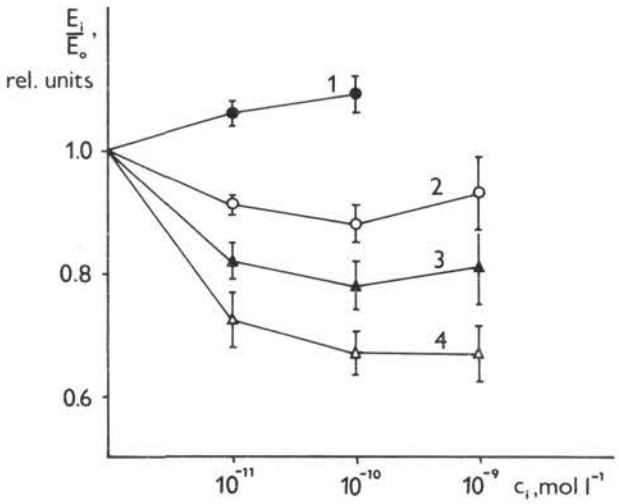

Fig. 5. The dependence of relative changes in modulus of elasticity E_i/E_o on insulin concentration; non-modified membranes (1,2) and liver membrane fragments-modified BLM (60 μg membrane proteins per 1 ml lipid solution) (3, 4) with various cholesterol contents: 11 mol % (2,4), 33 mol % (1,3).

to form BLM, on hormone receptor activity. This problem was paid special attention to by studying binding of mono- ^{125}I-(tyr A 14) -insulin, prepared according to Jørgensen and Larsen (1980), to liver plasma membranes in the presence of 50μl *n*-heptane per 500 μl membrane solution (100 μg membrane protein), using the method described by Schilling et al. (1979). The tests were performed with lipid solution containing egg lecithin, cholesterol (11 mol %) in *n*-heptane (20 mg.ml^{-1}) and liver membrane fragments. *n*-Heptane did not degrade insulin receptors; on the contrary, it induced an approximatively twofold increase in specific insulin binding. Fig. 6 summarizes the results and shows schematically total, specific and non-specific insulin binding to rat liver plasma membranes in the presence of *n*-heptane (dashed columns) and without the solvent (empty columns). Differences in total and specific insulin binding between the *n*-heptane containing and the solvent-free system were statistically significant (Student's *t*-test; $p < 0.005$ and $p < 0.001$ respectively). *n*-Heptane had no effect on non-specific insulin binding on membranes.

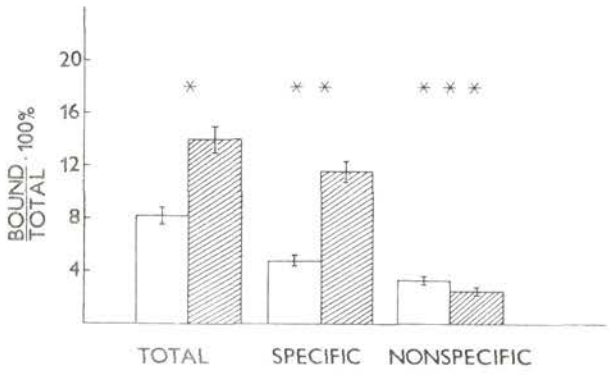

Fig. 6. Comparison of total, specific and non-specific insulin binding to liver plasma membranes without n-heptane (empty columns) and in the presence of *n*-heptane (hatched columns). Mean values and mean quadratic deviations for 5 measurements in each series are given. Statistical significance of differences: + = $P < 0.005$; + + = $P < 0.001$; + + + = statistically insignificant.

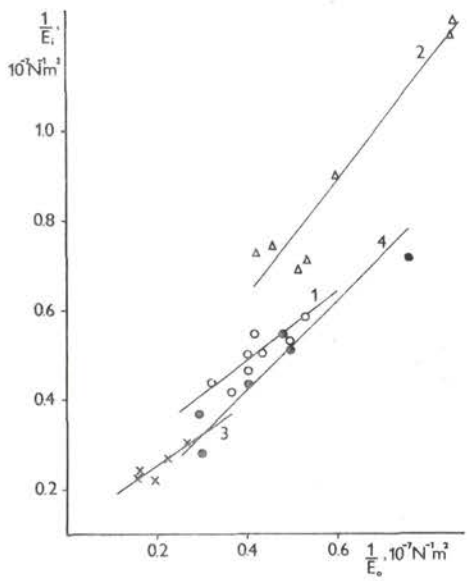

Fig. 7. The dependence of membrane deformability $1/E_i$ on initial deformability $1/E_o$, the former being measured 30 min following the addition of 10^{-10} mol.l^{-1} insulin to the electrolyte. Membranes of egg lecithin and cholesterol in *n*-heptane: 1 — without membrane fragments 11 mol % cholesterol, 2 — with liver membrane fragments (60 μg membrane proteins per 1 ml lipid solution), 11 mol % cholesterol, 3 — with liver membrane fragments (60 μg membrane proteins per 1 ml lipid solution), 33 mol % choresterol, 4 — with liver membrane fragments (60 μg membrane proteins per 1 ml lipid solution) treated by trypsin, 11 mol % choresterol.

Discussion

1. *Specificity of the insulin action on membrane*

The specificity of the insulin action on liver membrane fragments-modified BLM (containing hormone receptors) is suggested by relative changes in membrane moduli of elasticity E_i/E_o: the changes are by more than 20 % larger ($p < 0.001$) under insulin action as compared with non-specific insulin action (on non-modified membranes, Fig. 2). Nevertheless, a special analysis is required to confirm specificity of the insulin action on modified membranes. Substantial changes in E_i/E_o of modified BLM in the presence of large cholesterol concentrations in the lipid bilayer (33 mol %) also support the specificity of insulin action: at these concentrations, non-specific hormone action is entirely blocked. Trypsin offers another possibility to check the specificity: it digests peripheral membrane proteins including insulin receptor.

In our experiments, moduli of elasticity $E_\perp$ of BLM modified by liver membrane fragments with treated or untreated hormone receptors were measured. The protein concentration was $60\,\mu g.ml^{-1}$. At this concentration the insulin-induced decrease of E_i/E_o is maximal. Our experiments showed that changes in moduls of elasticity E_i/E_o of modified membranes with the hormone receptors treated by trypsin were effectively similar to the dependence of E_i/E_o on insulin concentration in the electrolyte as typical of non-specific insulin action (Fig. 3). A typical pattern observed with liver membrane fragments-modified BLM with treated receptors were considerably (2.5 times) larger values of initial modulus of elasticity E_o ($E_o = (4.7 \pm 0.5) \times 10^7$ Pa) as compared to the respective values of E_o of membrane fragments-modified BLM with preserved hormone receptors ($E_o = (1.7 \pm 0.1) \times 10^7$ Pa) (Fig. 7). These results suggest a considerable effect of proteins on mechanical properties of lipid bilayers. The increase in modulus of elasticity E_o of membrane fragments-modified BLM with treated protein receptors suggests that the protein receptor-induced change in structural state of lipid bilayer requires an entirely intact structure of the protein globule. Alterations of the arrangement of the protein-lipid complex due to trypsin-induced proteolytic digestion of peripheral membrane proteins results in a considerable change in the membrane state.

2. *Effects of the organic solvent*

The results obtained (enhanced specific insulin binding to hormone receptors in the presence of *n*-heptane) (Fig. 6) might be explained by a decreased parameter of ordering of the lipid bilayer due to the presence of the organic solvent (see Passechnik and Hianik 1978); as a result, the active centers of the protein receptor get a certain similarity with the earlier observations of enhanced

interaction of vesicular Ca-ATPase preparations following a preceding incubation with organic solvent (Passechnik et al. 1981).

It should be noted that similar results (enhanced activity of hormone receptors in the presence of *n*-heptane) have been reported by Zórad et al. (1987) who worked with whole cells (erythrocytes).

3. *Structural changes in the lipid bilayer*

The insulin-induced changes in mechanical characteristics of BLM suggest important changes to occur in the membrane. To analyze our results quantitatively, we employed the model of the occurrence of changed membrane structure around hormone receptors with hormone-interaction-induced conformational changes (see Hianik et al. 1985, 1987). According to this model, BLM contains two different structural regions: those with a changed structure (with a relative area (s) and Young modulus of elasticity E_M), and those with the initial, unchanged membrane structure (with a relative area (1-s) and modulus of elasticity E_o). Let us consider that relative area (s) also includes areas of altered lipid bilayer structure due to the effect of protein receptors of the modified membrane itself (without insulin) on BLM. According to Passechnik and Hianik (1978), modulus of elasticity of the membrane as a whole, E_i, can be related to E_M, E_o and (s) by

$$1/E_i = s/E_M + (1 - s)/E_o \tag{1}$$

Eq. (1) can be employed to evaluate areas of changed structure (s) around hormone receptors. The dependence of membrane deformability $1/E_i$ on initial deformability of the respective BLM was constructed for the membrane systems studied. Values of $1/E_i$ were expressed at time $t = 30$ min following the addition of 10^{-10} mol.l^{-1} insulin to the electrolyte, in which the membrane had been formed. It is obvious that the relationship $1/E_i$ ($1/E_o$) can be represented by a straight line $1/E_i = m/E_o + b$. The least squares method was used to express parameters m, b, and correlation coefficient r. The results are shown in Table 1. Two peculiarities are typical of membranes with a low cholesterol concentration (11 mol %): following inequalities hold for parameters b and m of BLM without receptors: $m < 1$, $b > 0$; while $m > 1$ and $b < 0$ for membrane fragments-modified BLM. The latter shows that, for the given case, the non-homogenous membrane model described by relationship (1) does not hold. Indeed, for BLM with membrane fragments the values of (s) become negative due to insulin action, and modulus of elasticity of changed regions E_M is larger than that characterizing areas of changed membrane structure arising a result of non-specific insulin action. However, this conclusion does not agree with the experi-

Table 1. Parameters of relationship $1/E_i = m/E_o + b$ for BLM of various composition in the presence of 10^{-10} mol.l^{-1} insulin, calculated by the least squares method. r — correlation coefficient, n — number of membranes in individual experimental series. Mean quadratic error of parameter m is given.

Membrane protein concentration in lipid solution μg. ml^{-1}	m ± SE	b m^2/N	correlation coefficient r	number of membranes
egg lecithin + cholesterol 11 mol %				
0	0.81 ± 0.05	0.16	0.86	8
40	1.57 ± 0.10	−0.13	0.86	7
60	1.27 ± 0.07	−0.12	0.96	7
80	1.35 ± 0.14	−0.01	0.79	7
160	2.18 ± 0.12	−0.49	0.93	5
egg lecithin + cholesterol 33 mol %				
60	0.68 ± 0.07	0.12	0.87	5

mentally obtained results. This paradox may be associated with the following process. During insulin binding to receptors, the vacant sites in active receptor sites have been occupied, further insulin molecules continuing to interact with the lipid bilayer start being incorporated into already changed regions. These arise around receptors due to conformational changes resulting from insulin binding to active receptor center. This process results in qualitatively new interactions in the membrane: various changed membrane regions may interact with each other as insulin can be expected to induce additional changes in the lipid bilayer as result of non-specific interaction of insulin with already changed membrane structures. Non-specific insulin action on non-modified membrane (see Hianik et al. 1987) suggests that a similar process may occur. As arises from the above paradox, mutual interactions between changed structure regions can no more be described by the two region model (changed and unchanged membrane structure). Obviously, the present case requires the development of more complex models of interaction mechanism in the membrane, accounting for several stages of mutual interaction between the individual structures of the lipid bilayer. Also, the associated inhomogeneity of the membrane should be considered.

The presented hypothesis concerning mutual interactions of various types of changed structure regions around protein receptors following insulin binding (specific interaction) and around insulin molecules incorporated in the lipid bilayer (non-specific interaction) requires both theoretical and experimental verification. These considerations are supported by our results showing changes in mechanical characteristics of membranes with large cholesterol content

during insulin action. As shown above, a large cholesterol content in the membrane (33 mol %) entirely suppresses the process of specific interaction of insulin with BLM, resulting in a decreased effectivity of specific interactions of insulin with modified membranes. Thus, employing a modified membrane with a large cholesterol content abolishes the non-specific interaction of insulin with the lipid bilayer when all the active centers of hormone receptors have been occupied by insulin molecules. Indeed, in this case, the parameters of the straight line are as usual: $m = 0.681$; $b = 0.12 \times 10^{-1}\,\mathrm{Pa}^{-1} > 0$ (see Table 1). These parameters allow to evaluate the relative area of the changed membrane structure occurring due to insulin binding-induced conformational changes of the protein receptor: $s = 1\text{-}m = 0.32$. Hence, the specific interaction of insulin with modified membrane with a large cholesterol content results in a change of approximatively 32 % of the membrane structure. This value represents almost a twofold of the corresponding area of changed membrane structure typical of non-specific insulin action (Hianik et al. 1987). In view of a considerable effect of cholesterol in membrane mechanical characteristics (see Hianik et al. 1986), an even much more significant enlargement of changed membrane area can be expected to occur around hormone receptors at low cholesterol contents in BLM.

In conclusion it can be stated that, as compared to non-specific interactions, the specific interactions in lipid bilayers result in much greater structural changes in lipid bilayers involving large membrane areas. The considerable effect of cholesterol in insulin interaction with the lipid bilayer during both specific and non specific action of the latter agrees well with experimental results obtained with the aid of spin and fluorescent spin probes (Aksentsev 1983), and confirms the hypothesis concerning the inhibitory cholesterol action on the aggregation process and on structural receptor changes due to a decreased mobility of membrane lipids (Gibbons et al. 1982).

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