Biochimica et Biophysica Acta, 318 (1973) 326–334

BBA 76416

STUDIES OF EXCITABLE MEMBRANE FORMED ON THE SURFACE OF PROTOPLASMIC DROPS ISOLATED FROM *NITELLA*

II. TENSION AT THE SURFACE OF PROTOPLASMIC DROPS

TETSUO UEDA, ISAO INOUE and YONOSUKE KOBATAKE
Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo (Japan)
(Received March 23rd, 1973)

SUMMARY

A protoplasmic drop isolated from an internodal cell of *Nitella* became electrically excitable in a solution containing 0.5 mM NaCl, 0.5 mM KNO_3, 1 mM $Ca(NO_3)_2$ and 2 mM $Mg(NO_3)_2$. A thermodynamic property of the excitable membrane was characterized in terms of tension at the surface of the protoplasmic drop. This was determined by the compression method and/or by the sessile-drop method. The surface tension of the membrane was obtained as a function of the composition of the salts in the external solution, and of the time during the formative period of the excitable surface membrane. The results are summarized as follows:

1. The surface tension of the protoplasmic drop increased with time starting from 0.003 dyne/cm and approached a steady value of about 0.1 dyne/cm within 1 h after the drop was placed in the test solution described above. The membrane became electrically excitable when the surface tension attained the steady value.

2. Increase of concentration of either Na^+ or K^+ in the solution induced a sudden decrease of the surface tension, which followed a suppression of the excitability. The critical concentration of Na^+ or K^+ was about 10 mM.

3. The surface tension remained constant at about 0.1 dyne/cm in a Ca^{2+} concentration ranging between about 0.1 and 10 mM. At this concentration the drop was excitable. Below and above this range of Ca^{2+} concentration, the surface tension changed sharply with concentration, and the excitability disappeared. At about 0.1 mM Ca^{2+} concentration a discrete variation of the surface tension was observed.

4. The surface tension of the drop stayed constant at 0.1 dyne/cm in the range between 1 and 10 mM of Mg^{2+} concentration. Above and below this range of Mg^{2+} concentration, the surface tension increased sharply with the variation of Mg^{2+} concentration.

These results indicate that the protoplasmic drop retains its excitability in a limited range of salt composition in the external solution. This implies that the excitable membrane of the drop must be very labile in its structure against external perturbations such as electrical stimulus and/or slight variation of salt composition in the solution.

INTRODUCTION

What is the characteristic nature of the excitable membrane? This question

may be answered when the basic data on the physicochemical and biochemical as well as physiological properties of the membrane are accumulated. Intact nerve membranes of living organisms have great disadvantages for carrying out biochemical and physicochemical measurements, because they are covered with extremely thick connective tissue. These adherent tissues are considered to be indifferent to the process of excitation of the cell membrane.

As shown in the previous papers[1,2], the protoplasmic drop isolated from an internodal cell of *Nitella* is a suitable semi-artificial cell for investigating the relation between the structure and functions of the membrane, because the drop has no connective tissue around the surface, and is electrically excitable in an appropriate salt composition in the external solution. In Part I of this series[2], we discussed the structure of the membrane of the protoplasmic drop of *Nitella* determined by measuring the refractive index of the surface and by studying the effects of enzymes on the electrochemical properties of the drop. It was shown that the formation of the lipid–protein complex in the membrane is essential for the appearance of excitability, and that the process of formation of the excitable membrane was very much affected by the ionic composition of the external solution.

Before proceeding with investigations on the molecular mechanism of the process of excitation in the living cell, it is necessary to understand the physicochemical properties of the membrane. This paper describes the results obtained from the measurements of tension at the surface of the protoplasmic drop of *Nitella* in varying compositions of the external solution. The implications concerning the surface tension of the cell membrane are discussed in connection with the electrical properties of the membrane.

EXPERIMENTAL

Materials and conditions

All the materials and the experimental conditions were the same as those employed in Part I of this series[2]. The protoplasm in an internodal cell of *Nitella* was isolated in the basal solution containing 70 mM KNO_3, 50 mM $NaNO_3$, 50 mM $NaNO_3$ and 4 mM $CaCl_2$. Then the external solution was replaced by the test solution composed of 0.5 mM KNO_3, 0.5 mM NaCl, 1 mM $Ca(NO_3)_2$ and 2 mM $Mg(NO_3)_2$ with a pH of 6.3–7.0 adjusted by Tris–acetate buffer (1 mM). The osmotic pressure of the test solution was kept isotonic by adding recrystallized mannitol. The sizes of the drops used in the present study were 250–300 μm in diameter. All experiments were carried out at a room temperature of 17 ± 1 °C.

Determination of tension at the surface of the protoplasmic drop

The tension at the surface, γ, was determined by the use of the compression method[3] and/or the sessile-drop method[4,5]. The compression method is applicable for the case in which γ is relatively large, while here, the sessile-drop method is useful in the case where γ is less than 0.01 dyne/cm.

When the external force, F, is applied to a spherical drop, the drop is deformed as is shown schematically in Fig. 1b. Then the surface area of the drop expands by ΔS. Since the work exerted on the drop during deformation must be balanced by the change in surface energy, we have the following equation: $F\Delta Z = \gamma \Delta S$, or

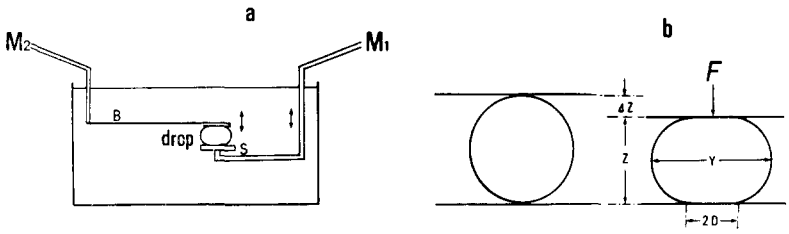

Fig. 1. Schematic illustration for measuring tension at the surface of the protoplasmic drop by the compression method. (a) Arrangement for compressing the drop. *B*, bending balance, end of which is attached by a small piece of cover glass coated with Teflon. *S*, Teflon stage attached to a stiff glass rod. M_1 and M_2, micromanipulators. (b) Deformation of the drop by compression viewed from side. *F*, external force. $2D$, diameter of the contact area. *Z*, thickness of the compressed drop. ΔZ, amount of deformation along the external force. *Y*, diameter of the compressed drop.

$\gamma = F/(\mathrm{d}S/\mathrm{d}Z)$, where ΔZ is the amount of deformation along the external force as illustrated in Fig. 1b, and γ is the surface tension. Here, γ is assumed to be uniform and constant irrespective of the extent of deformation of the drop. The value of $(\mathrm{d}S/\mathrm{d}Z)$ can be determined by measuring Y, D, Z and ΔZ in the figure, under conditions so that the volume of the drop is not changed by the deformation.

When the difference between the densities of the protoplasmic drop and the external medium is large and the surface tension is small, the drop deforms appreciably under the influence of the force of gravity (Fig. 2). In this case the surface tension is calculated by the following equation as explored by Dorsey[6], and utilized by Kamiya[4] for the measurements of γ of the protoplasmic drop of *Nitella:*

$$\gamma = g(d-d_0)r^2\left(\frac{0.0520}{f} - 0.12268 + 0.0481f\right)$$

where f is defined by

$$f = \frac{CB}{r} - 0.41421.$$

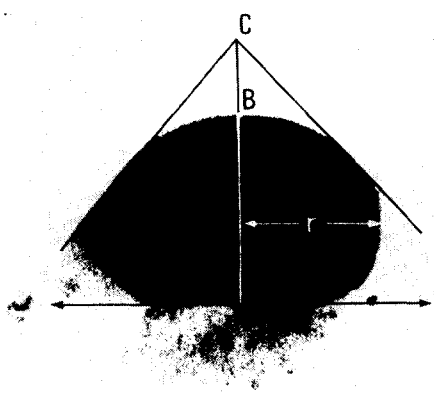

Fig. 2. A flattened sessile drop. *r*, radius of the maximum horizontal section. A pair of tangents drawn on the drop make 45° with the horizontal plane.

Here, g is the acceleration due to gravity, r the radius of the maximum horizontal section of the flattened drop, and $(d-d_0)$ the difference in the densities of the protoplasmic drop, d, and of the medium, d_0. CB is the length indicated in Fig. 2. These equations are applicable only in the case where γ is independent of the extension of the surface. This condition is assumed to be satisfied to the first approximation in the naked protoplasmic drop in this investigation. The density of the protoplasmic drop was determined to be 1.017 ± 0.001 g/cm^3 by the isopycknotic method[5]. Here the osmotic pressure in the density gradient tube was kept identical with that of the internodal cell of *Nitella* (250–260 mosM) by changing the ratio of mannitol and sucrose.

Measurements of γ

A schematic diagram of the experimental arrangement is shown in Fig. 1a. A small piece of cover glass (0.5 mm $\times$ 0.5 mm in size) was coated with Teflon and used as an upper deck, while a Teflon sheet (3 mm $\times$ 3 mm in size and 0.5 mm in thickness) served as a compressing plate. The Teflon sheet was attached to a stiff glass rod (1 mm in diameter) serving as a lower stage. The cover glass was attached to a slender glass bar having a circular cross section (10–20 μm in diameter, and about 5 cm in length) which worked as a bending balance for measuring the compressing force, F. The displacement of the action point of the slender bar, ΔZ, was measured by a microscope with an eyepiece micrometer. The bending characteristics of the bar had been determined by loading with a series of small pieces of paraffin paper (0.1–3 mm^2), the weight of which was calculated (0.003–0.1 mg) from the weight of the unit area of the paper or determined directly by using a micro balance. The stage and the balance were so mounted on micromanipulators as to make the surface of the upper and lower plates strictly horizontal.

The concentration of the solution in the vessel was graded by placing the basal solution in the upper portion, and the test solution in the lower part. The internodal cell of *Nitella* was held vertically as illustrated in Part I (ref. 2) and was amputated in the basal solution. The effused protoplasmic drop fell slowly onto the Teflon stage in the graded concentration of solution in the vessel. Then the solution was exchanged slowly. The drop flattened at first, and gradually became protuberant with time. Finally, the shape of the drop changed to a sphere within about 1 h. As will be shown later, the shape of the drop did not become spherical in the basal solution or in a test solution containing a high concentration of Na^+ or K^+. When the drop was deformed under the influence of gravitational force, the surface tension was determined by the sessile-drop method by taking a photograph of the drop as illustrated in Fig. 2.

The exchange of the solution in the vessel was performed slowly by the following method. When a denser solution was replaced by a lighter one, the latter solution was allowed to flow into the vessel from the upper inlet and flowed out from the lower outlet of the vessel, or *vice versa.*

RESULTS AND DISCUSSION

Time course of the variation of γ in the test solution

Fig. 3 shows the time course of variation of tension at the surface of the

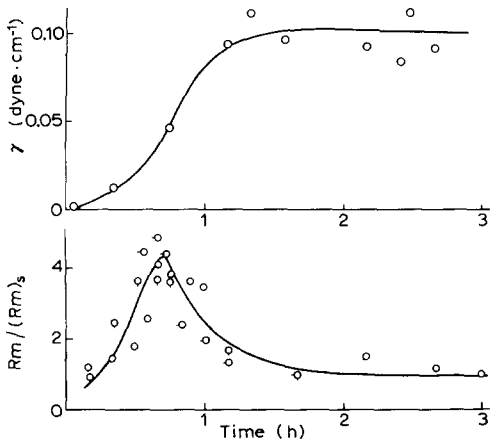

Fig. 3. Time course of the tension at the surface (γ) and the relative membrane resistance ($R_m/(R_m)_s$) (ref. 2) of protoplasmic drops after the onset of perfusion with the test solution. $(R_m)_s$ is the membrane resistance in the steady state.

protoplasmic drop of *Nitella* placed in the test solution. Determination of γ was carried out on different drops at different time intervals after the external solution was replaced by the test solution. Thus, we need not worry about the after effect of deformation on the process of formation of the excitable membrane. Tension at the surface of the drop increased greatly with time starting from 0.003 dyne/cm, and attained the steady value of about 0.1 dyne/cm within about 1 h after the test solution was replaced. In the lower half of the figure, the time course of the relative electric resistance of the drop membrane is shown for the purpose of comparison. As discussed in Part I (ref. 2), the drop became electrically excitable when the electric resistance fell from its peak value. We also showed in Part I (ref. 2) that the refractive index and the membrane potential decreased monotonously and attained their respective steady values within about 1 h after the drop was placed in the test solution. Thus the time course of γ and its steady value of 0.1 dyne/cm is considered to be an another manifestation of the formation of the excitable membrane.

Effect of concentration of univalent cation on γ

Fig. 4 shows the steady value of γ as a function of univalent cations, Na^+ or K^+, where the ionic composition is fixed at the same level of divalent salt as in the test solution. Measurements were performed after the value of γ attained the steady value in the test solution. The observed γ attained the steady value within 30 min after the composition in the external solution was changed with a new solution. It was noted that the variation of γ with concentration was reversible. As seen in Fig. 4 the value of γ decreased appreciably when the concentration of Na^+ or K^+ was raised to 10 mM. It may be important to note that the surface tension of the drop is closely related to the membrane potential. Fig. 5 illustrates the relationship between γ and the membrane potential, $\Delta\varphi$, measured simultaneously with γ in a variety of K^+ concentrations. Since the excitability of the drop was observed only when the membrane potential decreased between -50 and -100 mV (*cf.*

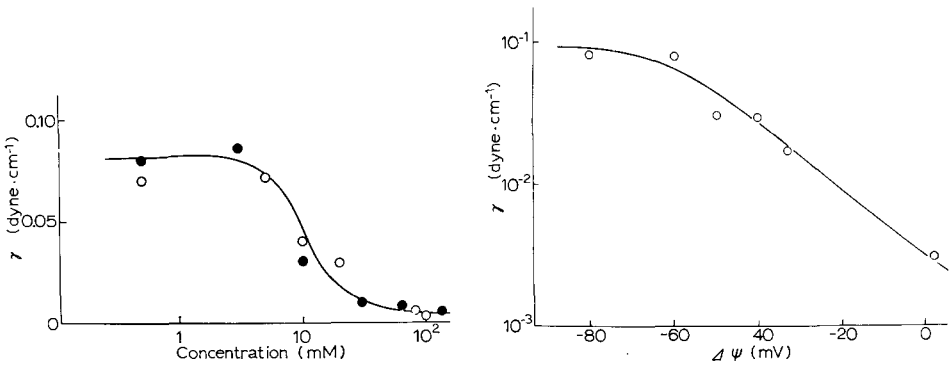

Fig. 4. Concentration dependence of the tension at the surface of protoplasmic drops. ○—○, KNO_3; ●—●, NaCl. Concentrations of other components were fixed at 1 mM $Ca(NO_3)_2$ and 2 mM $Mg(NO_3)_2$. The excitability was observed in the concentration range lower than about 10 mM of univalent cations.

Fig. 5. Relationship between tension at the surface (γ) and membrane potential ($\triangle\phi$), where the concentration of KNO_3 was changed. Other compositions were: 0.5 mM NaCl, 1 mM $Ca(NO_3)_2$ and 2 mM $Mg(NO_3)_2$.

Part I, ref. 2), the value of γ for the functional membrane must be about 0.1 dyne/cm, which corresponds to the low ionic concentration region in the γ *vs* log C relationship given in Fig. 4. Actually, the ecxitation in response to an external electrical stimulus occurs only when the concentration of Na^+ or K^+ is lower than 10 mM.

As pointed out in Part I (ref. 2), the drop is not excitable in the basal solution containing 70 mM KNO_3, 50 mM $NaNO_3$, and 5 mM $CaCl_2$ *i.e.* a high ionic strength media. Considering the reversible variation of γ against the change in salt concentrations in the external medium, the conformation of lipid and protein in the functional membrane of the drop surface[2] is formed or destroyed by the ionic composition in the medium which is contiguous with the drop surface. For the purpose of comparison, Table I summarizes the physical properties obtained so far of the drop membrane both in high and low ionic strength media.

TABLE I

SOME PROPERTIES OF THE PROTOPLASMIC DROP MEMBRANE

Property	*High ionic concentration*	*Low ionic concentration*
Membrane potential (mV)	−10 – −20	−50 – −100
Electrical resistance (kΩ·cm²)	below 0.2	0.35 – 4
Capacitance (ref. 7) (μF/cm²)	1.27	1.23
Refractive index	1.47	1.42
Tension at the surface (dyne/cm)	10^{-3} – 10^{-2}	10^{-1}
Excitability	none	yes

Effect of Ca^{2+} concentration

Fig. 6 illustrates the relation of γ as a function of Ca^{2+} concentration. Open circles show the case where the ionic composition, except Ca^{2+}, was kept constant in the test solution. Tension at the surface of the drop remained at a constant value of 0.1 dyne/cm in the range of Ca^{2+} concentration between 0.1 and 10 mM, where the drop was found to be excitable. Decrease in Ca^{2+} concentration induced a discrete variation of γ at about 0.1 mM and further decrease in Ca^{2+} concentration followed a continuous decrease in γ. On the other hand, the increase of Ca^{2+} concentration higher than 10 mM produced a continuous increase in γ. The dependence of γ on Ca^{2+} concentration at high concentrations is approximately the same as that at low concentrations, and γ seems to follow the same straight line when $\log \gamma$ is plotted against $\log C_{Ca^{2+}}$ as seen in Fig. 6. Furthermore, it is important to note that the observed membrane potential diminishes in the region where the linear relation between $\log \gamma$ and $\log C$ holds, and also that the excitability of the drop membrane disappears in these two regions. These will be discussed in detail in a forthcoming paper (Ishida, N., Inoue, I. and Kobatake, Y., unpublished).

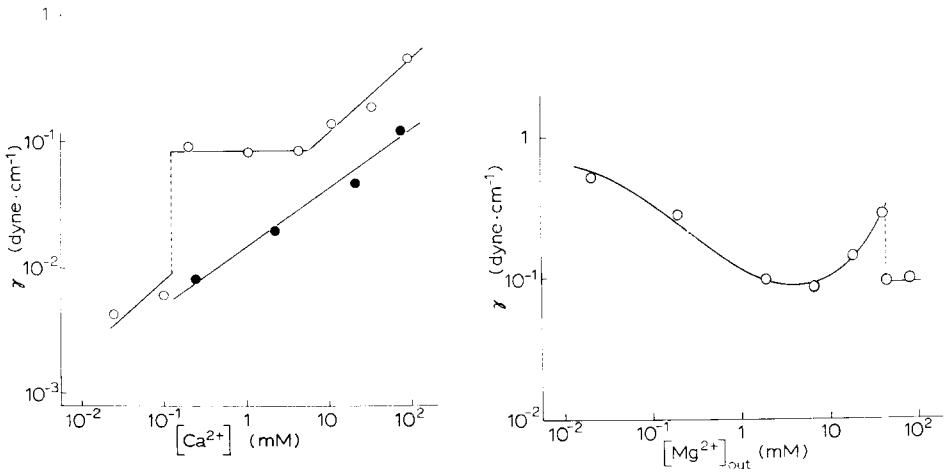

Fig. 6. Ca^{2+} concentration dependence of tension at the surface of protoplasmic drops. ○—○, 0.5 mM NaCl, 0.5 mM KNO_3 and 2 mM $Mg(NO_3)_2$. ●—●, 30 mM NaCl, 0.5 mM KNO_3 and 2 mM $Mg(NO_3)_2$. In the case of low ionic strength of the univalent cation as indicated by open circles, drops were excitable in the concentration range between 0.1 and 10 mM of $Ca(NO_3)_2$.

Fig. 7. Mg^{2+} concentration dependence of tension at the surface of protoplasmic drops. Other compositions were fixed at 0.5 mM NaCl, 0.5 mM KNO_3 and 1 mM $Ca(NO_3)_2$.

Closed circles in Fig. 6 indicate the case where the concentration of NaCl was fixed at 30 mM and the other composition was kept the same as that of the test solution except for the Ca^{2+} concentration. Under this condition (high concentration of Na^+) the excitability of the drop was not apparent as pointed out above. Contrary to the case of low ionic strength media, the tension at the surface varied monotonously with the concentration of Ca^{2+} in the whole range of the concentrations studied. Here, no discrete change in γ was observed.

Complete removal of Ca^{2+} from the external solution, by rinsing the drops with Ca^{2+}-free solution, or by adding EDTA, leads to an immediate disruption of the drop in low ionic strength media. Thus the presence of Ca^{2+} in the external solution is essential for maintaining the functional structure of the protoplasmic drop membrane.

Effect of Mg^{2+} concentration

Fig. 7 shows the dependence of γ on Mg^{2+} concentration in the external solution, where the concentration of $Mg(NO_3)_2$ was changed successively, the other components of the test solution being kept constant. As seen in the figure, γ exhibits a downward convex curve, the minimum region of which lies between 1 and 10 mM of $Mg(NO_3)_2$. In this concentration range of Mg^{2+} the drop was found to be excitable. Contrary to the case of univalent cations or of Ca^{2+}, the tension at the surface of the drop does not decrease to less than 0.1 dyne/cm both in high and low concentrations of Mg^{2+}. These results, together with the effect of Ca^{2+}, imply that an appropriate combination of univalent and divalent cation species is necessary for the appearance of excitability of the drop membrane. At present, the role of Mg on the structure and functions of the surface membrane of the drop is not yet clear.

CONCLUDING REMARKS

The excitability of the protoplasmic drop appeared in the limited range of salt composition in the external solution, where the surface tension of the drop membrane was characterized by about 0.1 dyne/cm. Beyond this region of the salt composition, the surface tension value either increases or decreases according to the variation of the salt composition, and leads to a suppression of the excitability of the drop membrane. Outside the optimum range of the salt composition, the electrochemical properties of the membrane, such as membrane potential and electrical resistance, diminish rather discontinuously. This fact implies that the structure or the conformation of the excitable membrane can be changed easily by external stimuli or perturbations, *i.e.* an electrical stimulus and/or slight variation of the salt composition in the external solution. Therefore, the notion that 'the process of excitation is accompanied by a conformational change in the membrane structure'[8,9] does not seem unreasonable, at least for the present semi-artificial excitable cell made of protoplasm of *Nitella*. At this stage, it is important to notice that the term 'structure' of the membrane should not be restricted to that in the sense of equilibrium thermodynamics. The concentration of univalent cations in the protoplasmic drop is known to be 100 mM or more, while the ionic strength of univalent cations in the external solution is limited to less than a few mM when the membrane becomes electrically excitable. This big difference in the ionic strength in the two sides of the membrane may be responsible for the formation of an appropriate conformation in the functional membrane at the surface of the drop. Under this condition, the difference in potential across the membrane arrives between -50 and -100 mV as pointed out in Part I (ref. 2). Therefore, it may be said that the formation of the surface membrane and the occurrence of the excitability are restricted to the non-equilibrium condition across the membrane. The generalized

thermodynamics of non-equilibrium systems tells us that the 'dissipative structure' can be formed only when the system is far removed from the equilibrium condition[10]. At present, we cannot specify whether the structure of the functional membrane of the drop surface is either the dissipative structure or the equilibrium one, or both. However, it is evident that the occurrence of the process of excitation in response to external stimuli is attributed to the labile structure of the surface membrane formed in the limited salt composition in the external medium.

REFERENCES

1 Inoue, I., Ishima, Y., Horie, H. and Takenaka, T. (1971) *Proc. Jap. Acad.* 47, 549–553
2 Inoue, I., Ueda, T. and Kobatake, Y (1973) *Biochim. Biophys. Acta* 298, 653–663
3 Yoneda, M. (1964) *J. Exp. Biol.* 41, 893–906
4 Kamiya, N. and Kuroda, K. (1958) *Proc. Jap. Acad.* 34, 435–438
5 Kamiya, N. and Kuroda, K. (1957) *Proc. Jap. Acad.* 33, 403–406
6 Dorsey, N. E. (1928) *J. Wash. Acad. Sci.* 18, 505–509
7 Miyake, M., Inoue, I. and Kobatake, Y. (1973) *Biochim. Biophys. Acta* submitted
8 Tasaki, I. (1968) *Nerve Excitation*, Charles C. Thomas, Springfield, Ill.
9 Kobatake, Y., Tasaki, I. and Watanabe, A. (1971) *Adv. Biophys.* 2, 1–31
10 Glansdorff, P. and Prigogine, I. (1971) *Thermodynamic Theory of Structure, Stability and Fluctuations*, John Wiley and Sons, London