Insulin Effects on the Resistance of Dipalmitoylphosphatidylethanolamine-succinyl Bilayer Membranes.

Vitaliy Kapishon[1] and Roger R. Lew, Biology Department, York University

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OBJECTIVE

The molecular interaction between insulin and dipalmitoylphosphatidylethanolamine-succinyl (DPPE-succinyl) membrane bilayers was studied using a planar bilayer lipid membrane system to measure the effect of insulin on the ionic conductance of the membrane. Changes in membrane resistance (the inverse of conductance) were measured by voltage clamp (in the range –200 mM to +200 mV) for 20 minutes prior to, and 20 minutes after the addition of 1 μg ml^{-1} insulin. The *cis* and *trans* bathing solution was phosphate buffered saline. Insulin caused an increase in the bilayer resistance of about 15 GΩ, while control treatments with PBS alone caused a decrease in resistance of about 75 GΩ (which is explained by membrane thinning effect). The difference between insulin and control treatments was statistically significant (two-tailed ttest, $P = 2.9 \bullet 10^{-5}$). We conclude that insulin is capable of increasing membrane resistance, possibly by intercalating itself into hydrophobic part of the membrane, thereby inhibiting ion movement through the membrane.

[1] RAY (Research at York) Research Assistant. Experiments were performed 01MAY2008 through 30NOV2008 in the Lew Laboratory and were funded in part by a NSERC (Natural Sciences and Engineering Research Council) Discovery Grant to RRL.

INTRODUCTION

The electrical characteristics of lipid bilayers —consisting of naturally occurring phospholipids such as phosphatidyl-choline, -serine, -ethanolamine, etc.— have been studied by monitoring the electrical resistance of the membrane. The electrical resistance of the single or mixed phospholipid bilayers depends directly on the structural and electrical properties of the phospholipids as well as composition of the solvent solution used to form a bilayer [1]. The addition of the naturally-occurring sterols (often cholesterol) and various proteins increase the bilayer resistance by decreasing fluidity, thus lowering ion leakage through the membrane. Despite the enormous amount of research done towards understanding electrophysiological properties of lipid bilayers in relation to biological membranes, very little attention has been to lipid bilayers composed of synthetic phospholipids. The synthetic phospholipids are of interest because of their potential use in liposomal drug delivery systems. In support of this rapidly developing area of biomaterial engineering, we studied the interaction of a lipid bilayer made of particular type of synthetic lipid, dipalmitoylphosphatidylethanolamine-succinyl (DPPE-succinyl) with insulin in phosphate buffered saline. Insulin is used as a model protein to determine how blood proteins will interact with the liposomes used in the drug delivery system.

METHODS AND RESULTS

Due to the absence of any previous electrophysiological characterization of DPPE-succinyl bilayers, the initial goal of this study was to successfully form DPPE-succinyl bilayer membranes and characterize the electrical properties (capacitance and electrical resistance) after bilayer formation. Then, the effect of insulin on the electrical resistance of the DPPE-succinyl bilayer was determined.

Bilayer chamber setup. The bilayer cuvette (internal diameter of 13 mm, with aperture for supporting the bilayer membrane of 0.1 mm diameter) (figure 1) was cleaned in hexane. It was inserted into the rear compartment of the bilayer chamber (Model BCH-

13A, Warner Instruments) so that the aperture faced the forward compartment. The cuvette and forward compartment were filled with phosphate buffered saline (138 mM NaCl, 2.7 mM KCl, pH 7.4 with 10 mM Na-phosphate). Electrical connections were made via salt bridges (U-shaped tubes containing 3 M KCl in 2% [w/v] agar, connecting the compartments to the Ag/AgCl half-cell chambers that were in turn connected to a head stage. The bilayer chamber was placed on top of a stir plate for mixing when insulin or control saline was added to the front compartment. Both head stage and bilayer chamber were shielded from electrical and vibrational interference by placing the entire assembly on an anti-vibration platform surrounded by a grounded metal cabinet (Faraday cage). The stir plate electrical outlet was also grounded when not in use.

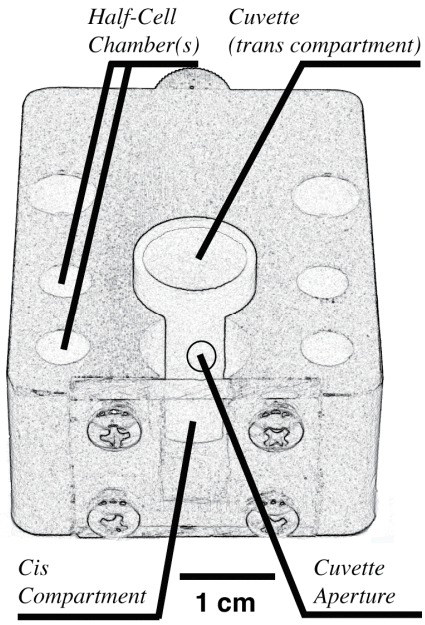

Figure 1

Apparatus for bilayer measurements. The bilayer was painted across a small aperture in the cuvette (at the center of the circle shown in the diagram). Electrical connectivity was achieved by connecting the *cis* and *trans* compartments to the half-cell chambers with 3 M KCl agar filled tubes (not shown). Silver chloride-coated silver wires were inserted into the half-cell chambers and connected to the headstage of the amplifier.

Electronics Set Up. The head stage was connected to bilayer clamp amplifier (BC-525C Warner Instrument Corp.) configured for voltage clamping. The amplifier applies a voltage across the membrane-supporting aperture of the cuvette and measures the clamping current required to maintain the voltage at the specified value. The data consists of two real-time analog channels (voltage and current), which were monitored and measured with a TDS 210 oscilloscope after low-pass filtering at 610 Hz. Prior to bilayer formation, the resistance of the aperture is very low (~1kΩ); in other words, the clamping

current was quite high with even the slightest voltage application. This is seen as a large magnitude square wave (~24 nA) under the capacitance test of the amplifier (a 30 mV triangular signal).

Bilayer Formation. The bilayer membrane was formed by applying 4 μl of 1 mg/ml DPPE-succinyl in benzene over the frontal side of the aperture using a 10 μl positive displacement micropipette. This was done during continuous capacitance measurements where the resulting current square wave showed an immediate decrease in capacitance from ~24 nF to ~26 pF. This is due to the formation of a bilayer. For the aperture of 0.1 mm diameter, a capacitance of ~26 pF lies within the "specific membrane capacitance" range of 24 – 30 pF (0.0019 – 0.0024 $pF \bullet cm^{-2}$) determined by previous BLM works on lipid bilayers and therefore is strong evidence of bilayer formation.

Bilayer Resistance Assay. Following membrane formation and confirmation of specific membrane capacitance, the bilayer resistance was measured by applying voltage clamps of ±50, ±100, ±150 and ±200 mV across the aperture while recording the clamping current on the oscilloscope. The data were plotted as current *versus* voltage along with a linear regression of the slope (the membrane conductance, inverse of resistance). The resistance measurements were performed at –20, –15, –10 and –5 min. After the –5 min measurement, 20 μl of 0.3 mg/ml insulin solution was pipetted into the front chamber and the solution stirred. Resistance measurements were then made 0, 5, 15, and 20 min. An example of measurements is shown in figure 2.

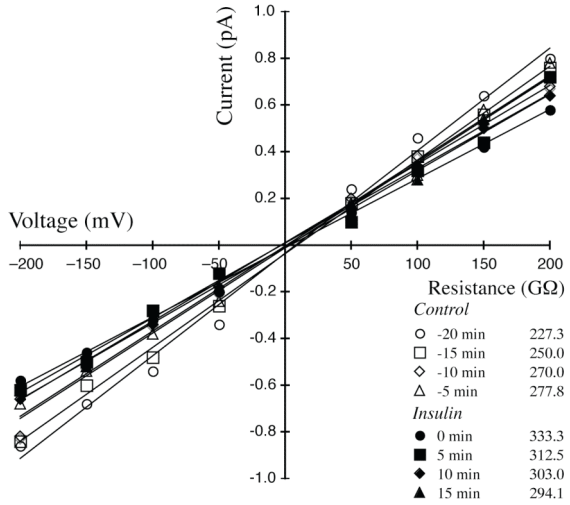

Figure 2

A single bilayer resistance assay generates 8 measurements in the form of 8 linear plots on current vs. voltage graph. The slope of the line is bilayer conductance, $G = \Delta I/\Delta V$, the resistance value for each measurement was calculated as the reciprocal of conductance, $R = 1/G$.

In order to account for the natural change in bilayer resistance after its formation, independent of an insulin effect on the bilayer, we also conducted a number of control assays. This was done by measuring resistances for a period of 40 minutes as described above but with saline addition rather than insulin addition at 0 min. A total of 17 resistance assays for insulin treatment and 10 controls were performed. All of the data are summarized in Figure 3.

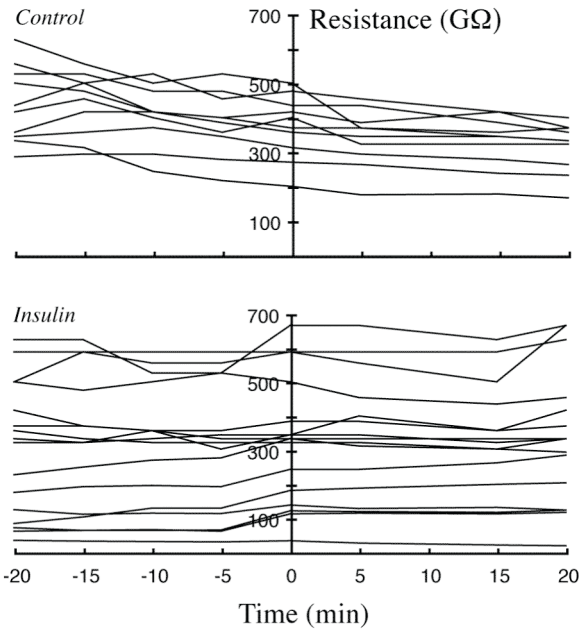

Figure 3

The y–axis (t = 0) separates bilayer resistance before and after insulin (or control) treatments. The absolute change in resistance was the difference between average resistances after and before insulin injection: ΔR = average R_{after} - average R_{before} for each experiment, or ΔR = average $R_{t=0,5,10,15}$ - average $R_{t=-20,-15,-10,-5}$.

Before comparative analysis of insulin assays and negative controls, we had to consider the consistency between the two sets of data. One major issue was the fact that the initial resistances (R_i) range for insulin treatments was much broader with a number of low values relative to the R_i range for the control data (figure 4).

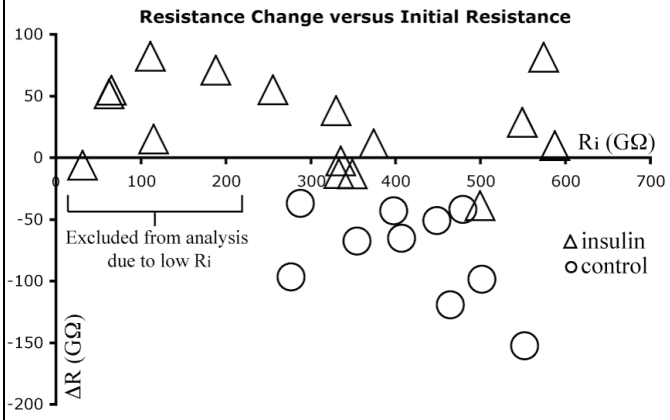

Figure 4

The change in resistance ($R_{after} - R_{before}$) versus the resistance before treatment ($R_{initial}$) graph shows that a subset of insulin experiments had a very low $R_{initial}$ (< 200 GΩ), while all of the controls had $R_{initial}$ ranging from 250–600 GΩ. The low initial resistance may reflect poorly formed bilayers which cannot be directly compared to higher resistance bilayers. Therefore, the measurements with $R_{initial}$ < 200 GΩ were discarded from further analysis.

After low resistant and disrupted membrane experiments were discarded, there were a total of 10 insulin and 10 control experiments. Statistical analysis of the adjusted data pool showed no significant differences between insulin and control experiments for $R_{initial}$ (two-tailed ttest, P = 0.95). The change in resistance was significantly different (two-tailed ttest, $P = 2.9 \bullet 10^{-5}$). For insulin treatments, there was an increase in resistance of 15 ± 36 GΩ; for control treatments, there was a decrease in resistance of –77 ± 38 GΩ (figure 5).

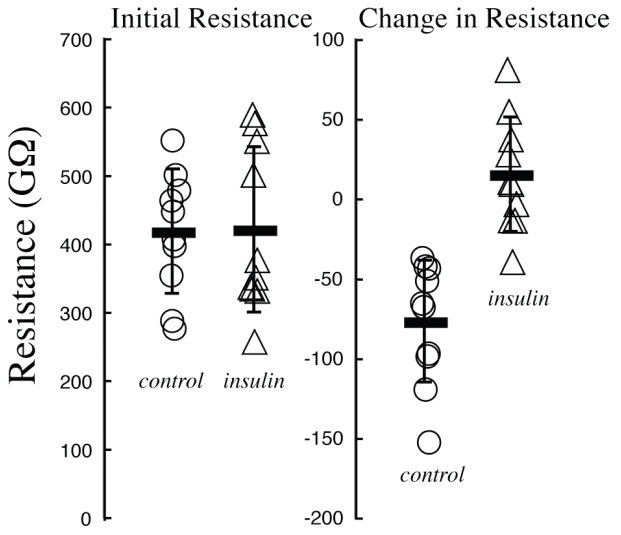

Figure 5

Left panel: the initial resistance for the two sets of experiments, (controls and insulin, as marked). Right panel: the change in resistance for controls and insulin treatments. The control resistance declined by –77 GΩ, the change for insulin treatments increased by 15 GΩ, a statistically significant difference compared to the control.

DISCUSSION

DPPE-succinyl bilayers were successfully formed. The initial electrical resistance was about 420 GΩ, which is about $3.3x10^7$ Ω•cm^2 (given an aperture radius of 0.005mm). These values are comparable to resistance measured for a variety of naturally occurring phospholipids (Table I).

Table I – Electrical resistances of naturally occurring phospholipids in comparison with DPPE-succinyl bilayer resistance [1]

Membrane Composition	Electrical Resistance (Ω•cm^2)
Phosphatidylethanolamine (A)	$2x10^5$
Phosphatidylcholine (A)	$8x10^5$
DPPE-succinyl (benzene)	**$3x10^7$**
Phosphatidylethanolamine and cholesterol (B)	$3x10^8$
Phosphatidylcholine and cholesterol (B)	$5x10^8$

(A) – solution of chloroform/methanol/tetradecane; (B) – solution decane;

From table I, it is clear that DPPE-succinyl phospholipids form a more resistant bilayer relative to phosphatidylethanolamine (PE) and phosphatidylcholine (PC) solvated in a

mixture of chloroform, methanol, and tetradecane. This can be explained by structural differences between the ethanolamine/choline and succinyl head groups. The positive charge located on nitrogen of both PE and PC is neutralized by the negative charge on phosphate giving an overall neutral molecule (zwitterion). However, in the case of DPPE-succinyl, the positive charge on the ethanolamine nitrogen is neutralized by formation of a peptide bond with one of the carboxyl ends of succinate, producing an overall –2 charge from phosphate and the free carboxyl on the succinate. Therefore, DPPE-succinyl bilayer forms two layers of negatively charged head groups enclosing the hydrophobic acyl groups from aqueous solution that create electrical barrier to the applied voltage resulting in higher resistance. Previous studies of bilayer formation report the existence of a time-dependent thinning process of a lipid bilayer which in turn results in a decrease in surface charge density upon thinning with resulting over time decrease in membrane resistance and increase in membrane fluidity [2]. This may explain the decrease in DPPE-succinyl bilayer resistance ($\Delta R_{control}$ = -77.2 GΩ) over time in negative control assays. Such membrane thinning and decrease in surface charge density allows more electric flow through the DPPE-succinyl bilayer and a smaller "electrical barrier" effect of negatively charged succinyl groups.

Table I also shows that the presence of cholesterol in PC and PE membranes results in a dramatic increase in electrical resistance of the bilayers, surpassing the DPPE-succinyl bilayer resistance. Cholesterol decreases membrane fluidity by compressing hydrophobic chains into higher ordered state [3]. Previous studies suggest that insulin, in its action on membranes, is similar to cholesterol. That is, it decreases membrane fluidity by inserting itself into hydrophobic core of the membrane. However, the extent of such penetration is expected to be lower because, unlike cholesterol, insulin is not entirely hydrophobic and thus does not completely penetrate the membrane. Even so, our results show that the introduction of insulin to the buffer surrounding DPPE-succinyl bilayer prevented a decrease in electrical resistance over time, actually increasing the resistance to a small extent (15 GΩ). This suggests that insulin penetration of the bilayer resulted in a more rigid membrane. Several studies involving insulin interaction with prokaryotic and eukaryotic membranes show that insulin decreases membrane fluidity [4, 5]. Our results

suggest that insulin can do the same to the synthetic lipid bilayer (DPPE-succinyl) which lacks natural mosaic of sterols and proteins found in membranes extracted from cells. In addition, insulin molecules, unable to penetrate the given small area of the bilayer due to full saturation, may aggregate at the surface of bilayer due to a tendency to expose their nonpolar regions to hydrophobic environment of membrane. Such aggregation would create additional impedance in series with the bilayer membrane.

CONCLUSION

We have demonstrated that it is possible to form stable pure DPPE-succinyl bilayers. The increase in resistance caused by insulin suggest a well-defined interaction between DPPE-succinyl and insulin. Our findings are a reference point for electrophysiological investigations of polymer grafted DPPE-succinyl bilayers designed for liposomal drug delivery systems.

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