Origin of the Membrane Potential in Plasmodial Droplets of Physarum polycephalum

Evidence for an Electrogenic Pump

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ABSTRACT

Spherical droplets, derived from Physarum plasmodia by incubation in 10 mM caffeine, seemed to be an excellent system for electrophysiological studies because they were large (≤300 μm in diameter) and because they tolerated intracellular electrodes filled with 3 M KCl and 10 mM EDTA for a few hours. Intact plasmodia, by contrast, gave valid records for only a few minutes. Under standard conditions ([K+] o = 1 mM, [Na+] o = 5 mM, [Ca+++] o = 0.5 mM, [Mg++] o = 2 mM, and [Cl−] o = 6 mM at pH 7.0), the potential difference across droplet membranes was −80 to −120 mV, interior negative. The membrane potential was only slightly sensitive to concentration changes for the above-mentioned ions, and was far negative to the equilibrium diffusion potentials calculated from the known internal contents of K, Na, Ca, Mg, and Cl (29.4, 1.6, 3.7, 6.5, and 27.8 mmol/kg, respectively). Variations of external pH did have a strong influence on the membrane potential, yielding a slope of 59 mV/pH between pH 6.5 and 5.5. In this pH range, however, the equilibrium potential for H+ (assuming 6.2 ≤ pHi ≤ 7.0) was >75 mV positive to the observed membrane potential. Membrane potential was directly responsive to metabolic events, being lowered by potassium cyanide, and by cooling from 25 to 12°C. This ensemble of results strongly indicates that the major component of membrane potential in plasmodial droplets of Physarum is generated by an electrogenic ion pump, probably one extruding H+ ions.

INTRODUCTION

Plasmodia of the slime mold Physarum polycephalum exhibit a rhythmical fluctuation of the electrical potential difference along their length, with a period of a few minutes (Watanabe et al., 1937). The potential difference was later found to correlate closely with the rhythm of motive force responsible for their characteristic protoplasmic streaming called “shuttle streaming” (Kamiya and Abe, 1950). However, little is known up to the present either about the origin of the membrane potential or about its role in motility.

The reason few analytical studies have been carried out since then on the electrical properties of Physarum is probably the difficulty encountered in...
sustaining intracellular recordings. Their plasma membranes quickly reseal over microelectrodes, so that it is practically impossible to measure the membrane potential with standard glass microelectrodes (Tauc, 1954; Rhea, 1966; Miller et al., 1968). Rhea (1966) succeeded somewhat by using microelectrodes filled with 3 M KCl containing ethylenediamine tetraacetic acid (EDTA), showing the membrane potential itself to change rhythmically, but even then long-term recordings were not feasible. A way around this problem was afforded by the discovery of Hatano (1970) that a brief incubation of plasmodia in 5–10 mM caffeine solution produced membrane-limited spherical droplets of cytoplasm. These droplets displayed cytoplasmic movements in the presence of a low concentration of Ca ion (Hatano, 1970; Hatano and Oosawa, 1971; Matthews, 1977). But more importantly for the present purposes, droplets 150–300 μm in diameter have been found to possess large and stable membrane potentials that can be studied for 1–3 h and behave much like the membrane potentials of intact plasmodia. Additional advantages of the membrane-limited droplets for electrophysiology include their regular spherical geometry and the absence of coating mucopolysaccharides that create a thick, fixed-charged, unstirred layer around plasmodia. Caffeine-induced droplets of Physarum thus seem to correspond to thin-walled spheroplasts of bacteria and plant cells.

The experiments reported below, using caffeine-induced droplets, were designed to demonstrate the essential mechanisms for generating membrane potential in Physarum. Potential was measured as a function of the extracellular concentrations of K⁺, Na⁺, Ca²⁺, Mg²⁺, Cl⁻, and H⁺. In addition, the effects of metabolic inhibitors and low temperature were examined, and the results suggest that an electrogenic proton pump is responsible for the major component of the resting membrane potential of Physarum droplets.

MATERIALS AND METHODS

Plasmodia

Stock cultures of plasmodia of Physarum polycephalum were maintained on wet oatmeal at 18–20°C in the dark (Camp, 1936), and could be kept a month or more by refreshing the oatmeal daily. 2 or 3 d before use, a small plasmodial mass was removed from the stock culture and placed on the surface of an agar plate (3%) containing no added salts or nutrients.

Media

Compositions of all media used in the present experiments are listed in Tables I and II. To measure the membrane potential of intact plasmodia, standard P medium was used. In a series of experiments designed to test the effects of external cations, sucrose was added to maintain the iso-osmolarity of media.

Solutions for creating plasmodial droplets (DF medium) and for electrical recording from droplets (standard T medium) were designed in imitation of the mucoid layer that covers normal plasmodia (Kuroda and Kuroda, 1980). These concentrations were established in DF medium and standard T medium by addition of chloride salts. The Na concentration of these media (5 mM) was somewhat higher than in the mucoid layer, because NaOH was used to neutralize piperazine-N,N'-bis(2-ethane
sulfonic acid) (PIPES). Mannitol was used for adjusting the osmolarity of all recording media to ~110 mosmol, at which the droplets maintained the spherical shapes and stable membrane potentials for the longest period. Because of the unknown mode of action of caffeine and its uncertain long-term effects, many attempts were made to preserve droplets in the absence of caffeine. All such attempts failed, however, and within 5 min of caffeine removal the droplets reverted to nonspherical form, apparently on the way toward reformation of plasmodia. All media for droplets, therefore, contained 10 mM caffeine. In certain media, the larger and therefore presumably less permeant anion methanesulfonate$^-$ (MeSO$_3^-$) was substituted for Cl$^-$. In the early stage of present experiments, Tris maleate buffer and sucrose were used instead of

| TABLE I | COMPOSITIONS OF MEDIA FOR INTACT PLASMODIA |
|---------|------------------------------------------|
|         | K$^+$ Na$^+$ Ca$^{++}$ Cl$^-$ Tris maleate Sucrose |
| Standard P medium | 0 3 1 2 3 0 |
| 33 K-P medium | 33 0 1 32 3 0 |
| 3 K-P medium | 3 0 1 2 3 60 |
| 33 Na-P medium | 0 33 1 32 3 0 |
| 3 Na-P medium | 0 3 1 2 3 60 |

Values are presented in millimolars. The pH of these media is 6.9-7.1.

| TABLE II | COMPOSITIONS OF MEDIA FOR DROPLETS |
|----------|------------------------------------|
| K$^+$ Na$^+$ Ca$^{++}$ Mg$^{++}$ Cl$^-$ PIPES MeSO$_3^-$ Tris Caffeine Mannitol |
| DF medium | 1 5 0.5 2 6 5 0 0 10 0 |
| T media Standard | 1 5 0.5 2 6 5 0 0 10 80 |
| 30 KCl | 30 5 0.5 2 35 5 0 0 10 27 |
| 30 K | 30 5 0.5 2 5 5 30 0 10 27 |
| 0 K | 0 5 0.5 2 5 5 0 0 10 82 |
| 30 Na | 5 30 0.5 2 5 5 30 0 10 27 |
| 10 Ca | 1 5 10 2 5 5 20 0 10 55 |
| 0 Ca | 1 5 0 2 5 5 0 0 10 81 |
| 10 Mg | 1 5 0.5 10 2 5 20 0 10 59 |
| 30 Cl | 1 5 0.5 2 30 5 1 25 10 30 |

Values are presented in millimolars. The pH of all the above media is 6.9-7.1.

PIPES buffer and mannitol, respectively. There was no systematic difference in the behavior of the membrane potentials between the medium containing Tris maleate and sucrose and that containing PIPES and mannitol.

Preparation of Droplets

A small fragment of plasmodium was cut off by a hair loop from the advancing margin of a plasmodium on an agar plate and transferred to DF medium. Within 10-30 min, at ~23°C, the surface of the plasmodium “budded” in all directions, releasing many spherical droplets into suspension. Droplets were then sucked up gently by a pipette whose tip had been fire-polished, and were transferred to standard T medium.
Droplets were incubated in this medium until use. In standard T medium, the droplets maintained their spherical shapes and cytoplasmic movements for the maximal time of 3 h.

**Experimental Chambers**

A flat lucite chamber of ~3-ml vol was used for recording from intact plasmodia. A short straight strand was cut off from plasmodia, together with a small agar block, and placed on the bottom of the chamber.

A different chamber, fitted to the stage of a horizontal microscope, was used for experiments with droplets. An agar block, on which several hemispherical cavities 150–300 μm in diameter were molded, was put on the bottom of this chamber, and a plasmodial droplet was loaded into a cavity of appropriate size by dropping gently from a pipette. The diameter of each droplet was estimated with an ocular micrometer.

In both experimental chambers, fluids were added through a channel bored near the bottom and were removed through a channel near the top. Slightly more than 3 min was required to exchange fluids. Most experiments were conducted at the ambient temperature of 23 ± 3°C. The temperature of the medium was monitored by a copper-constantan thermocouple placed next to the droplet.

**Electrodes**

With standard 3 M KCl-filled glass microelectrodes, stable intracellular recordings were impossible on intact plasmodia (Fig. 1 a) or on droplets (Fig. 1 c), because *Physarum* quickly resealed off the inserted electrodes by surrounding the tip and outer wall with newly formed membrane. Therefore, tribasic K salt of EDTA was added to the electrode-filling solution. These electrodes permitted a few minutes of recording from intact plasmodia (Fig. 1 b), and a few hours of continuous recording from single droplets (Fig. 1 d).

In most cases, 3 M KCl containing 0.1 M EDTA was used in electrodes for intact plasmodia, and 3 M KCl containing 0.01 M EDTA was used for plasmodial droplets. Electrodes were selected with 8–15-MΩ resistances. The standard reference electrode consisted of a glass pipette filled with 30 mM KCl and 3% agar. Each electrode was connected to the amplifier via a 3-M KCl bridge and an Ag-AgCl half cell.

**Electrical Measurements**

Because the membrane of plasmodial droplets proved too fragile to admit more than a single microelectrode, resistance measurements had to be carried out by passing current through the single voltage-recording electrode. For this purpose, an FET operational amplifier (model 1009; Teledyne Philbrick, Dedham, Mass.) with a bootstrap circuit was used. As arranged, the amplifier had an effective input resistance of >10^8 Ω, and a leakage current of <10 pA. The voltage displacement across the electrode itself was balanced out with an impedance bridge before insertion of the electrode into the droplet. The output of the amplifier was led to an oscilloscope (model 5103N; Tektronix Inc., Beaverton, Oreg.) and a chart recorder (model RM-20; Nihon Kohden, Tokyo; or model MC6601; Watanabe Instruments Corp., Tokyo, Japan).

Membrane resistivity was calculated from the voltage displacement produced by injection of rectangular pulses of inward (hyperpolarizing) current, and the surface area of the droplet calculated from the measured diameter, assuming spherical geometry.
Other Methods

For electron microscope examination of plasmodial droplets, droplets were fixed with 2% glutaraldehyde-0.1 M potassium cacodylate buffer (pH 7.4), and then postfixed with 0.1% OsO₄-0.2 M sucrose-0.1 M cacodylate buffer (pH 7.4). After dehydration in a graded series of chilled ethanols, the droplets were embedded in Epon 812 (Shell Chemical Corp., N. Y.). The specimens were sectioned, stained with uranyl acetate and lead citrate, and examined in a JEM-7 electron microscope (JEOL, Tokyo, Japan).

The CI ion concentration in plasmodia was estimated as follows. Whole plasmodia, with no added solution, were centrifuged for 60 min at 100,000 g at 4°C, which broke the plasma membrane and left two fractions: a cytoplasmic gel pellet and a clear supernatant fluid (called the “soluble fraction”), as described previously (Kuroda and Kuroda, 1980). The CI ion concentration in the soluble fraction was electrometrically measured by the method of Mailman and Mullins (1966), and the value obtained was tentatively regarded as the CI ion concentration in plasmodia. The CI ion concentration in tap water was measured by the same method.

RESULTS

Intracellular Ion Concentrations

The cationic concentrations of Physarum plasmodia were estimated from our earlier data on the cationic contents of the soluble fraction of plasmodia.
(Kuroda and Kuroda, 1980) to be as follows (in mM): K, 29.4 ± 3.7 (SD); Na, 1.6 ± 0.6 (SD); Ca, 3.7 ± 1.7 (SD); and Mg, 6.5 ± 2.1 (SD), assuming that the specific gravity of the soluble fraction was unity. The Cl ion concentration in the plasmodia was estimated 27.8 ± 1.8 (SD) mM. For technical reasons, the internal ionic composition of droplets has not yet been measured. However, it is not likely to be much different from that of plasmodia, since the droplets are formed by direct "budding" of the plasmodia.

Intracellular pH was previously reported to be ~6.3 from measurements with antimony microelectrodes (Gerson and Burton, 1977). Although we have been unable to repeat those measurements because of rapid membrane rescaling, the internal pH of plasmodia can be crudely estimated by the color of endogenous pigments. Seifriz and Zetzmann (1935) suggested that the native yellow pigment in slime mold plasmodia might be a good indicator of internal pH. They tabulated the color of extracted pigment against pH, and the resultant chart indicates that both the droplets and the intact plasmodia used in the present experiments must have internal pH's of 6.2–7.0 (light yellow to sulphur color).

Membrane Potentials of Intact Plasmodia

During exposure to tap water, in which the concentrations of all the ions are very low (e.g., [Cl⁻] =0.5 mM), membrane potentials of plasmodia ranged from −90 to −130 mV, interior negative. Therefore, if passive ion diffusion were responsible for the measured membrane potential, efflux of intracellular cations rather than influx of extracellular anions would be implicated. In Physarum plasmodia, as well as in most other cells, K⁺ is the most abundant cation, and seemed the most likely ion to contribute to the resting membrane potential. To test this possibility, membrane potential was measured as a function of varied external K⁺ concentration ([K⁺]₀) (Fig. 2 a). As controls, similar measurements were made with varied external Na⁺ ([Na⁺]₀) (Fig. 2 b). The two salient features of these results from intact plasmodia were (a) that the membrane potential lay far negative to the equilibrium potentials of both K⁺ (E_K) and Na⁺ (E_Na); and (b) that the slopes of membrane potential vs. log [K⁺]₀ or log [Na⁺]₀ were small: ~20 mV/log unit K⁺ and 15 mV/log unit Na⁺. It is evident, therefore, that neither K⁺ diffusion nor Na⁺ diffusion can be responsible for the major fraction of the resting membrane potential in intact plasmodia.

For the sake of completeness, the possible contribution of Ca²⁺ diffusion to the resting membrane potential of plasmodia was also checked. At external Ca²⁺ concentrations of >0.1 mM, Ca had little effect on the membrane potential, although at >5 mM it accelerated the "sealing off" reaction (see Materials and Methods), and made recording unfeasible. Removal of Ca, as by addition of EGTA (ethylene glycol bis [β-aminoethyl ether] N,N',N"-tetraacetic acid), diminished the membrane potential and made it unstable (Fig. 3 a). In general, the effects of Ca in stabilizing and increasing the membrane potential are similar to those observed in Neurospora (Slayman, 1965a).
As noted in Materials and Methods, caffeine induced the formation of plasmodial droplets, but its own effect on the membrane potential had not been previously investigated. Although droplets were continuously formed at the growing ends of plasmodia in 10 mM caffeine, plasmodial branches did not significantly change shape. The membrane potential, therefore, could be recorded from strands before, during, and after the addition of 10 mM caffeine. Occasionally, transient depolarization occurred on addition of caffeine (Fig. 3 b), but the normal steady membrane potential was quickly restored. Apparently the electrical characteristics of the pre-existing membrane are not greatly affected by caffeine.

**Presence of a Membrane on the Droplets**

As shown in Fig. 1 d, stable internal potentials of -80 to -120 mV were recorded beginning a few seconds after the insertion of electrodes into droplets. These stable internal potentials did not change by advancing electrodes deeper. This, along with the smooth-surfaced spherical shape of droplets (Fig. 4), suggested the presence of an enclosing membrane on the droplet surface, and subsequent electron microscope studies indeed confirmed the existence of
a well-formed surface membrane (Fig. 5). The internal potentials recorded by microelectrodes, therefore, should be regarded as the membrane potentials of droplets.

Effects of External Ions on Membrane Potentials in Droplets

The droplet membrane potential was determined as a function of $[K^+]_o$, using Cl⁻ or MeSO₃⁻ as the anion (Fig. 6). Assuming the internal K⁺ concentration ($[K^+]_i$) to be 29 mM, the membrane potential was again found to be more negative than $E_K$ at least for $[K^+]_o$ of >0.5 mM. This finding clearly precludes the possibility that K⁺ diffusion could be mainly responsible for the membrane potential. As with intact plasmodia, the slope of membrane potential vs. log $[K^+]_o$ was much smaller than the Nernst value, for single-ion diffusion, of 59 mV/log unit (at 23°C). The data plotted in Fig. 6 yield least-squares slopes of 9.9 mV/log unit KCl and 4.9 mV/log unit KMMeSO₃. We have not attempted to interpret the implied difference between anions, but the fact that the less permeant anion yields the smaller slope presumably means that anion diffusion per se is not involved. Thus, both the absolute value of membrane potential and the insensitivity of the potential to changes of $[K^+]_o$ imply that K⁺ diffusion does not significantly contribute to the resting membrane poten-
**FIGURE 4.** Light micrograph of a droplet. A microelectrode is seen in the figure. Bar indicates 50 μm.

**FIGURE 5.** Electron micrograph of a cross-section of a droplet. Note the membrane-like structure on the surface of the cytoplasm, and the absence of external fibrils (slime) outside the membrane. Bar indicates 0.5 μm.
tial of Physarum droplets. In this respect the slime mold membranes resemble the true mold Neurospora (Slayman, 1965a) or the charophyte algae Nitella (Kitasato, 1968) more than they do the excitable membranes of animal nerve and muscle cells.

Concerning the other ions tested, extracellular Cl\(^-\), Na\(^+\), Mg\(^{++}\), and Ca\(^{++}\), all had only slight effects on the resting membrane potential of droplets. Fig. 7 shows that the transition from 6 to 30 mM [Cl\(^-\)]\(_o\) (with constant [K\(^+\)]\(_o\)) reversibly hyperpolarized droplets by 7 mV; shifting from 5 to 30 mM [Na\(^+\)]\(_o\) (with changing [K\(^+\)]\(_o\) from 1 to 5 mM) depolarized by 12 mV; and 2 to 10 mM [Mg\(^{++}\)]\(_o\) depolarized by 10 mV. Ca\(^{++}\) was examined more system-

![Figure 6. Effects of external K\(^+\) on the membrane potential of droplets. Media were prepared by mixing 30 KCl-T medium (circles, solid circles, and open circles represent different droplets), or 30 K-T medium (squares), and 0 K-T medium (see Table II). The diagonal straight line represents equilibrium potentials for K\(^+\) (E\(_K\)), assuming [K\(^+\)]\(_i\) = 29 mM. The straight lines: V\(_m\) = 9.9 log [K\(^+\)]\(_o\) - 100.2 for circles, and V\(_m\) = 4.9 log [K\(^+\)]\(_o\) - 109.7 for squares, were fitted by the method of least squares (V\(_m\) = membrane potential).](image-url)

atically (Fig. 8), but it too affected the membrane potential only slightly, depolarizing by 8.0 mV/log unit between 0.1 and 10 mM [Ca\(^{++}\)]\(_o\).

In all of these experiments, the resting membrane potential in standard T medium varied from -80 to -120 mV, which should be compared with the following ionic equilibrium potentials, calculated from the ionic concentrations in intact plasmodia (see Intracellular Ion Concentrations): E\(_{Na}\) = +29 mV; E\(_{Mg}\) = -15 mV; E\(_{Ca}\) = -26 mV; E\(_{Cl}\) = +39 mV. Thus, all single-ion diffusion potentials are more positive than the observed membrane potential, and the slope sensitivity of the resting potential is much less than would be predicted for simple diffusion regimes.

These results indicate that the membrane potential of Physarum droplets cannot be accounted for by simple diffusion of K\(^+\), Na\(^+\), Ca\(^{++}\), Mg\(^{++}\), or Cl\(^-\) ions.
FIGURE 7. Changes of membrane potential with shifts of external Cl⁻ (a), Na⁺ (b), and Mg²⁺ (c). Standard T medium containing 6 mM Cl⁻, 5 mM Na⁺, and 2 mM Mg²⁺ was used in the control period before and after the intervals with 30 mM Cl⁻ (30 Cl⁻-T medium) (a), 30 mM Na⁺ (30 Na⁺-T medium) (b), or 10 mM Mg²⁺ (10 Mg²⁺-T medium) (c).

FIGURE 8. Effect of external Ca²⁺ on the membrane potential. Media were prepared by mixing 0 Ca⁻-T medium and 10 Ca⁻-T medium. At 0 mM [Ca⁺⁺]₀, 1 mM EGTA was added to 0 Ca⁻-T medium. Note that removal of Ca⁺⁺ diminishes the membrane potential, just as with intact plasmodia (Fig. 3). The diagonal straight line represents the equilibrium potentials for Ca⁺⁺ ($E_{Ca}$), assuming [Ca⁺⁺]₀ = 4 mM. Different symbols represent different droplets. The straight line of $V_m = 8.0 \log [Ca^{++}]_0 - 90.0$ was obtained by the method of least squares.
Proton Sensitivity of the Membrane Potential

External hydrogen ions, by contrast, had a remarkable effect on the membrane potential (Fig. 9). As the pH of the external medium (pH₀) decreased, the membrane potential became sharply less negative, so that between pH 6.5 and 5.5, the curve of the membrane potential vs. pH₀ fitted the theoretical Nernst slope of 59 mV/log unit. To determine whether this sensitivity might reflect a pH-dependent shift of permeability for other ions, membrane potential and membrane resistance were determined simultaneously during stepwise changes of the pH₀ (Fig. 10). Despite large changes in membrane potential,

the membrane resistance was essentially constant between pH 6.6 and 5.6. It is likely, therefore, that the pH sensitivity of membrane potential in Physarum droplets does not result from changes of ionic permeability. Although the steep slope of membrane potential vs. pH₀ might suggest that passive diffusion of protons produces the resting membrane potential, there is still a significant discrepancy between the actual membrane potential (~80 to −120 mV in standard T medium) and the equilibrium potential for protons (~−47 mV ≤ E₉ ≤ 0 mV in standard T medium, assuming 6.2 ≤ pHᵢ ≤ 7.0). An internal pH of ~5 would be required to give a diffusion potential of −120 mV, but such a low pHᵢ should be easily detected by a dark yellow color of the intrinsic
dye (Seifriz and Zetzmann, 1935). It is therefore necessary to seek a mechanism to explain both the level and the slope of membrane potential as a function of pHo.

The circumstance in Physarum droplets is very similar to that observed for Nitella (Kitasato, 1968; Spanswick, 1972), which suggests that an electrogenic proton pump may be involved.

**Effects of Metabolic Inhibitors**

The existence of electrogenic H\(^+\) pumps has also been postulated in several kinds of plant cells, whose membrane potentials are sensitive to the state of metabolism (Slayman, 1965b and 1974) and/or to the intracellular ATP concentration (Slayman et al., 1973; Shimmen and Tazawa, 1977; Keifer and Spanswick, 1979; Kishimoto et al., 1980).

We therefore examined the effect of some metabolic inhibitors on the membrane potential of Physarum droplets. As shown in Fig. 11, cyanide had a significant effect on the membrane potential. Low concentration of KCN (1 mM) induced a small depolarization (Fig. 11a, lower curve). The specific membrane resistance (upper curve) was found to be nearly constant during the time required for depolarization, and then a reversible increase of membrane resistivity was observed after the shift of membrane potential. A higher

![Figure 10. Simultaneous recording of the membrane potential and resistance, with the changes of pHo. At the arrows, pHo was changed as indicated in the figure. Note that the membrane resistance (squares) changes only slightly despite the large change in membrane potential (circles).](image-url)
concentration of KCN (10 mM) brought about a quick and large depolarization from $-90$ mV to $-20$ mV (Fig. 11 b), indicating that the contribution of metabolism to the membrane potential might be estimated at $\sim 70$ mV. The total membrane resistance, indicated in Fig. 11 b by the voltage displacement, however, was revealed to increase remarkably as depolarization progressed. As KCN was washed away, both membrane potential and total membrane resistance returned to about the original levels.
This result implies that the major portion of membrane potential of Physarum droplets is generated through an energy-consuming system, which is similar to that observed in Neurospora (Slayman, 1965b and 1974; Slayman et al., 1973). The membrane resistance will be discussed later.

Among other inhibitors tested, 1 mM 2,4-dinitrophenol (DNP) caused a small transient depolarization, and 5 mM NaF had no effect. 1 mM iodoacetate had a small depolarization effect, but also produced an irreversible rise in membrane resistance.

Effects of Low Temperature

Furthermore, to examine the relation between the membrane potential and metabolism in droplets, the effect of low temperature was tested (Fig. 12). Temperature down-shift from 25 to 12°C resulted in depolarization and increase of the membrane resistance without any time delay. Upon rewarming, both membrane potential and membrane resistance recovered coincidently with the temperature change.

The effect of temperature on membrane potential in Physarum droplets, taken together with the effects of the external pH and cyanide, suggests that an electrogenic H⁺ pump contributes the major portion of the resting membrane potential.

DISCUSSION

Evidence for an electrogenic proton pump as the primary origin of the resting membrane potential in Physarum droplets comes from four groups of results:

(1) None of the common inorganic cations and anions can support the normal resting membrane potential by simple diffusion, because that potential is relatively insensitive to variations of the external concentrations of these ions (K⁺, Na⁺, Ca²⁺, Mg²⁺, and Cl⁻; see Figs. 6-8), and because the normal membrane potential (−80 to −120 mV) is far negative to the equilibrium potentials for all the ions.

(2) The fact that the droplet membrane is polarized negative inside, coupled with the indications for an electrogenic pump, which are provided by ionic (Figs. 6-10) and metabolic (Figs. 11 and 12) experiments, implies that either a cation must be pumped outward or an anion must be pumped inward. But it is easy to find conditions in which K⁺, Na⁺, and Mg²⁺ are maintained at

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**Figure 11 (opposite).** Effect of KCN on the membrane potential and resistance. (a) 1 mM KCN; a small membrane depolarization (circles) was followed by the delayed increase of specific membrane resistance (squares). (b) 10 mM KCN: a large and quick depolarization, and a nearly simultaneous increase of membrane resistance were brought about. Voltage displacements imposed on the curve of membrane potential (upper figure) were produced by injecting rectangular pulses of hyperpolarizing current (duration of 400 ms) (lower figure). The amount of displacement is proportional to the total membrane resistance of the droplet. Specific membrane resistances before KCN application, at the maximum depolarization, and after extensive washing are 7.4, 100, and 6.2 kΩ-cm², respectively.
higher concentrations in the cytoplasm than in the external solution, which indicates that they are not actively pumped out. The membrane potential of \(-95\ \text{mV}\) is sustained in a \(\text{Cl}^-\)-free solution (data not shown), so that inward pumping of \(\text{Cl}^-\) should also be negligible.

(3) Variations of external \(\text{pH}\) have a strong effect on the membrane potential of droplets. Especially between \(\text{pH} 6.5\) and 5.5, the curve of the membrane potential vs. \(\text{pH}_o\) fits the theoretical Nernst slope, but the normal internal \(\text{pH}\) (probably 6.2-7.0) appears too high to give a membrane potential of \(-80\) to \(-120\ \text{mV}\) only by passive \(\text{H}^+\) diffusion. Studies with internally perfused, tonoplast-free \textit{Chara} cells have indicated that the \(\text{H}^+\) pump itself is

**Figure 12.** Effect of low temperature on the membrane potential and resistance. (a) Changes of the membrane potential (\textit{upper figure}) with change of temperature of the perfusing medium (\textit{lower figure}). At the arrow, the electrode was inserted into the droplet. The voltage displacements from the membrane potential were produced by the same current pulses (\textit{middle figure}) as Fig. 11 \(b\), and they are proportional to the resistance. Note that the time-course of the change in membrane potential is the same as that of the change in temperature. (b) Changes of the resistance of the electrode itself (\textit{upper figure}) with change of temperature (\textit{lower figure}) after recording (a). Since the resistance of electrode itself increases with decreasing temperature, it should be subtracted from the resistance recorded in (a) in order to calculate the membrane resistance of the droplets. The specific membrane resistances, thus, are 7.9 \(\text{k}\Omega\cdot\text{cm}^2\) at 25°C, and 22.1 \(\text{k}\Omega\cdot\text{cm}^2\) at 12°C.
sensitive to external pH (Kawamura et al., 1980). On the other hand, Kitasato (1968) has suggested the possibility that the passive $H^+$ influx is affected by external pH, whereas the activity of $H^+$ pump remains constant. These possibilities are now being examined in Physarum droplets. Thus the quantitative importance of diffusion vs. electrogenic pumping has not been settled, but pH sensitivity of the membrane potential indicates that either $H^+$ diffusion or electrogenic pumping is paramount.

(4) It is, finally, the effect of metabolic inhibitors that argues most clearly for the existence of an electrogenic pump. The membrane potentials of droplets were sharply reduced by cyanide (Fig. 11), and diminished in proportion to decreases of the ambient temperature (Fig. 12). Here we should comment on the increase of membrane resistance by KCN and lowered temperature. In various kinds of cells that possess the electrogenic pump, for example, Neurospora (Slayman, 1965b) and Chara (Keifer and Spanswick, 1979; Kishimoto et al., 1980), the depolarization of membranes is accompanied by an increase of membrane resistance (in other words, the decrease of membrane conductance). Two alternative explanations are possible for this phenomenon. One is that the pump has a large conductance under normal conditions, and the resistance increase is integral to any mechanism for pump inhibition (Keifer and Spanswick, 1979; Kishimoto et al., 1980). The other one is that the metabolic inhibition evokes separate, secondary increase of resistance in the passive ionic pathways through the membrane. In Physarum droplets, both a high concentration of KCN (10 mM) (Fig. 11 b) and a lowered temperature (12°C) (Fig. 12) bring about nearly simultaneous depolarization and increased resistance of the membrane. These results support the former explanation. However, when a low concentration of KCN (1 mM) is applied (Fig. 11 a), the membrane resistance is nearly constant during the time required for membrane depolarization. Moreover, although Keifer and Spanswick (1979) have shown membrane conductance to be a function of membrane potential in Chara cells, the results of Figs. 11 b and 12, recorded from the same droplet, are at variance. These facts may be taken as evidence against the notion that the pump has a large conductance. Thus, we cannot yet firmly attribute the resistance changes, which accompany “metabolic” depolarization, to primary rather than secondary events.

An important next step in the study of electrogenic ion pumping in Physarum droplets should be to determine whether the putative $H^+$ pump is fueled by ATP. We have not yet measured the ATP concentrations of droplets, because of difficulties in preparing large quantities of droplets, but instead have measured ATP in microplasmodia, which were grown in liquid medium. The results, to be published in detail elsewhere, show that ATP decreases drastically in plasmodia treated with 10 mM KCN. The decrease is well correlated with membrane depolarization, confirming the existence of an electrogenic pump in Physarum plasmodia. We conclude, therefore, that the same system is present in both droplets and plasmodia.

We undertook the present experiments as a first step to elucidate the mechanism for generating rhythmic fluctuations of membrane potentials in intact plasmodia (Watanabe et al., 1937; Kamiya and Abe, 1950; Rhea,
That fluctuation is expected to be closely related to shuttle streaming of the protoplasm. Since the results strongly suggest that an electrogenic H⁺ pump generates the major component of the resting membrane potential, it is reasonable to ask whether rhythmical changes of the electrogenic pump may cause the rhythmical variations of membrane potential in Physarum plasmodium. Since the H⁺ pump of Physarum appears to be an ATPase, like those of Neurospora (Slayman et al., 1973; Kuroda et al., 1980) and Chara (Shimmen and Tazawa, 1977), it is of great interest that the concentrations of possible regulatory factors for ATPases, e.g., ATP (Sakai, 1977) and free Ca²⁺ ion (Ridgway and Durham, 1976), also change rhythmically in Physarum plasmodia.

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