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

V. EFFECT OF THE CHEMICAL MODIFICATION OF MEMBRANE PROTEINS WITH SH REAGENTS ON EXCITABILITY

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SUMMARY

Two SH reagents, *p*-chloromercuribenzoate and *N*-ethylmaleimide, and one reducing agent, dithiothreitol, were applied extrinsically to the surface membrane of protoplasmic droplets isolated from *Nitella*. The electrophysiological properties of the membrane and the tension at the surface of the droplets were examined in conjunction with the chemical modifications. The results obtained are summarized as follows:

1. The excitability of the drop membrane was suppressed irreversibly when the drop was treated either with 0.2 mM *p*-chloromercuribenzoate or 1 mM *N*-ethylmaleimide. All droplets treated with SH reagents were disrupted within an hour when the period of the treatments exceeded 5 min. During the process of disruption, the membrane potential and the electric resistance increased. The surface tension decreased temporarily by a factor of 10^{-3} (from 10^{-1} to 10^{-4} dyne/cm), then increased and approached 10^{-1} dyne/cm in the *p*-chloromercuribenzoate solution without recovery of the excitability.

2. Application of 4 mM dithiothreitol against the *p*-chloromercuribenzoate-treated droplet led to a gradual but complete restoration of the membrane potential, electric resistance and excitability.

3. The excitability which was suppressed by the treatment with SH reagents could also be recovered by means of alternative exchanges of the external media with dilute and concentrated salt solutions.

These results imply that the processes of depolarization and repolarization caused by the external media are accompanied by a considerable variation in the composition of molecules constituting the surface membrane of the protoplasmic droplets.

INTRODUCTION

This series of papers purports to study the molecular architecture of the surface membrane of the protoplasmic droplets of *Nitella* in connection with the process of

Abbrevation: PCMB, *p*-chloromercuribenzoate.

excitation [1–6]. In the preceding papers, we showed that the drop membrane was liable to change between two conformational states, the resting and excited (or depolarized) states, when the external salt composition was varied and/or when an electric current was applied across the membrane [1, 2, 5]. The macroscopic structure of the two states were discussed on the bases of the measurements of the electrophysiological and physical properties of the membrane, i.e. the refractive index and the tension at the surface of the droplet [3, 6].

In order to reveal the details of the molecular conformation of the excitable membrane, however, it is desirable to determine the chemical properties of the membrane. A powerful method for this purpose would be the use of an adequate chemical modification of the membrane molecules [7], which may furnish further information about the molecular mechanism underlying the excitation phenomena. In the present study, two SH reagents and one reducing agent were employed in the chemical modification of the drop membrane. The effect of these reagents on the electrical properties and the tension at the surface of the membrane are examined. The labile character of the excitable membrane of the protoplasmic droplets and the dynamic feature of the membrane conformation are discussed.

EXPERIMENTAL

Materials and procedures

The materials and experimental procedures were the same as those used throughout this series of studies [2–6]. An internodal cell of *Nitella flexilis* was amputated in a basal solution containing 75 mM KCl, 45 mM NaCl and 5 mM $CaCl_2$ in a vessel. The effused protoplasm which flowed down from the opening of the cell formed a sessile drop of about 300 μm in diameter on the bottom of the vessel [2]. After several droplets were formed, the external basal solution was changed with a testing solution by means of perfusion, whereby the droplets became electrically excitable with a time lag of about 1 h. The membrane potential and electric resistance at this stage were about −90 mV and 1–4 kΩ/cm^2, respectively [2, 5]. The salt composition of the testing solution was 0.5–2 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$ and 2 mM $MgCl_2$; the pH of the solution was adjusted between 6.3 and 6.7 with 1 mM Tris–acetate buffer. Each solution used was kept isotonic with the protoplasm of the internodal cell of *Nitella* by adding purified mannitol.

For measuring the electrophysiological properties of the drop membrane, two glass microelectrodes filled with 3 M KCl were inserted into the droplet. One was used for recording the transmembrane potential and the other for supplying the electric current.

The measurement of tension at the surface was performed by the same method as reported in Part II of this series of papers [3]. When the surface tension was larger than 10^{-2} dyne/cm, the compression method [9] was used, while the sessile drop method was employed when the surface tension was smaller than 10^{-2} dyne/cm [10]. All measurements were carried out at room temperature, 22$\pm$1 °C.

Chemical reagents

Two SH reagents, *p*-chloromercuribenzoate (PCMB) and *N*-ethylmaleimide, and one reducing agent, dithiothreitol, were purchased from Nakarai Chem. Co., Kyoto, Japan.

20 mg of PCMB, which is hardly soluble in water at neutral pH, was dissolved in 0.5 ml of 1 M NaOH aqueous solution. The PCMB solution thus obtained was diluted 500 times by the testing solution and neutralized by HCl, and then the pH of the final solution was adjusted to 6.5 with Tris–acetate buffer. The final concentrations of PCMB and Na^+ were 0.2 mM and 2 mM, respectively. This sodium concentration caused no change of the electrophysiological properties of the drop membrane as shown in the preceding paper [5]. *N*-ethylmaleimide was dissolved in the testing solution immediately before use, although the *N*-ethylmaleimide is known to be comparatively stable in an aqueous solution of which the pH is lower than 7.0. The concentration of *N*-ethylmaleimide used was 1 mM. The dithiothreitol solution was prepared by dissolving 12 mg of the chemical in 20 ml of the testing solution. The concentration of dithiothreitol was 4 mM.

Each reagent was applied by perfusing the external testing solution containing the respective reagent for a predetermined period, e.g. 5–20 min. The flow rate of the perfusion of the external solution was 2 ml/min, which could change the whole external solution within 2 min.

RESULTS AND DISCUSSION

Effect of SH reagents

Fig. 1a illustrates the effect of PCMB on the membrane potential (top) and the electric resistance (bottom). The abscissa shows the time in minutes after the onset of the perfusion of the PCMB solution. The marks of PCMB and T in the figure

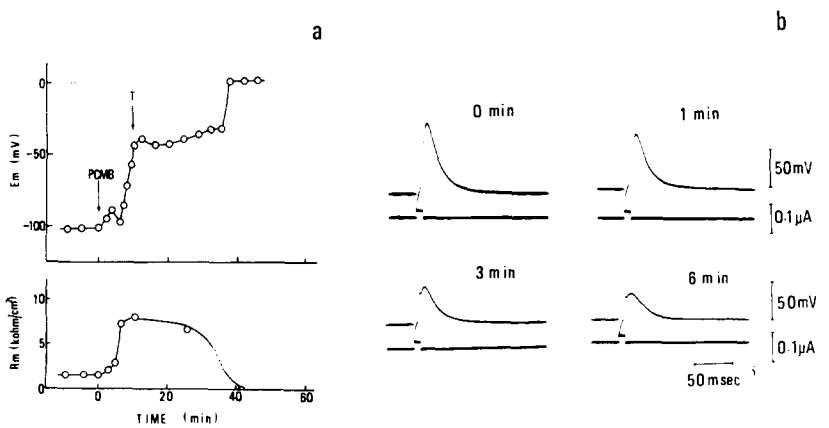

Fig. 1. (a) Effects of PCMB on the membrane potential, E_m, and on the electric resistance, R_m PCMB and T show the time when the 0.2 mM PCMB solution and the testing solution were perfused, respectively. (b) Process of suppression of the action potential in the PCMB solution. The upper traces indicate the action potential of the droplet and the lower traces show the outward current pulse applied.

indicate, respectively, the times when the PCMB solution and the testing solution were switched. In this specific case, the droplet was treated with 0.2 mM PCMB for 10 min. The membrane potential stayed at the resting level for a while after the application

of PCMB, followed by a swift depolarization of about 50 mV. After this, the membrane potential stayed at the same level for about 30 min. At the end of this stage, disruption of the droplet took place and the membrane potential diminished. The electric resistance increased sharply several times with almost the same time-course as the membrane potential after the application of PCMB, and stayed at a constant level until the disruption of the droplet was caused and the resistance became zero. These time-courses of the variations of the membrane potential and the electric resistance were not altered by the removal of PCMB from the external testing solution. Every droplet which was treated with PCMB for more than 5 min was found to be disrupted within an hour. Furthermore, the time-courses of the electrical properties during the process of disruption of the droplets were not affected appreciably by the length of the applied time of the PCMB solution. In some cases, the depolarization occurred without pursuing two steps, i.e. the membrane potential became 0 mV directly, and even in these cases, the electric resistance stayed at the high value of a few $k\Omega/cm^2$ until the droplet was disrupted.

The excitability of the drop membrane was suppressed in a relatively early period after an application of PCMB. Fig. 1b shows typical examples of the process of suppression of the observed action potential. The droplet was the same as that in Fig. 1a. The upper oscillograph trace in each figure shows the action potential and the lower trace shows the outwardly directed brief-current pulse applied externally. The amplitude of the action potential induced by the same current intensity was decreased with time in the PCMB solution, and almost vanished within 6 min after the application of PCMB, where the resting potential and the membrane resistance were -80 mV and 2.5 $k\Omega/cm^2$, respectively. In other words, the swift depolarization of the membrane potential occurred after the loss of the excitability. A conditioning current in the hyperpolarizing direction did not give rise to an appreciable effect on the excitability of the membrane. Furthermore, no restoration of the excitability was observed by the removal of PCMB from the external solution.

Fig. 2. shows the effect of PCMB on the tension at the surface of the droplet. A large and temporal decrease in the surface tension was observed in the early period

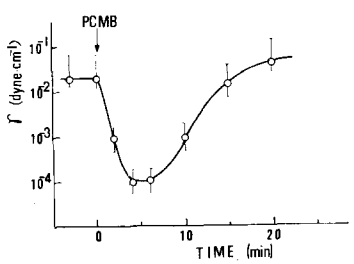

Fig. 2. Effect of PCMB on the tension at the surface of the protoplasmic droplet. The abscissa shows the time in minutes after the onset of perfusion of the 0.2 mM PCMB solution. The vertical bars show the possible error involved.

after the application of the 0.2 mM PCMB solution. The surface tension decreased from $0.2 \cdot 10^{-1}$ dyne/cm to about 10^{-4} dyne/cm in this specific droplet. After this, the surface tension increased and almost approached the same level as that in the

resting state. The electrical properties of the final state of the droplet, however, are entirely different from those in the original state (see Fig. 1).

It is interesting to note that the membrane potential of the droplet remained at the resting level in the early period after the treatment with PCMB, while the suppression of the excitability, the increase in the membrane resistance, and the decrease in the surface tension had largely occurred during this early stage. Namely, the time-course of the membrane potential in the PCMB solution was different from those of the other membrane properties.

Similar results, as shown in Figs 1 and 2, were observed when the droplets were treated by 1 mM *N*-ethylmaleimide solution instead of 0.2 mM PCMB solution.

Restoration of the excitability by dithiothreitol

The excitability and other electrical properties of the membrane which were suppressed by the treatment of PCMB could be restored by the application of a reducing agent, dithiothreitol. Fig. 3 shows an example of the restoration of the

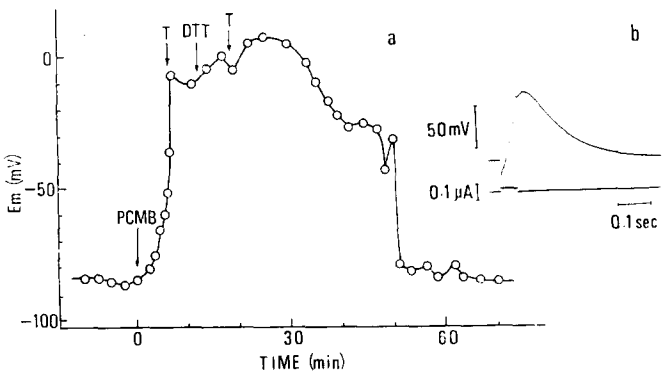

Fig. 3. (a) The restoration of the membrane potential of the droplet, which was pretreated by PCMB, by an application of dithiothreitol. PCMB, T and DTT indicate the times when the 0.2 mM PCMB solution, the testing solution, and the 4 mM dithiothreitol solution were perfused, respectively. (b) Action potential observed at the stage where the membrane potential was recovered by treatment with dithiothreitol.

membrane potential and the excitability caused by an application of 4 mM dithiothreitol solution. The presence of dithiothreitol in the testing solution had no effect on the electrical and physical properties of the drop membrane when the drop was not pretreated with PCMB. In the case given in Fig. 3a, dithiothreitol was applied for 7 min after the membrane potential had been depolarized to almost 0 mV in the PCMB solution. By the application of dithiothreitol for 7 min, not only the disruption of the droplet was prevented, but also the membrane potential was repolarized gradually and finally reached the value of its original resting state (−90 mV in this specific droplet). At this stage, the excitability was recovered completely. An example of the action potential observed with the recovered droplet is shown in Fig. 3b.

These results imply that the blockage of the excitability by the treatment of PCMB occurs at the membrane and not in the protoplasm inside the droplet. In fact, the protoplasmic streaming in the droplet was not suppressed by the application of PCMB.

Restoration of excitability by means of alternative exchange of the external media

Without application of the reducing agent such as dithiothreitol, the excitability of the surface membrane of the droplet which was suppressed by treatment with SH reagents could be restored. The following observations demonstrate the restoration of the membrane properties without the use of dithiothreitol.

In previous papers of this series, two distinguishable conformational states of the membrane, i.e. the resting and depolarized (or excited) states, were demonstrated by the measurement of the physical and electrophysiological properties of the membrane. We showed that the transition between these two states could easily be induced by a variation of the salt composition of the external medium [3, 5, 6]. As shown below, the transition between the two states of the membrane could be induced even in a drop membrane treated by PCMB, although each state did not correspond exactly to the resting or excited state as described above. By way of an example, Fig. 4a shows the time-courses of the membrane potential and of the electric resistance for a PCMB-treated droplet by alternative changes of the external media with the testing and the basal solutions. PCMB, T and B in the figure indicate the time when the 0.2 mM PCMB solution, the testing solution and the basal solution, respectively,

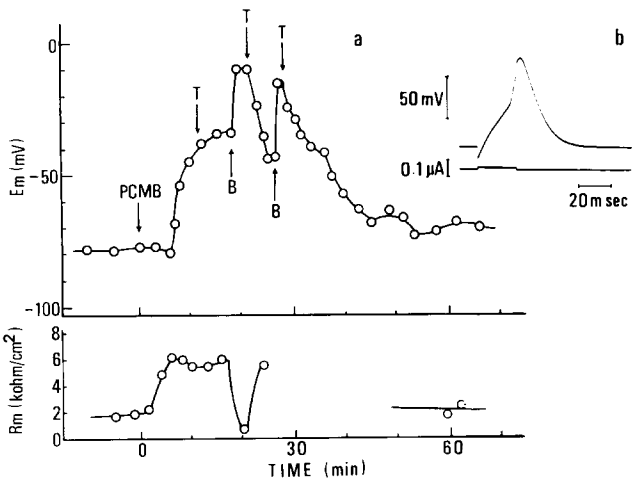

Fig. 4. (a) Restoration of the membrane potential, E_m, and the electric resistance, R_m, of the PCMB-treated droplet by means of alternative changes of the salt composition in the external media with the testing and the basal solutions. PCMB, T and B indicate the times when the 0.2 mM PCMB solution, the testing solution and the basal solution were perfused, respectively. (b) Action potential recorded at the stage where the membrane potential and the electric resistance returned to the original resting level.

were perfused externally. The pH of the basal solution used in this study was adjusted to 6.5 with 1 mM Tris–acetate buffer. The droplet was treated by PCMB solution for a period of 12 min in this specific case. As seen in the figure, the drop membrane underwent a discrete depolarization in the basal solution, where the membrane potential and the electric resistance were observed to be about -10 mV and 0.5 kΩ/cm^2, respectively. One may notice that the membrane potential in the testing solution is repolarized to a more negative level with every successive depolarization of the mem-

brane by the basal solution. Finally, the membrane potential in the testing solution returned to the same level as that of the original resting stage, where the electric resistance of the membrane also returned to the original value. At this state, a complete restoration of the excitability was observed (Fig. 4b). A similar result was obtained from droplets treated with 1 mM *N*-ethylmaleimide instead of PCMB. An example is shown in Fig. 5.

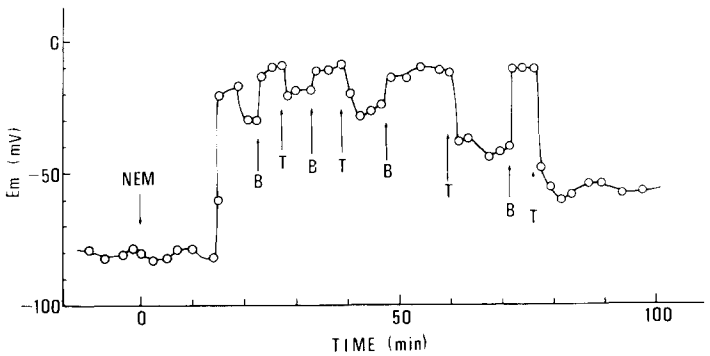

Fig. 5. Restoration of the membrane potential of the droplet which was treated by 1 mM *N*-ethylmaleimide solution, by means of alternative changes of the salt composition of the external media with the testing and the basal solutions. NEM, T and B indicate the times when 1 mM *N*-ethylmaleimide solution, the testing solution and the basal solution were perfused, respectively.

As shown in the previous papers, the depolarized state of the protoplasmic drop membrane is considered to be a lipid-rich structure, while the resting state of the membrane is characterized by a lipid–protein complex or lipoprotein state [2, 3, 6]. From these results together with the results shown above, we may infer that a part of the modified proteins due to SH reagent is released from the membrane by an alteration of the external medium from the testing to the basal solutions. The proteins existing in the protoplasm, which are not modified by the SH reagent applied externally, may be absorbed into the membrane and reconstitute the membrane structure in the testing solution. Thus, the SH-modified proteins in the membrane can be changed with new proteins by the alternative exchange of the external media with the basal and the testing solutions. When a greater part of the modified proteins are replaced by new proteins, the conformation of the membrane returns to the original resting state, where the membrane potential, the electric resistance, and the excitability of the drop membrane are recovered. This dynamic picture of the surface membrane is consistent with the formative processes of the protoplasmic drop membrane discussed in Part I of this series [2].

From the results shown in this paper, we may consider that the processes of discrete depolarization and repolarization caused by the ionic environment are accompanied by a drastic change in the membrane structure which involves the variation of the chemical composition of the membrane macromolecules. Such drastic changes in molecular composition seems to take place to a greater or lesser extent during the process of excitation not only in the excitable membrane of the protoplasmic droplets but also in that of nerves. In fact, it has been reported that

an appreciable difference in the protein composition of the squid axon membrane is observed between the resting and depolarized states [11].

As has been stressed repeatedly, the excitability of the membrane of the protoplasmic droplet of *Nitella* is essentially attributed to the lability of the surface membrane. The membrane can easily be transformed to a new state by a weak external perturbation, e.g. application of chemical reagent as demonstrated here, electric current [1, 2], temperature change [6], or change in the salt composition of the external medium. This lability of the membrane structure itself seems to be an indispensable character of the excitable membrane.

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