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Ion-Diffusion Potentials and Electrical Rectification Across Lipid Membranes Activated by Excitation-Induced Material

(membrane conductance/sphingomyelin/D-α-tocopherol)

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ABSTRACT **Membranes synthesized from sphingomyelin–tocopherol mixtures and treated with the protein, "excitation-inducing material," to increase their electrical conductance were tested for applicability of the Nernst equation and changes in electrical rectifying behavior with temperature. Above pH 7, and in the temperature range 303–323°K, ion-diffusion potentials created by salts of monovalent cations showed that areas of the membrane activated by excitation-inducing material were only permeable to cations. Results also indicated that a parallel current with an activation energy of 1.00 eV per molecule passed through unactivated areas of the membrane. Below pH 7, the ratio of measured diffusion potentials to predicted values decreased steadily.**

Electrical rectification was exhibited by membranes activated by excitation-inducing material with positive current consistently passing preferentially from the side rich in excitation-inducing material to the opposite side. For a given membrane, the current–voltage curves did not change from 312 to 323°K; but below 312°K, the membrane suddenly increased its conductance and became ohmic. This phenomenon was independent of salt concentration and pH, and resembled a phase transition.

Membranes formed by the spontaneous thinning of lipid films separating two aqueous regions are model systems whose examination will hopefully reveal mechanisms for some of the processes occurring at living-cell surfaces. In the present study, a system was selected that shows a particularly close behavioral resemblance to excitable cell membranes. This was the sphingomyelin–tocopherol membrane activated by the protein, excitation inducing material (EIM), first reported by Mueller and Rudin (1). The membrane not only has the same electrical capacitance as excitable membranes, indicating a similar lipid thickness, but also has been shown to exhibit zero-current potentials in the presence of salt concentration gradients, delayed rectification of electrical current, and rhythmic action potentials after exposure to certain proteins (2, 3).

This investigation was originally started to determine the temperature dependence of parameters in the rectification equation for EIM-activated membranes proposed earlier (3),

$$I_m = I_0 \{\exp[(ne/kT)(V_m - V_0)] - 1\} \qquad [1]$$

where $V_0 = V_m$ at $I_m = 0$ and I_0, n = constants. Surprisingly, I_0 did not show a significant temperature dependence, even though rectification disappeared and the membrane became ohmic at lower temperatures, e.g., 300–305°K. It was found, however, that the zero-current potential, V_0, in the presence of a salt-concentration gradient was very dependent on temperature, suggesting that closer examination of this phenomenon might provide a clearer view of conduction processes occurring at this membrane.

Abbreviation; EIM, excitation-inducing material.

MATERIALS AND METHODS

The arrangement of apparatus used in this investigation is shown schematically in Fig. 1. Electrometer V_1 was a Keithley model 602 and V_2 was a Keithley model 600A. The signals measured on both electrometers and the thermocouple were recorded simultaneously on a strip-chart recorder (Argonaut Assoc., model MC6113H), and the thermometer was checked periodically to verify that the aqueous solutions inside and outside of the polyethylene cup were at the same temperature. This temperature was maintained constant within 1°K by means of a magnetic stirrer–hot plate. pH was measured with a Beckman model GS pH meter, and experimentally determined curves were used to correct for the change of pH with temperature.

Membranes were formed from mixtures of stearoyl sphingomyelin (Pierce Chemical Co.) and D-α-tocopherol (Eastman Kodak Co.) in ratios varying from 1:10 to 1:40 by weight. Each mixture was diluted with an equal volume of chloro-

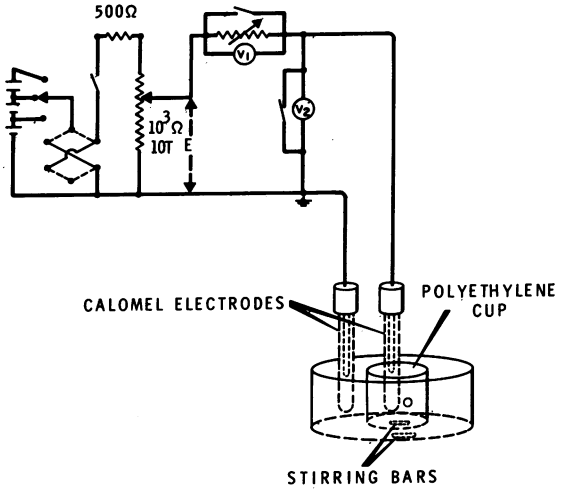

FIG. 1. Apparatus used for measurement of membrane potentials, conductances, and capacitances. Meter V_2 measured the membrane potential, V_m, while meter V_1 measured the membrane current, I_m. E, potential difference.

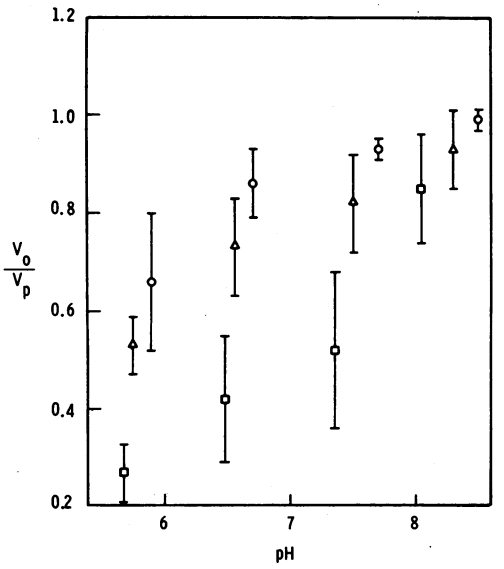

FIG. 2. Ratio of observed to predicted membrane potential as a function of pH at; O, 303°K; △, 313°K; □, 323°K. The *vertical lines* through the averages express twice the standard deviation of the measurements.

form–methanol (3:2) to form a painting solution. Doubly distilled water was used to prepare buffer solutions of DL-histidine (Sigma Chemical Co.), Tris [tris(hydroxymethyl) aminomethane, Sigma Chemical Co.], and PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid), Calbiochem], as well as solutions of reagent-grade inorganic salts. EIM was obtained from the Eastern Pennsylvania Psychiatric Institute.

Solutions that were 5.0 mM in the buffer salt and 10.0 mM in an inorganic salt were boiled to remove dissolved air, cooled rapidly to 313°K, adjusted to their initial volume with distilled water, and poured on both sides of the polyethylene cup. A thin film of the sphingomyelin–tocopherol solution was painted across the aperture in the cup connecting these sides, and this film gradually and spontaneously thinned until its surface took a gray metallic sheen. Apertures were

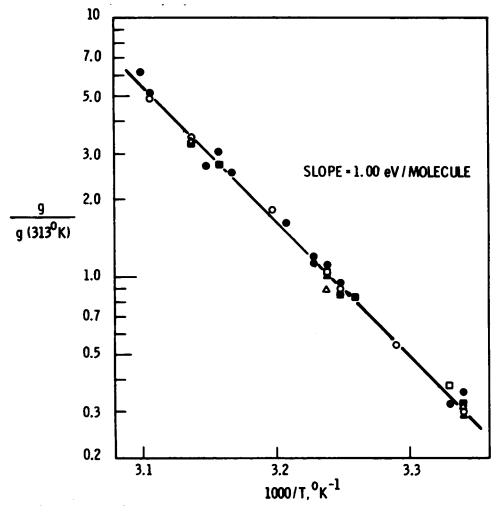

FIG. 3. Variation in specific conductance of unactivated sphingomyelin–tocopherol membranes with temperature. Data on replicate membranes were normalized at 313°K and are represented by different symbols. *g*, conductance.

TABLE 1. *Ratios of observed ion-diffusion potentials, produced by various salts and corrected for current through the unactivated membrane, to predicted potentials at 313°K, if we assume that the membrane is only permeable to cations*

Salt	Buffer	pH	V_0'/V_p ± SD	Determinations
KCl	PIPES	7.7	0.87 ± .01	3
KCl	Tris·HCl	7.7	0.93 ± .04	2
KCl	Histidine	7.5	0.94 ± .06	8
LiCl	"	7.2	0.93 ± .04	4
Li_2SO_4	"	7.3	1.00 ± .04	4
NaCl	"	7.5	0.91 ± .05	6
KF	"	7.5	0.95 ± .05	5
K_2SO_4	"	7.5	0.94 ± .09	5
CsCl	"	7.3	0.95 ± .06	9
NH_4Cl	"	7.6	0.99 ± .05	2
$(CH_3)_4NCl$	"	7.3	0.90 ± .04	5

used varying from 1.2 to 2.1 mm² in area, and the flatness of the membranes formed across them could be readily detected by the pattern of reflected light.

Electrical capacitance of the membrane was determined from the time constant for the charging curve at 313°K. The electrical resistance was then measured over the range 298–325°K. After these measurements, the temperature was readjusted to 313°K, and small quantities of EIM were added as an aqueous solution to the inside compartment. The membrane resistance decreased rapidly as the protein interacted with the lipid barrier; this process was accelerated if a positive electrical current was directed from inside to outside. Additional inorganic salt of the type used in preparing the original solution was then added to the inside compartment in the form of a concentrated solution. During this operation, care was taken to keep the membrane flat by addition of sufficient amounts of the original buffer solution to the outer compartment until the membrane was observed to be flat. These volume changes were too small to affect the inorganic salt concentrations within the precision of this investigation.

After the concentration of salt in the inside compartment was increased to a new value, ranging up to 80 mM, the open-circuit potential-difference across the membrane was measured by reading the applied potential difference, *E*, required to nullify the circuit current. This could always be measured to the nearest mV. Data for a complete current–voltage characteristic curve was then taken at 313°K, and the process was repeated at temperatures about 10°K lower and higher.

These measured potentials were compared with the ion-diffusion potentials predicted by the Nernst equation, i.e.:

$$V = (t_+ - t_-)\ (kT/ne)\ \ln\ (a^\circ/a) \qquad [2]$$

where: V = ion diffusion potential across the membrane, t_+ = transport number for the cation, t_- = transport number for the anion, a° = activity of permeable electrolyte on the grounded side of the membrane, and a = activity of permeable electrolyte on the other side of the membrane.

In addition, complete current–voltage characteristic curves were obtained for the membranes to determine their electrical rectifying behavior as a function of temperature and pH.

The pH of the initial buffer solution used on both sides of the membrane was varied in approximately single pH-unit increments, over the range 6–9, and the open-circuit potentials as well as current–voltage curves were obtained at the three temperatures. Various 1-1 and 2-1 electrolytes were investigated including LiCl, NaCl, KCl, CsCl, NH_4Cl, $(CH_3)_4NCl$, KF, and K_2SO_4. Usually histidine was used as the buffer; some checks were also made with the other buffers mentioned.

RESULTS AND CORRELATIONS

Before interaction with EIM, sphingomyelin–tocopherol membranes were not found to develop ion-diffusion potentials in the presence of LiCl, NaCl, KCl, or CsCl even when concentration ratios of up to 8:1 were used across the membrane. Differences in hydrogen-ion (or hydroxide-ion) concentration across the membranes, however, did create membrane potentials. If we assume that these potentials were governed by the Nernst equation, the transport number of the hydrogen ion was calculated to be 0.94 ± 0.06 on the basis of measurements on five membranes at temperatures ranging from 303°K to 323°K. Within this same temperature range, capacity measurements on 70 membranes gave an average value of 5.0 ± 0.6 $\mu F/cm^2$ and were independent of salt concentrations and composition of painting solution.

After EIM interacted with the membranes, concentration gradients of all the listed salts produced ion-diffusion potentials. In each case, a negative potential developed on the side of the membrane exposed to the higher salt concentration. It was found that the zero-current potential, V_0, in the presence of a salt-concentration gradient was very dependent on temperature, decreasing by as much as 30 mV from 303°K to 323°K. In order to understand this decrease, the potentials predicted by Eq. [2] for $t_+ = 1.00$, were calculated and the ratios of V_0 to V_p, the predicted potential, were plotted as a function of pH for three different temperatures as shown in Fig. 2. In this set of data, KCl was used as the inorganic electrolyte and histidine was used to buffer the solution. It is evident from the graph that the standard deviations for data taken at 303°K are the smallest. This is also the temperature at which the conductance of the unactivated lipid membrane was very low. Examination of the data also indicated that the smallest values of V_0/V_p at a given pH were always obtained at the highest temperature, especially in cases where the resistances of the activated and unactivated membranes were nearly equal. This suggested that there were two mechanisms for current flow in the EIM-activated membrane. One mechanism was that present in the unactivated film; the other was due to salt conductance after activation with EIM.

The conductances of unactivated sphingomyelin–tocopherol membranes were found to be quite sensitive to temperature, as shown in Fig. 3. For membrane potentials between ±50 mV, Ohm's law was obeyed; but conductance values ranged from 2×10^{-8} to $2 \times 10^{-6}\ \Omega^{-1} \cdot cm^{-2}$ with most values near $2 \times 10^{-7}\ \Omega^{-1} \cdot cm^{-2}$. Despite this variation in absolute values, the dependence of relative conductance on temperature was the same for all membranes and could be represented by $g/g_0 = \exp(-\Delta E/kT)$, where $\Delta E = 1.00$ eV per molecule (96 kJ/mol).

If the current through the bare lipid regions of the membrane is in parallel with that through the EIM-activated regions, a simple circuit analysis showed that the correct ion-diffusion potential, V_0' should be the measured value of V_0 divided by a factor $(1 - x)$, where x is the ratio of the resistance of the EIM-activated membrane at zero current to that of the unactivated membrane. The correction due to this factor was 5% or less at 303°K, 10–20% at 313°K, and sometimes as much as a factor of 3 at 323°K. Fig. 4 shows the results when these corrections are made and supports the view that there are two parallel conductive pathways.

When this correction is made, the temperature dependence of the corrected values appears to be simply that of the

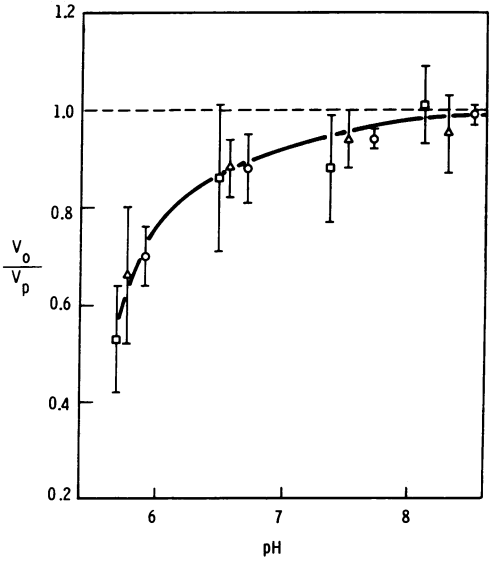

FIG. 4. Ratio of observed membrane potential, corrected for current through the unactivated membrane, to predicted membrane potential as a function of pH at: ○, 303°K; △, 313°K; □, 323°K. The *vertical lines* through the averages express twice the standard deviation of the measurements.

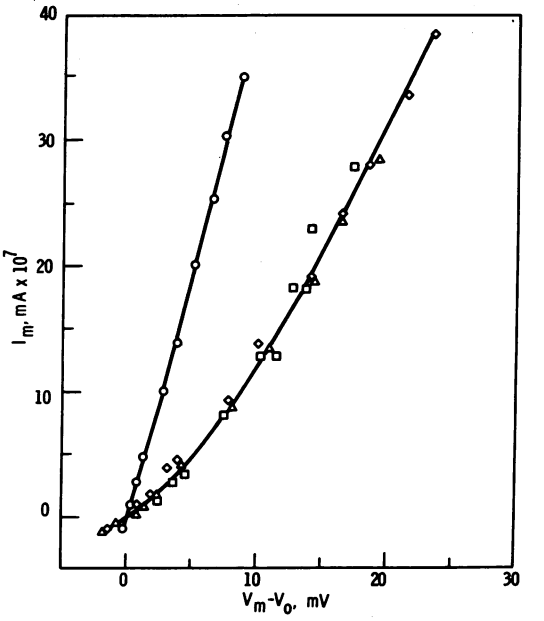

FIG. 5. Current–voltage characteristic curves for the same membrane at temperatures of: ○, 308°K; △, 312°K; ◇, 315°K; □, 319°K. The pH of the buffer was the same and near 7.5 on both sides of the membrane. The ratio of KCl activities on the two sides, $a°/a$, was near 8.

Nernst equation. Also, the value of V_0'/V_p is 1.0 at pH 8.0 but falls off to about 0.5 near pH 5.0. The data shown in Fig. 4 represent average values of 4–9 observations at 313°K, 2–7 observations at 303°K, and 2–5 observations at 323°K.

Although not as much data was taken for the other salts and buffers, there was no evidence of any difference in their behavior greater than the experimental error. Data was taken on some of these salts over the entire pH and temperature ranges shown, but most values were obtained at 313°K and near pH 7.5 (Table 1).

Graphs of I_m against V_m were made not only to obtain the resistance of the activated membrane at $I_m = 0$ but also to determine the range of the constants I_0 and n in Eq. [1]. Early measurements showed that these latter quantities were far from constant. I_0 was found to range from 4.0×10^{-7} to 1.6×10^{-4} mA/cm² although most values were between 5×10^{-6} and 5×10^{-5} mA/cm². The values of n ranged from 1 to 6, with most of the values around 2 or 3. Careful examination of the data from many experiments indicated that this variation was due to different extents of interaction between EIM and the lipid membranes. This interaction, as measured by the increase in membrane conductance and rapidity of delayed rectification, was found to be strengthened by repeated pulses of positive current in the rectifying direction and weakened by current in the reverse direction. Both effects were enhanced at higher temperatures.

When the extent of interaction had stabilized, values of I_0 and n were found to be independent of temperature from 323°K to 312°K; but the membrane conductance changed precipitously below 312°K. This is illustrated in Fig. 5. Somewhere between 312°K and 308°K, the conductance of the membrane not only increased, but became almost ohmic. This behavior has been observed with many membranes although the temperature range for the transition was not always defined so closely. Although KCl was used as the electrolyte in most of the experimental investigations of this phenomenon, initial measurements with NaCl, CsCl, and $(CH_3)_4NCl$ as the electrolyte showed similar changes in membrane conductance. Also, this behavior was independent of pH in the range 6.0–9.0.

DISCUSSION

This investigation has provided additional evidence to aid in understanding the complexity of electrical conduction at biochemical membranes. In particular, the importance of alternative pathways for conduction has been demonstrated. Measurements of ion-diffusion potentials revealed that sphingomyelin–tocopherol membranes activated by EIM, while permeable to inorganic cations, are essentially impermeable to inorganic anions. Above 300°K, however, the extent of this discrimination could only be realized if the conduction through the unactivated portions of the membrane was also considered. Otherwise, the decrease in ion-diffusion potential with increasing temperature might be assumed to indicate an increasing permeability toward inorganic anions. The quantity V'_0/V_p is equivalent to the term $(t_+ - t_-)$ in the Nernst equation; and since the values shown in Table 1 for this quantity average 0.94, the average transport number for the cations represented is 0.97.

The decrease in ion-diffusion potentials with temperature is primarily due to the high temperature coefficient of conductance in the unactivated membranes. This has also been shown for sphingomyelin–tocopherol membranes by Kauffman and Mead (4), who found an activation energy of 1.07 eV per molecule in comparison with our value of 1.00 in the temperature range under consideration. This temperature sensitivity is much higher than that for ionic conductances in aqueous solutions, and indicates that the conduction mechanism is not simply the flow of ions through continuous aqueous pores in the membrane. It is tempting to attribute the conductance of the unactivated membrane to the transport of hydrogen ions because of their small size, multiplicity of transport modes, and because a concentration differential of these ions induced diffusion potentials. However, the poor reproducibility in the value of conductance for a series of carefully prepared membranes—an observation noted by several investigators using membranes with various lipid compositions (5, 6)—leaves the answer in doubt.

The sudden increase in conductance of EIM-activated membranes as the temperature falls below 312°K and the simultaneous loss of rectification suggest that a relatively high-conducting configuration of the membrane components is frozen into place at this temperature. Other investigators working with EIM have suggested that this protein-like material forms ion-conducting channels across lipid bilayers but that the population of these bridges fluctuates with temperature (2, 7). If this view is correct, the present results indicate that in sphingomyelin–tocopherol membranes, these channels are established in a rigid form at 312°K, the transformation resembling a phase transition.

Since the chemical structure of EIM is so vague as yet, its interaction with the lipid molecules of the membrane cannot be identified specifically. The change in ion-diffusion potentials across these membranes when the pH is decreased equally on both sides is another phenomenon that indicates an alteration in the EIM–lipid or EIM configuration. Both this effect of pH and the changes in relative importance of conductive pathways with temperature illustrate the multiplicity in control mechanisms possible at a biochemical membrane in response to fairly mild changes in the external environment.

We thank Drs. P. Mueller and D. O. Rudin of the Eastern Pennsylvania Psychiatric Institute for generously donating the EIM used in this work. This work was supported by the Battelle Institute under its Life Sciences Program.

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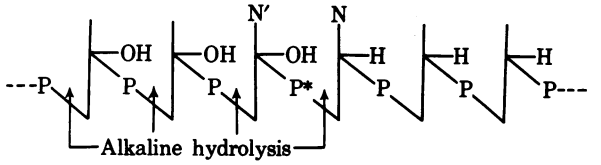
Correction. In the article "RNA-Linked DNA Fragments *In Vitro*," by Sugino, A. & Okazaki, R., which appeared in the January 1973 issue of *Proc. Nat. Acad. Sci. USA* **70,** 88–92, due to errors made in the *Proceedings* Office, the following corrections should be made.

On page 89, left-hand column,



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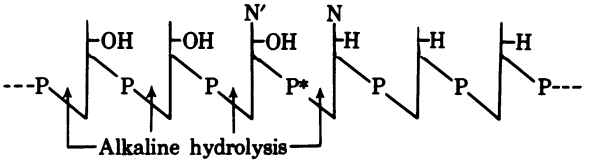

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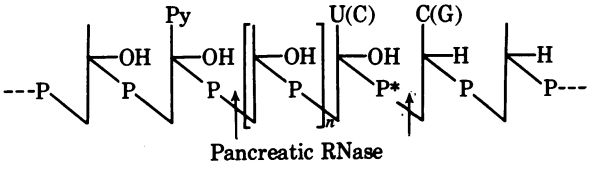

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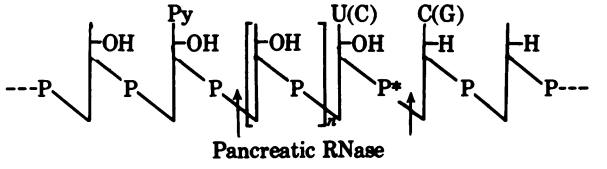

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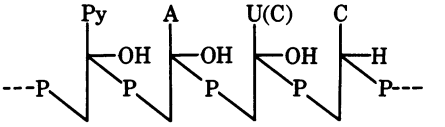

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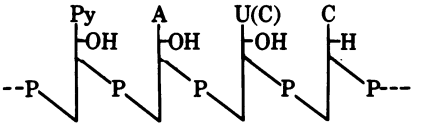

Correction. In the article "A Single Subunit from Avian Myeloblastosis Virus with Both RNA-Directed DNA Polymerase and Ribonuclease H Activity," by Grandgenett, D. P., Gerard, G. F. & Green M., which appeared in the January 1973 issue of *Proc. Nat. Acad. Sci. USA* **70,** 230–234, the following correction should be made. On page 230, column 2, line 8 from top (under *Enzyme Assays*), "50μ mM NaCl" should read: 50 mM NaCl.

Correction. In the article "Ion-Diffusion Potentials and Electrical Rectification Across Lipid Membranes Activated by Excitation-Induced Material," by Kalkwarf, D. R., Frasco, D. L. & Brattain, W. H., which appeared in the December 1972 issue of *Proc. Nat. Acad. Sci. USA* **69,** 3765–3768, page 3767, under *Results and Correlations*, column 1, line 13, the magnitude 5.0 ± 0.6 μF/cm^2 should read: 0.5 ± 0.06 μF/cm^2.