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ROLES OF Ca^{2+}, PHOSPHOLIPIDS AND PROTEINS IN THE EXCITABLE MEMBRANE OF PROTOPLASMIC DROPLET ISOLATED FROM *NITELLA*

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SUMMARY

Protoplasmic droplet isolated from *Nitella* became electrically excitable in appropriate salt solutions. Specific interactions among molecules constituting the surface membrane and ions in the surrounding media are responsible for the characteristic feature of the excitable membrane. Here, the roles of Ca^{2+}, phospholipids, and proteins for maintaining the excitability of the droplets were examined by modifying the surface membrane by means of various inorganic cations. The following results were obtained:

1. Sr^{2+} could substitute for Ca^{2+} in the external solution without loss of excitability. However, the membrane was in the resting state of about −50 mV in the membrane potential in media where the Sr^{2+} concentration was higher than 3 mM, whereas Ca^{2+} maintained the excitable membrane structure with about −100 mV in the resting potential in the concentration range between 0.1 and 10 mM.

2. The following order was found for the critical salt concentration at which an abrupt depolarization of the membrane potential was induced, when the following cations were added to the test solution; $UO_2^{2+} < Eu^{3+} < Nd^{3+} < Ca^{2+}$. Tension at the surface of the droplet increased remarkably with an increase of the concentration of the respective ion species.

3. The action of Hg^{2+}, Cd^{2+} and Ag^{+} gave rise to a temporal increase of the membrane resistance, and a decrease of tension at the surface. Then followed a depolarization of the membrane potential at the concentration as low as 10^{-6}–10^{-5} M.

4. Cu^{2+} acted on the membrane with a decrease in the membrane resistance and the tension at the surface, and followed by a depolarization of the membrane potential at about 10^{-5} M.

INTRODUCTION

It is well known that the production of an action potential in squid axon is accompanied by changes of other physical properties of the membrane such as light scattering, birefringence [1], extrinsic fluorescence [2], thermal properties [3, 4], ionic permeabilities [5], etc. These facts imply that the process of nerve excitation is an electrical reflection of a structural change in the membrane. Nevertheless, it is

extremely difficult to determine the structural features of the excitable membrane during excitation in living organisms, because thick adherent tissues prevent or make difficult the observation of the properties of a thin (about 100 Å thick) functional membrane.

Protoplasmic droplet isolated from *Nitella* [6] serves as a favorable system for studying the structure of the excitable membrane, because the surface membrane is contiguous to the external solution [7]. This fact permitted the determination of the interfacial properties of the droplet membrane such as the tension at the surface [8], the refractive index [9, 10] as well as the electrical properties [11, 12]. Variations of salt composition and temperature in the external medium induced more or less abrupt and discontinuous changes of the membrane properties. These observations corresponded exactly to those observed in squid axon under internal perfusion.

The next problem to be solved is to determine what kinds of molecular interactions are necessary for the maintenance of an excitable membrane structure. The technique of chemical modification of the membrane is one of the most powerful tools for this purpose [13]. In this paper we will report the effects of various inorganic cations on the membrane properties. Specific interactions of respective cations with some specific groups or molecules are well known in the field of lipid and protein chemistry. For instance, UO_2^{2+} has a strong affinity to the phosphate group in phospholipids [14]. Hg^{2+}, Cd^{2+} and Ag^{+} form complexes with the SH group in proteins [15]. Cu^{2+} inactivates the catalytic permeability of glycerol in erythrocyte membrane by protecting the histidine group in enzymes [16]. On the basis of experimental results obtained with these specific ions, discussion will be made on the roles of Ca^{2+}, phospholipids and proteins in the excitable surface membrane of the protoplasmic droplet isolated from *Nitella*.

EXPERIMENTAL

Materials and experimental procedures

The experimental procedures were essentially the same as those employed previously [5, 8]. The internodal cell of *Nitella flexilis* was amputated in an aqueous solution containing 70 mM KNO_3, 50 mM NaCl and 5 mM $Ca(NO_3)_2$. The effused protoplasmic droplet was allowed to stand in a test solution [0.5 mM NaCl, 0.5 mM KNO_3, 1 mM $Ca(NO_3)_2$ and 2 mM $Mg(NO_3)_2$; the solution was buffered at about pH 6.5 by Tris acetate and was isotonic by adding mannitol]. The protoplasmic droplet which reached the steady state in the test solution was found to be electrically excitable [8]. Variation of composition in the external solution was performed after the drop attained the steady state by adding respective salts to the test solution described above.

All experiments were performed at room temperature, 20 ± 2 °C.

The materials used were $UO_2(OAc)_2$, $EuCl_3$, $Nd(NO_3)_3$, $SrCl_2$, $Mg(NO_3)_2$, $Ca(NO_3)_2$, KNO_3, NaCl and Tris acetate of analytical grade. Mannitol was recrystallized from ethanol.

Determination of tension at the surface

Tension at the surface, γ, of the protoplasmic droplet was determined either by the sessile drop method [17, 18] or by the compression method [19]. Experimental details were described previously [7].

RESULTS AND DISCUSSION

Effect of substitution of Ca^{2+} by Sr^{2+} in the external media

It is well known that Sr^{2+} can substitute for Ca^{2+} with no loss of the physiological functions of living organisms such as the nerve and muscle fibres [20, 21]. Thus it may be interesting to examine the effect of substitution of Ca^{2+} by Sr^{2+} in the external solution on the properties of the surface membrane of protoplasmic droplets. Fig. 1a shows the dependence of membrane potential on Sr^{2+} concentration. Here, the salt composition in the external solution other than Sr^{2+} was kept at 0.5 mM NaCl, 0.5 mM KNO_3 and 2 mM $Mg(NO_3)_2$. For the sake of comparison, the dependence of the membrane potential on the Ca^{2+} concentration is also depicted in the same figure. By replacing Ca^{2+} with Sr^{2+} in the external solution, the membrane potential was depolarized slowly from a -100 mV level to about a -50 mV level without loss of excitability. A decrease of the Sr^{2+} concentration induced an abrupt depolarization to about zero mV at about 3 mM of Sr^{2+}, whereas an increase of Sr^{2+} did not induce any change in the membrane potential. These observations for Sr^{2+} constitute a sharp contrast to the case of Ca^{2+} in which the abrupt depolarization was elicited at two critical concentrations as shown in Fig. 1a. The lower critical concentrations differ remarkably between Ca^{2+} and Sr^{2+}; i.e., 0.1 mM for Ca^{2+} and 3 mM for Sr^{2+}. Results of the shallowing of the membrane potential from -100 to -50 mV and of an about 20-times rise of the lower critical concentration may be ascribed to the weakness of the interaction of Sr^{2+} with molecules constituting the membrane as compared with Ca^{2+}. Disappearance of the higher critical concentration in the case of Sr^{2+} is another difference between Ca^{2+} and Sr^{2+}. It was reported that the tension at the surface of the protoplasmic droplet was about 1 dyne/cm in 50 mM of Ca^{2+}. On the contrary, γ decreased to about 10^{-3}–10^{-2} dyne/cm in 50 mM of Sr^{2+}. At high concentrations of Ca^{2+}, the refractive index had a large value of about 1.6 [9], which implied that the surface membrane became rich in phospholipids. From the results given in Fig. 1a together with other characteristics of the membrane reported previously, we may infer that the high tension at the surface of the protoplasmic droplet prevents the penetration of proteins into the surface membrane, and which in turn induces the depolarization of the membrane potential in a

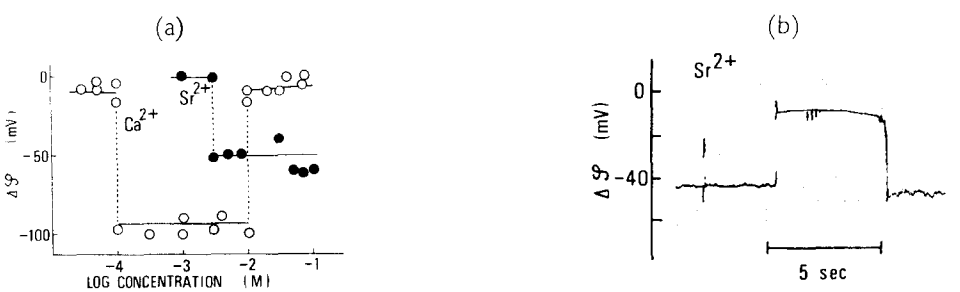

Fig. 1. (a) Effect of substitution of Ca^{2+} with Sr^{2+} in the external solution. Membrane potential is plotted against log concentration. ○ and ●, indicate the case of Ca^{2+} and Sr^{2+}, respectively. Other salt compositions were fixed at 0.5 mM NaCl, 0.5 mM KNO_3 and 2 mM $Mg(NO_3)_2$. (b) A pen-writing record of the rectangular action potential of long duration induced by an outward current pulse (0.1 s in duration). The external solution was free from Ca^{2+} and contained 7 mM of Sr^{2+}. The other salt compositions were the same as indicated in Fig. 1a.

high Ca^{2+} concentration media. This point will be discussed extensively in a later section of this paper.

The state of the -50 mV level of the membrane potential maintained by Sr^{2+} may be regarded as the resting state. Fig. 1b shows an oscillograph record of a transient variation of the membrane potential when a brief outward current pulse (0.1 s in duration) was applied to the droplet. A rectangular action potential of a long duration of a few sec was observed. During the production of an action potential, the droplet deformed appreciably. This observation indicates that the excited state of the droplet has low tension at the surface, which is consistent with the results that the depolarized state elicited by increasing the salt concentration of a monovalent cation or by decreasing the Ca^{2+} concentration has an extremely low surface tension (10^{-3}–10^{-4} dyne/cm) [7, 9].

Effect of UO_2^{2+}, Eu^{3+} and Nd^{3+}

Fig. 2 shows the concentration dependence of the membrane potential on various salt species where $UO_2(OAc)_2$, $EuCl_3$ and $Nd(NO_3)_3$ were added to the test solution. The following order is seen in the figure for cation species on the lowest (critical) salt concentration where either discontinuous or continuous depolarization of the membrane potential is induced; $UO_2^{2+} < Eu^{3+} < Nd^{3+} < Ca^{2+}$. Abrupt depolarizations of the membrane potential took place when the droplet was immersed suddenly in a high concentration of either Eu^{3+} or Nd^{3+}. Fig. 2b shows the time-course of the membrane potential when $5 \cdot 10^{-3}$ M of Nd^{3+}, the critical concentration of which was $7 \cdot 10^{-4}$ M, was added to the solution at the time indicated by an arrow in the figure. This indicates that no essential difference exists between discontinuous (e.g. UO_2^{2+}, and Ca^{2+}) and continuous but abrupt (e.g. Nd^{3+}, and Eu^{3+}) changes of the membrane potential at the critical concentration, and the difference may be attributed to how the external solution is exchanged.

Recent NMR and ESR studies on lecithin membrane showed that the affinity of multivalent cations to phospholipids gives the same sequence; $UO_2^{2+} < Eu^{3+} < Nd^{3+} < Ca^{2+}$ [22]. Good correspondence of the sequence described above indicates that the depolarization of the membrane potential caused by adding these ions may be ascribed to the specific interactions among phospholipids and respective cations. Typical effects are seen in the case of UO_2^{2+}. Fig. 3 shows the time-course of the membrane potential and membrane resistance when $2 \cdot 10^{-4}$ M of UO_2^{2+} was added to

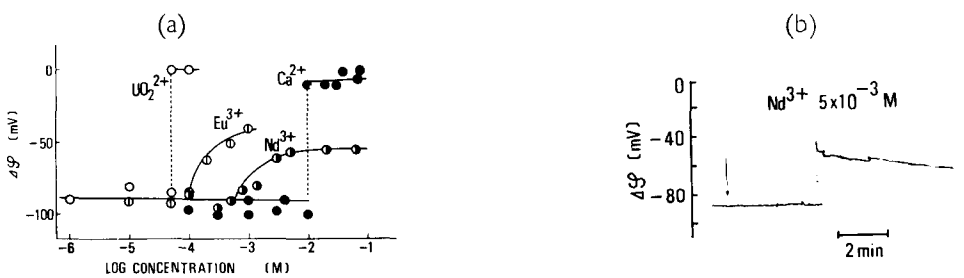

Fig. 2. (a) Dependence of membrane potential on the concentration of various cations. Each cation was added successively to the test solution. Marks of ○, ⓘ, ◑, and ● indicate $UO_2(OAC)_2$, $EuCl_3$, $Nd(NO_3)_3$ and $Ca(NO_3)_2$. respectively. (b) Time-course of the membrane potential when $5 \cdot 10^{-3}$ M of Nd^{3+} was added in the test solution at the time indicated by an arrow in the figure.

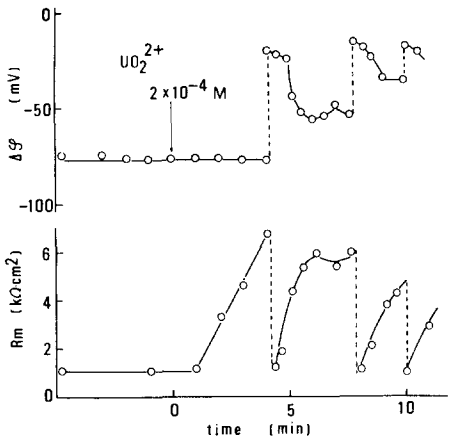

Fig. 3. Time-course of the membrane potential, $\Delta\varphi$, and resistance, R_m, when $2 \cdot 10^{-4}$ M of UO_2^{2+} was added in the test solution.

the external solution. The membrane resistance increased with time after the addition of UO_2^{2+} for several minutes by a factor of 7, while the membrane potential remained constant during this period of time. Then followed an abrupt depolarization of the membrane potential, which was accompanied by a discontinuous fall of the membrane resistance. Discontinuous changes of membrane potential and membrane resistance took place periodically as seen in Fig. 3. It was noticed that the tension at the surface of the protoplasmic droplet increased from 0.1 to several dyne/cm with an increase of the UO_2^{2+} concentration, where the membrane was depolarized.

Unexpectedly high values of tension at the surface, higher than 1 dyne/cm, in the presence of UO_2^{2+} seem to afford a key to explain the results given in Fig. 3. Studies on the monomolecular layer of phospholipids show that the strong affinity of UO_2^{2+} to phospholipids causes an aggregation of the phospholipids in the surface layer [14] and that the penetration of proteins from the subphase into the surface layer depends strongly on the surface pressure of the monolayer [23, 24, 25]. Similarly, an appropriate surface tension is necessary for the penetration of protein molecules into the lipid membrane. On this ground, the results shown in Fig. 3 may be explained in terms of the formation and destruction of lipid protein complexes in the surface membrane [9, 8]. The initial rise of the membrane resistance may be resulted from the exclusion of proteins from the surface, which is accompanied by a diminution of the path of movable ions. Nevertheless, a too rapid aggregation of phospholipids leads to the creation of a microscopic crack in the surface membrane. This interpretation is consistent with the discontinuous fall of the membrane resistance and with an abrupt depolarization of the membrane potential. Subsequent periodical changes of the membrane potential and resistance were explained as the competition of the formative and degradative processes of the surface membrane described above. The occurrence of the micro-crack in the membrane is also observed by the formation of small buds on the droplet surface under the presence of UO_2^{2+} in the medium. As seen in Fig. 3 the peak values of the membrane resistance decreased gradually with successive periodical changes. This result is also in accordance with the micro-crack picture of phospholipid membrane bound by UO_2^{2+} at the surface of the droplet.

Effect of Hg^{2+}, Cd^{2+} and Ag^{+}

Fig. 4. shows the time-course of the membrane potential and membrane resistance when $3 \cdot 10^{-6}$ M of $HgCl_2$, and $2 \cdot 10^{-5}$ and $6 \cdot 10^{-5}$ M of $CdCl_2$ were added to the external test solution. The membrane resistance increased irregularly by a factor of about two as compared with the original value. During this period, the membrane potential did not change appreciably but small fluctuations of the potential increased gradually with time. In each case the membrane resistance became very low when the membrane potential approached zero. In the case of Cd^{2+}, a gradual rise of membrane potential occurred with a time lag after the discrete fall of the membrane resistance. It seems to us that these various variations of the membrane properties caused by different multivalent cation species stem either from the difference in action points of these ions or from the different functions of the outer and inner surface of the membrane. Studies of the chemical modifications of membranes carried out either from the outer or from the inner sides of the droplet will be reported in a subsequent paper.

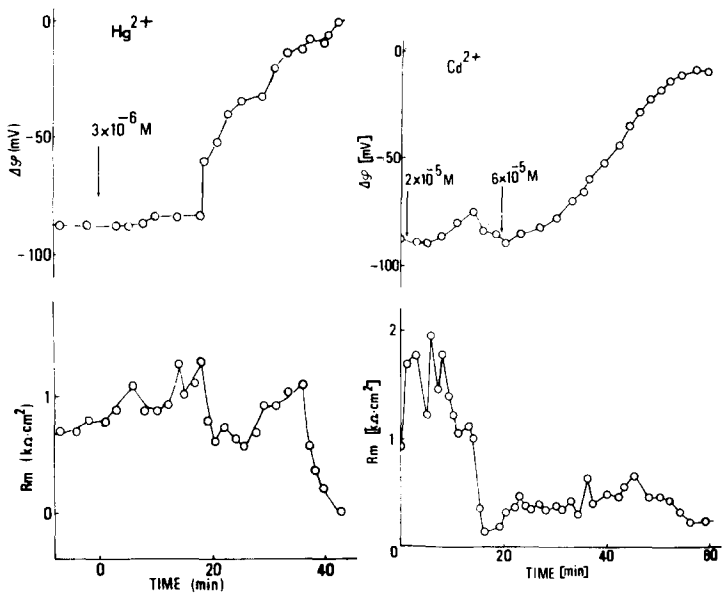

Fig. 4. Time-course of the membrane potential, $\Delta\varphi$, and resistance, R_m, after Hg^{2+} and Cd^{2+} were added in the test solution. Concentrations and the time of addition are indicated in the figure.

Fig. 5 shows an example of the tension at the surface, γ, of the protoplasmic droplet when the droplet was immersed in the test solution containing $3 \cdot 10^{-6}$ M of $HgCl_2$. γ decreased rapidly from 0.1 to the order of 10^{-4} dyne/cm within 10 min, and followed a recovery of the surface tension and gradual fall of γ again. Comparison between Fig. 5 and the upper trace of Fig. 4a reveals that the second fall of tension at the surface corresponds to the initiation of the irreversible depolarization of the membrane potential.

Degradation and formation of the membrane compete with each other under the presence of Hg^{2+}. The similar periodical and spike-wise changes of the membrane

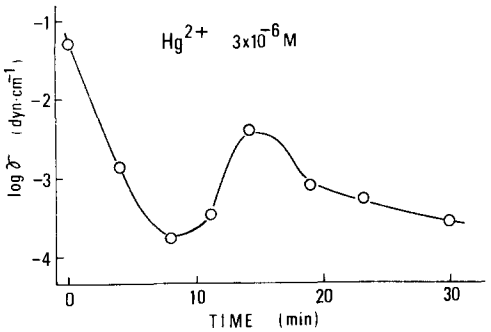

Fig. 5. Effect of Hg^{2+} on tension at the surface of the protoplasmic droplet. $3 \cdot 10^{-6}$ M of $HgCl_2$ was added in the test solution at time zero.

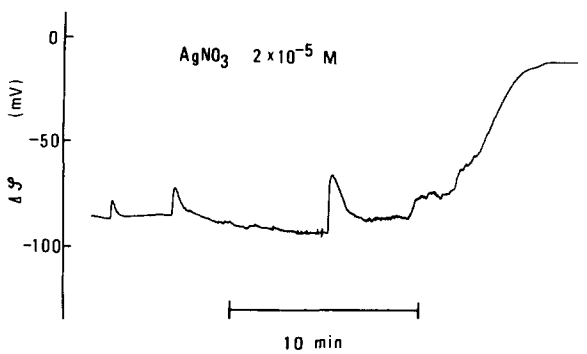

Fig. 6. Pen-writing record of fluctuation and spike-wise changes in membrane potential in the presence of $2 \cdot 10^{-5}$ M $AgNO_3$ in the test solution.

potential were observed when $2 \cdot 10^{-5}$ M of $AgNO_3$ was added to the external solution. At the same time, fluctuations of the membrane potential became larger and larger, and finally an irreversible depolarization was followed as illustrated in Fig. 6.

Effect of Cu^{2+}

The effect of Cu^{2+} constitutes a sharp contrast to those of Hg^{2+}, Cd^{2+} and Ag^{+}. Fig. 7 shows the time-course of the membrane potential and membrane resistance when $6 \cdot 10^{-6}$ and $3 \cdot 10^{-5}$ M of $CuCl_2$ were added successively to the external solution. A decrease of the membrane resistance, R_m, seems to induce a depolarization of the membrane potential. Moreover, a low value of R_m permitted the penetration of Cu^{2+} into the protoplasmic droplet. The protoplasmic movement became weaker, and then ceased from near the inner surface of the protoplasmic droplet. In the case of Hg^{2+} and Cd^{2+}, the protoplasmic movement continued even when the high concentration of respective ions were added in the external solution. The decrease of γ was observed initially in the presence of Cu^{2+} in the external solution. Nevertheless, a precise determination of γ seems to give little meaning, because the death of the droplet always followed.

Cu^{2+} is known to form a complex with an imidasol group in the histidine resi-

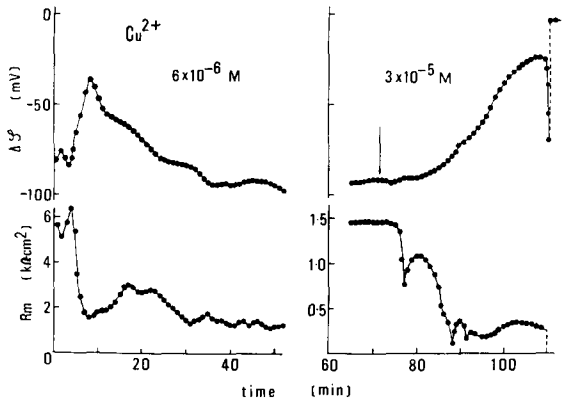

Fig. 7. Time-course of the membrane potential, $\Delta\varphi$, and resistance, R_m. The left-hand figure indicates the changes in the presence of $6 \cdot 10^{-6}$ M of Cu^{2+}, and the right one the further addition of $3 \cdot 10^{-5}$ M (arrow in figure).

due of proteins. Different effects of various inorganic cations may be attributed to the different modifications of the amino acid residue of proteins constituting the membrane.

CONCLUDING REMARKS

Results reported here indicate that the excitable surface membrane of the protoplasmic droplet is very liable to change its structure in response to the perturbation applied from the external solution, i.e., application of various inorganic cations. This fact gives rise to a warning against using other techniques in studying the membrane structure. For example, electronmicroscopy of the membrane can be taken only when the membrane is stained by electron-dense materials such as osmic acid or uranyl ions. This kind of staining or fixation inevitably induces variation of the structure of the functional membrane. Therefore, we feel that the structure which we can see in the fixed materials of the living organism is far from its living state. Note that the biological function and structure cannot be separated. In addition, we should not overlook the non-equilibrium factors for maintaining the membrane structure, because the membrane system is always in non-equilibrium conditions in its functional state: i.e., the differences in electrical potential and in ionic composition and their concentrations play essential roles in maintaining the structure of the excitable membrane.

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