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STRUCTURE OF EXCITABLE MEMBRANES FORMED ON THE SURFACE OF PROTOPLASMIC DROPS ISOLATED FROM *NITELLA*

I. CONFORMATION OF SURFACE MEMBRANE DETERMINED FROM THE REFRACTIVE INDEX AND FROM ENZYME ACTIONS

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SUMMARY

A protoplasmic drop isolated from an internodal cell of *Nitella* became electrically excitable in response to an external stimulus when the drop was placed in a test solution containing Ca^{2+}, Mg^{2+} and other appropriate ions. The structure of the surface membrane of the protoplasmic drop was determined by measuring the refractive index and the electrical properties of the membrane with and without enzymes in the external solution. The results obtained are summarized as follows.

1. The refractive index of the membrane, determined from the measurements of the Brewster angle, decreased with time from 1.47 ± 0.02 to 1.42 ± 0.02 within about 1 h after a drop was formed in the test solution.

2. The membrane potential decreased gradually with time and approached a steady value of about -90 mV or -50 mV, while the membrane resistance rose temporarily and then decreased and approached a steady value between 0.4 and 3 $k\Omega \cdot cm^2$ within about 1 h. The membrane became electrically excitable when the membrane potential and the resistance attained the steady values mentioned above.

3. The presence of proteases, *e.g.* trypsin and pronase, in the external medium during the formative period of the excitable membrane inhibited the decrease of the membrane potential and of the membrane resistance. The excitability of the membrane was not induced in this situation. Removal of the proteases from the external solution, however, led to the formation of the excitable membrane.

4. After the membrane became excitable, an application of proteases led to a depolarization of the membrane potential and an increase of the membrane resistance, and the excitability was suppressed.

5. Addition of phospholipase A, 0.05 mg/ml, caused an immediate destruction of the drop at any stage of membrane formation.

These results imply that the surface membrane of a protoplasmic drop is formed initially from phospholipids. Then proteins penetrate partially through the surface membrane and combine with the phospholipids in the membrane, leading to an appropriate conformation of the membrane macromolecules responsible for the excitability of the protoplasmic drop.

INTRODUCTION

Recent physicochemical studies on internally perfused squid giant axons suggest that the process of nerve excitation is accompanied by a conformational change in the macromolecules constituting the membrane[1]. This view is supported by transient changes in the optical and thermal properties of nervous tissues during excitation[2-4]. However, it is difficult to determine the physical and chemical structure of the functional membrane in nervous tissues and to detect a transient change in the conformation of membrane molecules during excitation, because the thickness of the membrane is merely 100 Å with overwhelmingly thick adherent tissues covering the plasma membrane in any sample of nervous tissue. It is urgently necessary to find a simple system in which the excitable membrane is contiguous with the external solution in order to determine the membrane structure and to clarify the relationship between physiological function and membrane conformation.

In preceding papers[5,6] we showed that a protoplasmic drop isolated from an internodal cell of *Nitella* became excitable in an aqueous solution of appropriate salt composition containing divalent cations (Ca^{2+},Mg^{2+}) as well as univalent cations (Na^{+},K^{+}). The electron microscopic study demonstrated that a thin membrane of 70–120 Å in thickness was formed on the drop surface. This drop membrane is suitable for studying membrane structure by use of biochemical and physicochemical techniques, since no connective tissue is observed outside of the surface membrane. The protoplasmic drop is also appropriate for investigations of the process of formation of the surface membrane and the growth of its excitability with time, because the surface membrane becomes electrically excitable with a time lapse of about 1 or 2 h after the drop is formed in the salt solution.

The present series of papers reports studies on the chemical and physical structure of the excitable membrane of a protoplasmic drop isolated from *Nitella*. This paper describes the experimental results obtained from measurements of refractive index of the surface membrane, and of the electrical properties of the membrane in the presence of proteases and a phospholipase in the external solution. The macroscopic conformation of the excitable membrane and the process of membrane formation are also discussed.

EXPERIMENTAL

Materials and conditions

Internodal cells of *Nitella sp.*, 300–500 μm in diameter and 30–50 mm in length, were used for the present study. The plant was obtained from a tropical fish shop and cultivated at 27 °C in a hothouse in Hokkaido University for more than half a year, and then stocked in artificial pond water at 22 °C for more than a month. The chemical composition of the artificial pond water was 0.025 mM KCl, 0.05 mM NaCl, 0.05 mM NaH_2PO_4, 0.2 mM $Ca(NO_3)_2$ and 0.1 mM $MgSO_4$. To proceed to the subsequent experiments on the protoplasmic drop, two artificial solutions were prepared. The first was called the 'basal solution' and was used for making a protoplasmic drop from an internodal cell of *Nitella*: it was slightly hypotonic in comparison with the osmotic pressure of the *Nitella* internodal cell in order to prevent plasmolysis. The approximate composition of the solution was 70 mM KNO_3, 50 mM

$NaNO_3$ and 5 mM $CaCl_2$. The second solution was the 'test solution', which was adjusted by adding mannitol to be isotonic with the protoplasm of *Nitella*. The ionic composition of the test solution was 0.5 mM KNO_3, 0.5 mM NaCl, 1 mM $Ca(NO_3)_2$ and 2 mM $Mg(NO_3)_2$. The pH value of the solution was kept at 6.3–7.0, adjusted by either 1 mM Tris–acetate or 1 mM histidine buffer. As pointed out in previous papers, this ionic composition was appropriate for the appearance of excitability of the protoplasmic membrane. The osmotic pressure of an internodal cell of *Nitella* was measured by the plasmolysis method[7], and was 250 to 260 mosmoles.

The isolation of the protoplasm from an internodal cell of *Nitella* was performed by the method proposed by Kamiya and Kuroda[8]. An internodal cell was held vertically in a holder filled with the basal solution, and a small portion of the lower part of the cell was submerged in the basal solution in a vessel (B) as shown schematically in Fig. 1a. The cell was amputated with a pair of small scissors at the position shown by the broken line in Fig. 1a. The protoplasm in the *Nitella* cell fell down from the opening of the cell in the form of a thread to the bottom of the vessel, where it formed a sessile drop as seen in Fig. 1b. When the diameter of the drop reached 300–500 μm, the internodal cell was removed from the vessel. Soon after the formation of the drop, the external solution was exchanged by starting a perfusion of the test solution, which overflowed from the outlet as illustrated in Fig. 1c.

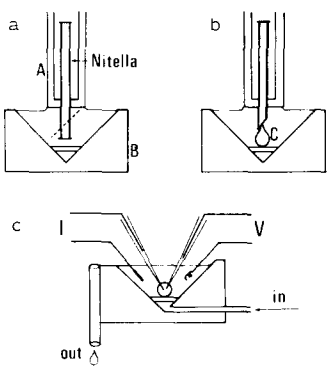

Fig. 1. Schematic illustration of the experimental procedures for making a protoplasmic drop and for recording the electrical responses of the drop membrane. (a) Arrangement for amputating *Nitella* internode. A, lucite holder; B, vessel. (b) Effusion of protoplasm from the lower opening of the cell. (c) Arrangement for measuring electrical responses. Two microelectrodes are inserted into the protoplasmic drop, one for potential recording (V) and the other for current supply (I). External solution is perfused from the bottom (in) and is overflowed from the top (out).

Enzymes

The enzymes used were two proteases, namely trypsin and pronase, and phospholipase A. Trypsin, prepared from bovine pancrease, was purchased from Sigma Chemical Co., St. Louis, Mo. Pronase, prepared from *Streptomyces griseus* K-I, was obtained from Calbiochem. Co., Los Angeles, and was a mixture of proteases with a wide range of side-chain specificity[9]. The optimum pH range of the proteases used was 7.0–8.5. Phospholipase A prepared from *Vipera tusselli* and from *Crotalus terr* were obtained from Sigma Chem. Co., St. Louis, and from Calbiochem., Los Angeles, respectively. Each enzyme was used as delivered with-

out further purification, and was dissolved in the external perfusing fluid at a concentration of between 0.05 and 1 mg/ml.

All experiments were carried out at room temperature of 20–27 °C. The temperature range in each experiment is noted in the relevant figure legends.

Measurements of the electrical properties

The experimental arrangement for measuring the electrical properties of the protoplasmic drop is shown schematically in Fig. 1c. Two glass microelectrodes filled with 3 M KCl solution were used as internal electrodes for potential measurement and for current supply, respectively. Chloridized silver wire electrodes of 100 μm diameter were used for the external electrodes. One was used for the potential reference and the other for external current supply. In order to minimize the effect of Ag^+ on the protoplasmic drop, the external electrodes were placed as close to the outlet of the solution as possible. The transmembrane potential was monitored by a beam of a synchroscope (Iwastsu Elec. Co., Type SS-5517) or a channel of a penwriting recorder (Nihon Kohden Co., Type WI-260) through a high input impedance d.c. preamplifier (Nihon Kohden Co., Type MZ-3B). Current pulses were delivered from a current clamp feedback amplifier of 60 db gain, and monitored by the other beam of the synchroscope or a channel of the penrecorder. The current–voltage relationship was obtained by plotting the steady value of the transmembrane potential as a function of the intensity of the applied current.

Determination of the refractive index of the membrane

The refractive index of the membrane was determined by measuring the Brewster angle. The protoplasmic drop was formed in the same manner as described above on the top of a small truncated cone made of lucite which was submerged in basal solution in a glass chamber (Fig. 2b). The experimental arrangement for measuring the Brewster angle is schematically shown in Fig. 2a. The glass chamber was placed in the center of a goniometer, and the center of the test drop was adjusted to coincide with that of the turntable of the goniometer. The light source, an 80-W tungsten lamp, was fixed on an arm (50 cm in length) attached on the turntable. The rays from the light source were converted into an approximately parallel narrow beam by means of lenses and an iris diaphragm and projected on a portion of the surface of the drop. A polarizer, Polaroid HNP'B film, was placed between the chamber

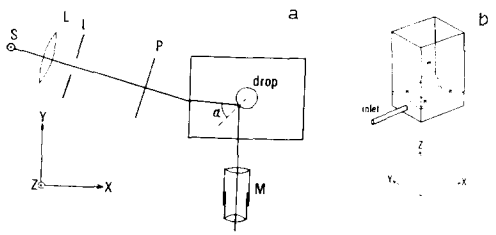

Fig. 2. Schematic diagram for measuring the Brewster angle. (a) Arrangement for measuring the Brewster angle. S, light source; I, iris; P, polarizer; M, traveling microscope; α, Brewster angle. (b) Glass chamber in which a small lucite truncated cone is placed. The drop is formed on the top of the cone.

and the iris, and the incident light was polarized in the direction parallel to the X–Y plane. The Brewster angle was determined by finding the angle where the intensity of the reflected light from the surface of the drop became minimum. The angle between incident and reflected beams was determined within an accuracy of two degrees.

The refractive index of the surface membrane, n, is calculated from the following equation by using the value of the Brewster angle, α, determined above:

$$n = \frac{\sin \alpha}{\sin (\pi/2 - \alpha)} \cdot n_s$$

where n_s is the refractive index of the external solution. The values of n_s of the basal solution and of the test solution were determined by a refractometer at 21.5 °C, and were 1.335 and 1.338, respectively.

RESULTS AND DISCUSSION

Time-course of formation of the excitable membrane

As pointed out in the previous papers[5,6], it required about 1 h for the protoplasmic drop to become electrically excitable after placing in the test solution. During this induction period the membrane potential and the membrane resistance changed with time. Fig. 3a shows the time-course of the variations of the membrane potential and the membrane resistance of the drops. The abscissa in the figure indicates the time in hours after the start of perfusion of the external solution with the test solution. It is seen that the membrane potential (upper trace) decreases rapidly after the start of perfusion to about −50 mV, remaining at this value for a period

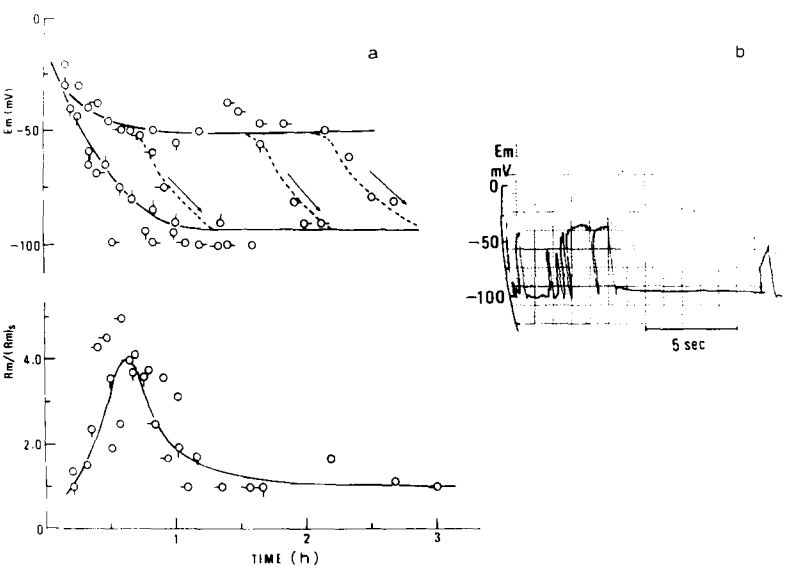

Fig. 3. (a) Time-course of the membrane potential, E_m, and the relative membrane resistance, $R_m/(R_m)_s$, of protoplasmic drops after the onset of perfusion with the test solution. $(R_m)_s$ is the membrane resistance in the steady state. Each different symbol indicates a different drop. (b) Flip-flop transition of the membrane potential between two steady potential levels. Temperature: 25 ± 1 °C.

of time, which varies from one drop to another, and then decreases again to approach the final level of -90 ± 20 mV as illustrated by arrows in the figure. Each different symbol in the figure refers to a different drop. Under the external condition studied here, the state characterized by the -90 mV potential level seems to be more stable than of the state of -50 mV, since the membrane potentials finally approach the lower level of -90 mV when the drops are allowed to stand for a few h. It is noted that spontaneous flip-flop transitions of the potential were sometimes observed between these two potential levels as shown in Fig. 3b. In contrast to the membrane potential, the electric resistance of the surface membrane increased temporarily after the start of perfusion, and then decreased and approached the final steady value. The absolute value of the membrane resistance in the final steady state lay between 0.4 and 3 $k\Omega \cdot cm^2$ for different drops. The lower half of Fig. 3a illustrates the variation of the membrane resistance, where the membrane resistance relative to that of the steady value is plotted against time. The symbols in the figure correspond to those in the upper half of the figure for the membrane potential plot, although some are lacking in the resistance measurements.

It was noted that the drops became electrically excitable only after the stage where the membrane resistance fell from its peak value, irrespective of the value of the membrane potential. In other words, at both the -50 mV and -90 mV levels of membrane potential the membranes were found to be excitable in response to an external electrical stimulus. Fig. 4 shows a typical example of the current–voltage relationships obtained for the surface membrane of drops in which the membrane potential and membrane resistance had reached their steady values. The transmembrane potential changed its value discontinuously when the intensity of electric current reached a certain value for each individual drop. Before and after the critical

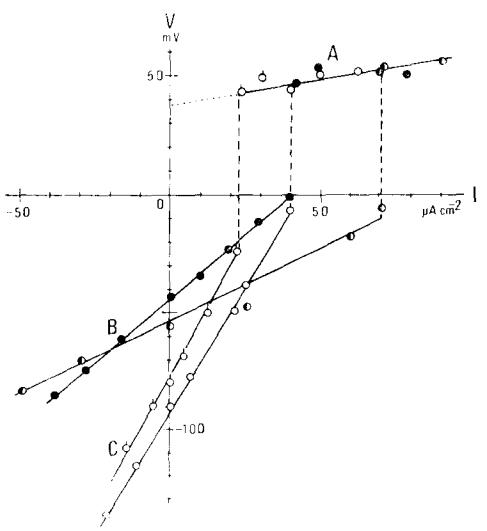

Fig. 4. Current–voltage relationship obtained from protoplasmic drops where the steady values of the transmembrane potentials are plotted against the applied current density. A, excited state; B and C, resting states characterized by the levels of the membrane potential of about -50 mV and about -90 mV, respectively, when no electric current passed through the membrane. Temperature: 23 ± 2 °C.

value of current density, the transmembrane potential varied linearly with current strength, but with entirely different slopes. In the figure, group B refers to drops having a membrane potential of about -50 mV, while group C refers to those of -90 mV when no electric current passed through the membrane. Note that the value of V for all the drops changed to about $+50$ mV with approximately $100\,\Omega\cdot cm^2$ in electric resistance when the current density exceeded their individual critical values. The critical current densities were different for different drop species. From the ordinate intercepts of the straight lines B, C and A in the figure, it is evident that the discrete variation of the transmembrane potential, V, accompanies the change in the membrane electromotive force. Comparing the values of potential and electric resistance of the surface membrane obtained above with those of living membrane, it can be inferred that state A in the figure is to be regarded as the so-called excited state of the membrane, while either B or C are to be regarded as the resting state. As pointed out above, membranes in either of the states B or C are excitable in response to an external electrical stimulus. Here, the term "excitation" is referred to as a transition from the resting state to the excited state with the application of external stimuli.

Various types of excitation were obtained from the protoplasmic membranes. Fig. 5a shows an action potential induced by a brief outwardly directed current pulse, and Fig. 5b illustrates the transition of state due to a fixed outward current. These various types of excitation process under different external and boundary conditions are also observed in squid giant axons[1].

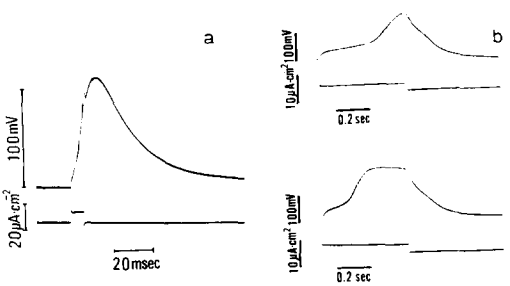

Fig. 5. Excitations recorded from protoplasmic drops. (a) Action potential induced by a brief outward current pulse. (b) State transition due to fixed outward current. The upper trace indicates the transmembrane potential and the lower trace indicates the applied current in each case. Temperature: 21 ± 1 °C.

Refractive index of the membrane

Fig. 6 shows the time-course of the variation of the refractive index of the membrane after the external solution was changed from the basal solution to the test solution. The refractive index, n, was observed to be 1.47 ± 0.02 in the basal solution, but it decreased monotonically and approached an average value of 1.42 ± 0.02 after changing the external solution. Although the two different states observed in E_m were not distinguished with the present refractive index measurements, the time-course of the variation of n is comparable to that of the membrane potential given in Fig. 3a. This fact implies that the decrease in n is closely related to the formation of the excitable membrane. The value of 1.47 for the refractive index is

approximately the same as those of phospholipids, which implies that the surface membrane is formed by phospholipids in the initial stage. The decrease in the refractive index with the growth of the excitable membrane is attributed to the decrease in the fraction of phospholipids in the surface membrane, probably stemming from penetration by protein molecules from inside the drop. This hypothesis of the formation of the functional membrane is also suggested by the study of enzyme actions as will be shown below.

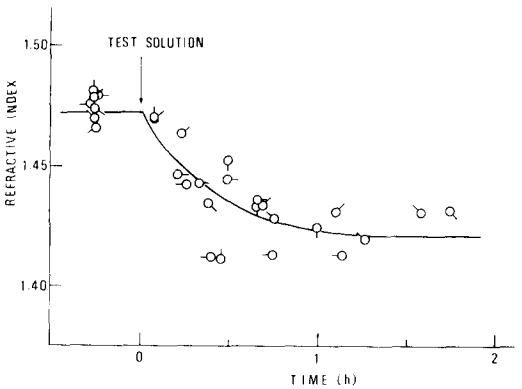

Fig. 6. Time-course of the variation of the refractive index of the membrane of protoplasmic drops. The abscissa shows the time in hours after the external solution is changed from the basal solution to the test solution. Each different symbol indicates a different drop. Temperature: 22 ± 1 °C.

Effect of enzymes

Fig. 7a shows the time-course of change in the membrane potential and the electric resistance in the presence of proteases. The test solution containing 1 mg/ml of pronase or 0.5 mg/ml of trypsin was perfused externally from the early stage of membrane formation. The open and closed circles in the figure show the effects of pronase and of trypsin, respectively. In both cases, the membrane potential decreased slightly after the onset of perfusion of enzyme solution, and approached zero as seen in the figure. However, if the external solution was switched to the test solution containing no proteases, the membrane potential decreased and approached the steady value of -50 mV or -90 mV. Corresponding variations of membrane resistance in the presence of pronase or trypsin are shown in the lower half of Fig. 7a. In these figures, the effect of removal of trypsin in the external solution after 25 min from the start of perfusion is shown as an example. In the case of pronase, similar variations of membrane potential and electric resistance were observed unless the enzyme was applied for more than 2 h. In this case, both the membrane potential and electric resistance increased continuously, and excitability of the membrane did not arise after removal of the proteases in the external solution. Fig. 7b shows the I–V relationship obtained from the drop 50 min after the removal of trypsin, where discrete variations in potential with a continuous increase of I was observed as in Fig. 4.

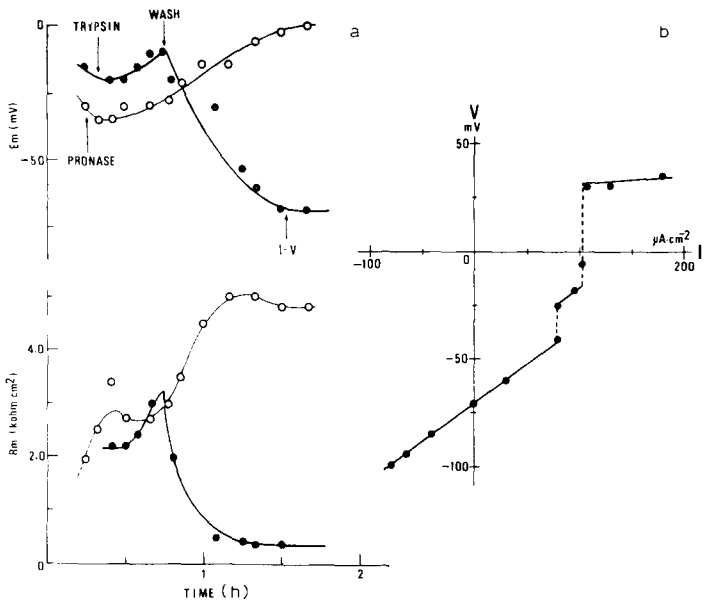

Fig. 7. (a) Time-course of the changes in the membrane potential and the membrane resistance in the presence of proteases in the formative period of the excitable membrane. Open and closed circles show the effects of pronase (1 mg/ml) and of trypsin (0.5 mg/ml), respectively. Marks of TRYPSIN and PRONASE show the time when the enzymes were applied, and the mark of WASH shows the time when trypsin was removed. The mark of I–V shows the time when the I–V relationship shown in Fig. 7b were measured. (b) Current-voltage relationship obtained from the drop after the removal of trypsin from the external solution. Temperature: 22 ± 1 °C.

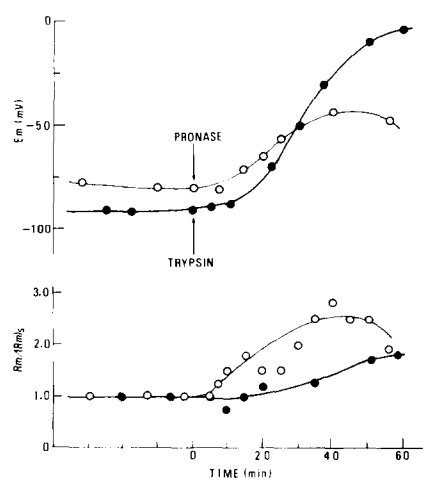

Fig. 8. Effect of proteases on the membrane potential, E_m, and the relative membrane resistance, $R_m/(R_m)_s$, after the drop became excitable in the test solution. Open and closed circles show the data treated by pronase (1 mg/ml) and by trypsin (0.5 mg/ml), respectively. The abscissa indicates the time in min after the application of proteases. Temperature: 25 ± 1 °C.

Fig. 8 shows the effects of application of proteases on the membrane potential and the electric resistance, where the enzymes were added to the external solution after the drop became excitable. The open and closed circles refer to the effects of pronase and trypsin, respectively, with the same concentration as in Fig. 7. The membrane potential was decreased and the resistance was increased by the protease treatments. The excitability of the drop was suppressed within 15 min after the application of protease, and almost at the same time both membrane potential and the electric resistance started to change. A slight difference between the action of trypsin and pronase is observed, as seen in Fig. 8. The implications of the difference between various proteases and phospholipases will be discussed in a subsequent article of this series.

In most cases the changes were irreversible at these enzyme concentrations. In some drops, however, the membrane potential decreased and increased periodically, and the excitability was correspondingly recovered in a periodic manner even in the presence of the enzymes in the external solution. This periodic variation was continued for two or three times after the application of enzymes. This behavior may be caused by competition between the rates of formation of the functional membrane and the degradation of the membrane by the proteases. The shape of the protoplasmic drop was not changed in the protease solution.

An addition of phospholipase A at 0.05 mg/ml in the external medium led to an immediate destruction of the drop at any stage of the formation of the surface membrane. This is an another item of evidence for the existence of the phospholipids in the surface membrane.

All the results described above imply that an appropriate conformation of phospholipids and proteins and/or of lipoproteins caused by the penetration of proteins into the surface membrane is essential for the appearance of the excitability of the membrane. At the same time, this conformation precedes the decreases in the membrane potential and in the electric resistance of the surface membrane of the protoplasmic drop. We can roughly estimate the fraction of proteins in the outer surface of the membrane from the results of the refractive index. This fraction was approx. 40%, which agrees with that evaluated from the chemical analysis of the plasmalemma fraction in living tissues[10–12].

CONCLUDING REMARKS

The surface membrane of a protoplasmic drop in the basal solution is considered to be formed by micelles or bilayers of phospholipids, which seem stable in the salt composition of the basal solution. When the external medium is exchanged for the test solution, proteins inside the drop are absorbed into the surface membrane and may form the desired conformation of phospholipids and protein and/or lipoprotein structure in the membrane as described above. Presumably this is the necessary condition for the appearance of the excitability. Enzyme actions studied here show that the proteins in the membrane play an important role in the generation of the resting potential and of excitability. However, it is hazardous to conclude that a change in properties of a specific protein itself in the membrane is responsible for the process of excitation. Further study is now in progress.

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