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Electric inhomogeneity in membranes of Characean internode influenced by light/dark transition, O_2, N_2, CO_2-free air and extracellular pH

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The electric membrane potential as functions of position and time of Characean internode has been studied using a modified water-film electrode technique. Between the low-conductance hyperpolarized region (called the H-region or acidic region) and the high-conductance depolarized region (D-region), there is a difference in the direction of responses to light-off and -on stimulations. In darkness the membrane potential becomes hyperpolarized in the D-region, whereas it is depolarized in the H-region at the steady state. The potential difference between D- and H-regions, ΔV_m, is increased by exposure to pure O_2, N_2, or CO_2-free air. When the amount of water surrounding the internode is limited, the formation of an electric pattern occurs rapidly. In contrast, the recovery is delayed. The membrane potential of the D-region is sometimes hyperpolarized significantly with lowering of the extracellular pH to 7.5, while the potential of the H-region is slightly depolarized. This seems to be an all-or-none type response. However, the electric profile is always homogenized with the pH of 6.8. Thus, the pH around 7.5 may be a threshold level to open/close putative OH^- (or H^+) channels of the D-region.

1. Introduction

In 1828, Bischoff already demonstrated that many kinds of aquatic plants including *Chara* developed $CaCO_3$ deposits. In 1897 and 1908 Migura showed that calcifications of Charophyte cells depended upon Ca^{2+} concentration, photosynthesis, light intensity and developmental state, as cited by Smith [1]. The calcifications are generally not uniform along the surface of a plant. An important key to clarifying the mechanism of this inhomogeneity was provided by Arens [2] by showing that the region for the dissolved inorganic carbon (DIC) uptake was spatially separated from that for OH^- extrusion. Subsequently, it was found that the alkalinization occurred even in the absence of Ca^{2+} and HCO_3^- [3].

Brown [4], on the other hand, developed an apparatus for measuring the electric polarity of the central axis of *C. vulgaris*; he found that the apex was more electropositive than the basal regions, and that the polarity was increased by red and blue illumination and by increasing the CO_2 concentration. From his data the electric potential distribution even in a single internode seemed to be inhomogenized. It was suggested in 1968 that such electric inhomogeneity of Characean internodes might be related to the active extrusion of protons [5]. This proposal was also supported by Spear et al. [6]. They suggested that light-induced Cl^- influx took place mainly in acidic regions and that coupled movement of Cl^- and H^+ influx might occur in the alkaline region. This alternat-

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ing configuration of acid and alkaline regions covers the whole circumference of the internode in a banding fashion in most cases.

In the present paper, we describe results of experiments using a modified water-film electrode technique [7,8]. The extracellular pH is shown to affect the formation of electric inhomogeneity. Experimental results on the kinetics also indicate that the electric inhomogeneity is a typical nonlinear phenomenon appearing far from equilibrium because the speed of D-region formation is delayed when the amount of surrounding water is large. It is pointed out that the electrogenic H^+ pumps could be one of the most important electric current sources, and that the assumption for the existence of putative electrogenic OH^- pumps may not be necessary to elucidate D-region formation.

2. Materials and methods

C. corallina used in this experiment was grown in a soil-extract culture medium containing 1 mM nitrilotriacetic acid (NTA), which filled a cylindrical container (90 cm depth, 18 cm diameter) at room temperature with a photoperiod of 12 h light (20 W/m^2 at the surface of the culture medium)/12 h dark.

Experiments were performed on two mature internodal cells in series, cut from the main axis of the vegetative shoot at least 24 h prior to use in each experiment. During this period the materials were kept in artificial pond water (APW) containing 0.1 mM K_2SO_4 and $CaCl_2$ with or without 1 mM $NaHCO_3$. The pH was adjusted by using 2 mM Hepes NaOH at pH 6.8, 7.5 and 8.2.

The membrane potential distribution along the length of the internode was measured using the water-film electrode originally developed by Ogata [7]; here a modified technique was applied [9]. The experimental setup is shown in fig. 1. In brief, two internodal cells, in series (one for the test and the other for the reference), were held vertically within a moist chamber so that the lower portion of the test cell could dip into APW (containing 0.01% Tween 80). Most of the end region of the upper cell was sealed into a silicone rubber cup which acted as the reservoir for APW for the reference cell. Before the experiment, the APW in this reservoir was gently replaced with 20 mM K_2SO_4-APW. It opened the K^+ channels in the plasma lemma of the reference cell, whose electric membrane potential and conductance were both almost insensitive to extracellular stimulations such as light and a physiological extent of change in the ionic strength. The potential difference between the upper reservoir and the water film could reflect the potential change of the membrane attached to the water film. Electrical contact with the external surface of the test cell was achieved by using an agar-encased (3%, w/v, in APW) ring-shaped Ag/AgCl wire, i.e., a loop; withdrawing the loop from the lower APW reservoir produced a thin film of solution that formed a bridge from the cell to the agar surface of the loop.

A constant bipolar sinusoidal current (5.0×10^{-9}A, 1.0 Hz) was applied via a 100 MΩ resistor between the lower APW reservoir and the loop electrode; this allowed the measurement of membrane conductance as a function of position along the length of the test cell. The specific resistance across the node between the cell sap of the test and the reference is 50-times smaller than that of the internodal cell plasmalemma [10]. Potential measurements were made between two micropipette electrodes (100 mM KCl in 3% agar) with one being positioned in the agar part of the loop and the other in the APW of the upper reservoir as the reference electrode. It should be noted that the imaginary component in the impedance locus could be neglected because of the low frequency of the sinusoidal current. Under the present ionic conditions, the series resistance R_s was estimated to be less than 10% of the total resistance, R_t, expected in the D-region. To estimate the apparent specific membrane resistance, the effective area of the membrane attached to the water film was assumed to be 0.06 cm^2.

The composition of the gas phase was manipulated via an inlet pipette inserted into the moist chamber. The gas (air, O_2, N_2 or CO_2-free air) was humidified before the inlet.

The influence of extracellular pH on the electric inhomogeneity along the test cell was investigated in O_2, CO_2-free air or N_2 because of the following reasons: the 0.03% of CO_2 in the air

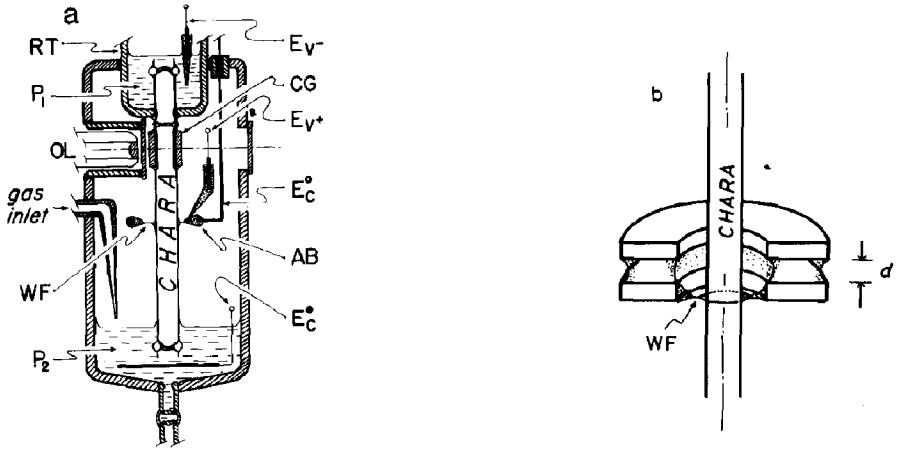

Fig. 1. Apparatus for measuring the distribution of electric potential and resistance along the length of a Characean internode (a) and the two disc plates for changing the amount of extracellular water (b). RT, silicone rubber teat; E_{V^-}, minipipette, agar-salt bridge/Ag/AgCl electrode for the reference; P_1, upper pool of artificial pond water containing 20 mM K_2SO_4; E_{V^+}, minipipette, agar-salt bridge/Ag/AgCl electrode attached to the agar part of the water-film electrode for the potential measurement; OL, objective lens of the microscope for checking the cytoplasmic streaming velocity; CG, pair of cover-glasses to obtain a better image of the cytoplasmic streaming; E_C°, ring-shaped Ag/AgCl electrode for one of the current electrodes; AB, agar part of the ring-shaped electrode; WF, water film with the same ionic composition as the lower pool P_2; $E_C^\bullet$, the other current electrode. The bipolar sinusoidal electric current was given between two current electrodes E_Cs. The operation of this apparatus in detail has been described [7,9].

lowered the pH from the given level significantly in the present buffering capacity. More than 10 mM of the pH buffer as Hepes should be required in order to maintain the given pH level/within 0.1 unit of pH drift. This amount of buffer, however, very often showed an unfavorable effect on the stability of the membrane potential especially for such a long-term study as the present experiment.

The pH of the lower APW reservoir was adjusted to 6.8, 7.5 or 8.2. Since the agar-encased loop was withdrawn for this reservoir, the extracellular pH surrounding the internode could be changed to the same value as in the reservoir.

When the effect of the water volume of the extracellular aqueous phase was studied in the kinetics of formation of electric inhomogeneity, the loop of agar-encased Ag/AgCl wire was replaced by a pair of parallel discs, made from 1 mm thick methacrylate resin plate. Each disc had a hole of 5 mm diameter. Between these two discs, APW was retained and the water film was formed in the hole of the lower disc (fig. 1b). The quantity of APW was controlled by carefully changing the clearance d between two discs. We adopted 30, 100 and 300 μl as the quantity of APW touching the internode.

The light source was an ordinary slide projector equipped with a tungsten lamp. The heat effect was minimized by a water filter of 10 cm length. Experiments were carried out at 22 ± 1°C.

3. Results

3.1. Membrane potential profiles along the length of the internode influenced by light / dark transition

The experiment was started with dark-adapted material. As demonstrated in fig. 2 showing the light-on transition, the magnitude of the membrane potential increased almost uniformly along the entire length of the test cell within several minutes prior to the growth of the D-regions. At several positions corresponding to D-regions, the membrane potential started to be depolarized by several tens of millivolts without inhomogeneity in

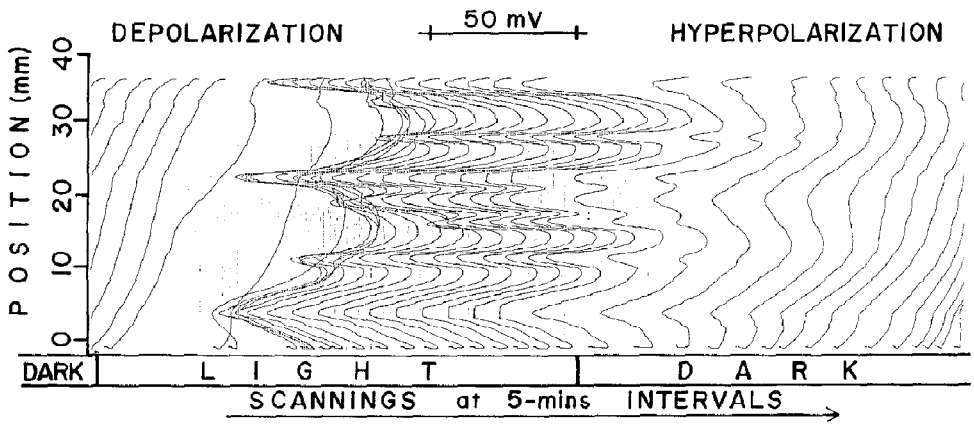

Fig. 2. Electric potential distribution along the length of the internode affected by the light/dark transition. The membrane potential along the length of the cell was almost uniformly hyperpolarized prior to the growth of several D-regions. In the present internode there was a membrane potential gradient, i.e., the upper region was depolarized more than lower. This kind of monotonic gradient was sometimes observed.

the membrane resistance along the cell: only the electric membrane potential along the cell turned out to be inhomogeneous (cf. fig. 6 of ref. 7). In this case, more efficient carbon fixation could possibly occur and then the cytoplasmic (ectoplasmic) level of OH^- around the D-regions would increase more than the neighboring H-regions. Therefore, the electromotive force for OH^- across the membrane increases in these regions. This would be one of the causes of the depolarization without any inhomogeneity in R_m. Sometimes, however, a localized increase in R_m was observed accompanied by depolarization. This may be considered to reflect the decrease in H^+-pump activity in that region.

After this period, remarkable depolarizations occurred and then several stable D-regions were established at -100 to -150 mV. In this case, as shown in fig. 3a, the depolarizations were associated with the decrease in membrane resistance. This membrane potential in the D-regions was almost at the same level as that estimated for the passive diffusion potential by the use of DCCD (dicyclohexylcarbodiimide) [11]; DCCD was assumed to be a specific inhibitor of the putative electrogenic proton pump. It is obvious, however, that the characteristics of the membrane differ completely because the apparent membrane resistance of the former was 10-times (or more) smaller than that of latter. Probably, the membrane of D-regions is permeable to OH^- (or H^+), as mentioned [12].

Each D-region did not begin to grow at the same time, depending on the position of the cell (fig. 2). Note that several small D-regions sometimes disappeared although the light intensity was constant, as can be seen from the behavior around 33 mm in fig. 2.

In the case of the light-off transition, as shown in fig. 3a, the potential of the D-region was hyperpolarized to almost the same level as that of the H-region within several tens of minutes. However, the time dependency of this initial hyperpolarization was not a single exponential and varied according to the position and depending on the cell itself for some unknown reason. In this time dependency, there seemed to be at least two time constants, i.e., a fast component $\tau_f \downarrow$ of several minutes and a slow component $\tau_s \downarrow$ of several tens of minutes. In this particular period, the membrane resistance of the D-region increased in a single-exponential fashion more rapidly than that of the H-region where the increase in membrane resistance was rather sigmoidal (fig. 3a and b). It may be possible that the activity of the putative H^+ pump is inhibited because of the intracellular pH being higher than 7.6. Actually, it has been reported that the ATP-dependent membrane

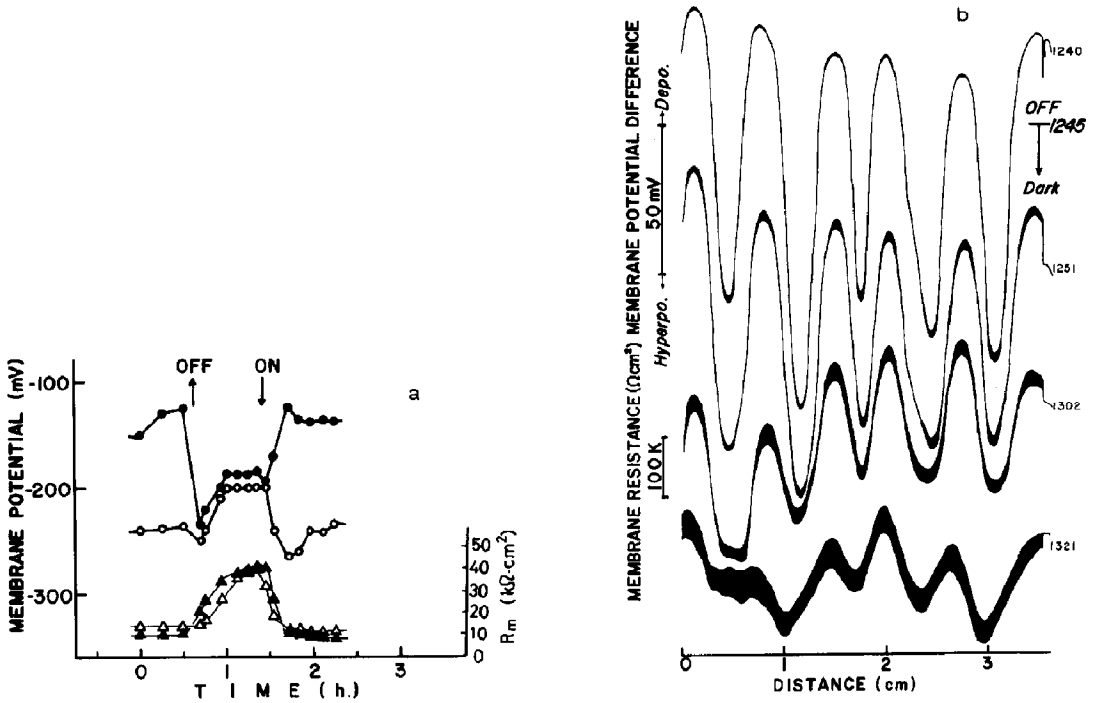

Fig. 3. Membrane potential and resistance of D- (● and ▲) and H- (○ and △) regions associated with the light off/on transition (a) and the typical profiles along the internode after the dark at 12:45 o'clock (b). (a) Membrane potentials of the D- and H-regions were hyperpolarized and depolarized by the light-off transition at the final steady states, respectively. Their potentials were depolarized and hyperpolarized respectively by the light-on transition. The time dependences of the membrane resistance R_m for the D- and H-regions differed from each other. Note that the R_m values seemed to change independently with the respective potential V_m values. (b) Width of each trace corresponds to the magnitude of the resistance. As soon as the light was switched off, the membrane resistance of the D-region increased more than that of the neighboring H-regions. In the profile obtained at 13:21, the membrane potential in the H-region at 5 mm from the left already reached a dark-adapted steady state.

potential decreased significantly when the cell was perfused intracellularly at pH higher than about significantly 7.6 [13]. On the other hand, the potential of the H-region was depolarized after a small hyperpolarization during this period. Thereafter, i.e., about 30 min after switching off, the potentials in both regions were gradually depolarized with a monotonic increase in membrane resistance up to 100 kΩ cm^2. In the dark-adapted steady state, the membrane-resistance profile along the length of the cell was almost uniform, when no calcification was observed on the surface of the material [7].

In the case of the light-on transition, the potential of the D-region was depolarized after a transient hyperpolarization. The transient hyperpolarization was dependent on both the position and the material itself. The potential of the H-region was hyperpolarized by about 75 mV and was then depolarized to the steady-state level at about −240 mV in the present material. Note that tin the H-region the difference in membrane potentials between the light and darkness was almost negligible at their steady-state level, while it sometimes depended upon the material. The slow component $\tau_s\uparrow$ was generally larger than that in an aqueous solution, as reported by Lucas [14]. The membrane potential in the H-region measured in the moist chamber was smaller than that measured in the aqueous solution (data not shown). It may be possible that H^+ pumped out of the cell acidifies the limited amount of solution on the surface of

the plasma lemma, which might inhibit the activity of the H^+ pump [15] more than in the cell in a solution.

The response of the membrane potential to the light-on/off stimulation seems to differ from the results on perfused and tonoplast-free model experiments obtained by Fujii et al. [16]. Their experiments have demonstrated monotonic depolarization and hyperpolarization. This difference might be derived from the fact that the materials in the present experiment were intact with tonoplasts. Furthermore, the cell was suspended in the moist chamber and was exposed to CO_2-free conditions, so that the cable problems related to the amount of water should be minimized and the extracellular pH drift caused by 0.03% CO_2 in air should not be expected [9].

3.2. Effects of gas composition (air, O_2, N_2 or CO_2-free air) and of DCMU on the electric membrane characteristics

The experiment was started in air under a constant illumination. Fig. 4 shows that pure O_2 increased the membrane potential difference between the D- and H-regions. Under this condition, 5×10^{-6} mol DCMU added to the lower reservoir decreased the membrane potential difference almost to zero. During this period the membrane potential of the H-region remained almost constant. The specific resistance of the D-region increased twice as much as that of the H-region reversibly with this drug. This concentration (5×10^{-6} mol) of DCMU inhibited the photoresponse with no significant effect on the membrane potential and conductance of the dark-adapted cell within at least several tens of minutes. This drug of 10-times more concentration is generally required to induce a depolarization and to decrease the specific membrane resistance in the dark.

When O_2 was replaced by air, the membrane potential difference between the D- and H-regions decreased, but only the specific resistance in the D-region increased significantly. Note that the behavior of specific resistance differs from the initial case (air) performed at the start of the experiment: when the cell was exposed to air for a long period, the profiles both in membrane potential and resistance along the cell tended to recover gradually to the original level, as reported elsewhere [7,8]. The air to O_2 exchange restored the potential and the resistance to their original levels within several minutes. The O_2 to N_2 ex-

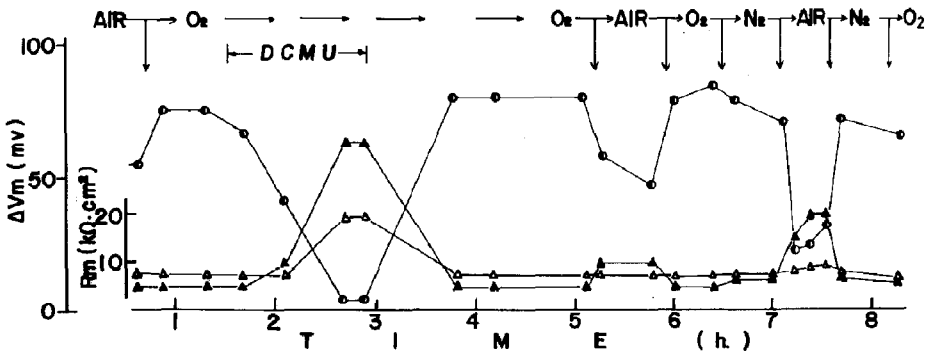

Fig. 4. Membrane potential difference ΔV_m between the D- and H-regions and specific members resistance R_m of each region affected by air, O_2, O_2 + DCMU and N_2. Symbols as in fig. 3. A pair of air-adapted cells was used. In the air-adapted cell, the membrane resistance of the H-region was generally larger than that of the D-region [7,8]. The magnitude of ΔV_m increased slightly by the air to O_2 exchange, and decreased significantly, to almost 0 mV on treatment with 5×10^{-6} M DCMU. The R_m of the D-region increased very much more than that of the H-region reversibly with this drug. The membrane potential of the D-region was hyperpolarized with this drug, whereas that of the H-region remained almost constant. Both the O_2 to air and N_2 to air exchanges induced a large decrease in membrane potential differences reversibly; this was caused mainly by hyperpolarization of the D-region. The effect of O_2 to N_2 exchange was not significant on ΔV_m but was so on R_m of the D-region, since R_m increased 1.5–2-times as much as in O_2.

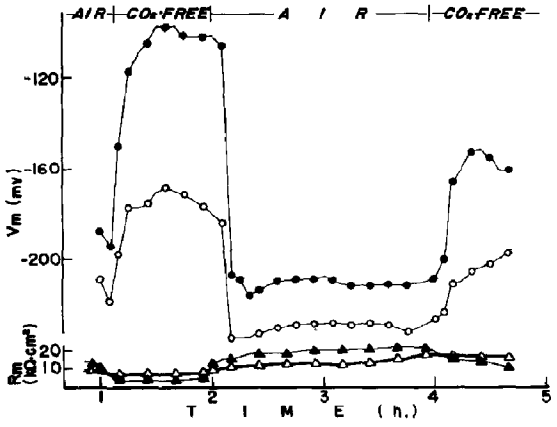

Fig. 5. Time dependence of membrane potentials V_m of the D- and H-regions and of specific membrane resistances R_m. Symbols as in fig. 3. These data were traced from a long-term experiment in which the cell was alternately exposed to air/CO_2-free air. The membrane potentials of the D- and H-regions were both hyperpolarized by the CO_2-free air exchange. However, the potential difference ΔV_m decreased remarkably. This cannot be explained by the acidification due to CO_2 in the air.

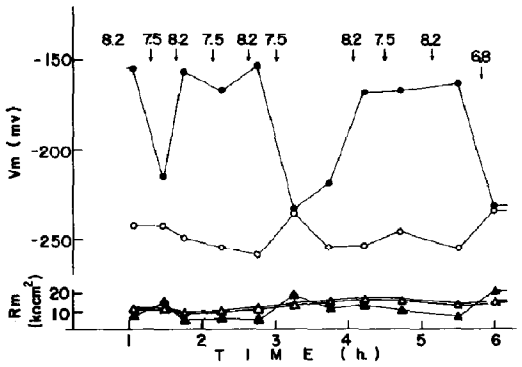

Fig. 6. Effects of extracellular pH on V_m and R_m values of both D- and H-regions. At pH 8.2, the membrane potential of the D-region was almost stable at the depolarized level whereas at pH 7.5, it showed two steady states: one was near the original depolarized level and the other around the level of the H-region which was slightly depolarized in this case. This slight depolarization of the H-region might be derived from the increase in OH^- concentration in the cytoplasm caused by shut-off OH^- (or H^+) channels. The small increase in OH^- might make the membrane potential depolarized, but maintain the membrane resistance remaining at almost a constant level. At pH 6.8, the membrane potential of the D-region was always hyperpolarized to the same level of the H-region. This experiment was carried out under CO_2-free conditions.

change, however, decreased the membrane potential difference little and increased the specific resistance of the D-region by about 30%. The N_2 to air exchange induced a significant decrease in the membrane potential difference from 70 to about 10 mV, and again only the specific resistance of the D-region increased remarkably during this period.

The air to CO_2-free air exchange decreased the membrane potential of the D-region more than that of the H-region. The specific resistance of the D-region decreased significantly but that of the H-region remained almost constant (fig. 5). By the CO_2-free air to air exchange, the membrane potentials of the D- and H-regions were hyperpolarized from -110 to about -210 mV and from -175 to -240 mV, respectively. The specific resistance of the D-region increased from 5 to 20 kΩ cm^2, whereas that of the H-region remained almost constant in this exchange. These tendencies of the membrane potential and the specific resistance are in accord with those observed in the O_2 to air and N_2 to air exchanges.

The decrease in membrane potential difference between the D- and H-regions with the exchange from O_2, N_2 or CO_2-free air to air, shown in figs. 4 and 5, cannot be explained by the acidification with 0.03% CO_2. This is because the response of the membrane potential of the H-region was opposite from the results of the pH experiment following (see fig. 6). This inhibition by air was more significant when the cell was exposed to a CO_2-free environment, i.e., in N_2, O_2 or CO_2-free air.

3.3. Effects of extracellular pH on the D-region (alkaline region) and H-region (acidic region)

As mentioned in section 2, this study was carried out under constant light intensity (20 W/m^2) with pure O_2. Using a pair of light-adapted internodes at pH 8.2, the membrane potentials of the D- and H-regions amounted to -150 and -250 mV, respectively. Generally at pH 8.2, the large potential difference continued for a long period, at least, for several hours. The extracellular pH was adjusted to pH 8.2, 7.5 or 6.8 with 2 mM

Hepes-NaOH. Each solution was saturated with pure O_2 prior to use.

As shown in fig. 6, in the D-region, the first and third treatments at low pH (pH 7.5) induced remarkable hyperpolarizations, whereas the effects were not significant in the case of the second and fourth treatments. It is not yet clear why the membrane potential in the D-regions behaves in such a manner of the all-or-none type. It may be possible, however, that there is a threshold level of pH to open/close the putative OH^- channels and that the level drifts for some unknown reason; when the extracellular pH is 7.5, the intracellular value is around the threshold level and may fluctuate. As another reason, it may be worth considering that the internode shows a kind of hysteresis of the change in intracellular pH. In the H-region, the membrane potential was depolarized with this low pH, but was rather stable to the change in extracellular pH.

Using a pH value of 6.8, however, always negated the electric potential difference between the D- and H-regions, as exemplified in fig. 6. Whenever the membrane potential of the D-region was hyperpolarized, the specific resistance of this region increased significantly, i.e., from a value less than 5 to 15–20 kΩ cm^2 (cf. 70–100 kΩ cm^2 in darkness; fig. 3b). It seems, therefore, that the pH of the extracellular bulk solution which is lower than a certain threshold level around 7.5 in this case can shut off the OH^- (or H^+) channels.

Lowering the extracellular pH might decrease the cytoplasmic pH below the threshold level to close the putative channels; the rising extracellular pH might increase the cytoplasmic pH to a value higher than this threshold level. Once the channels are opened, a large amount of OH^- extrusion (or H^+ influx) might occur according to the electrochemical gradient across the plasmalemma. The cytoplasmic pH is shifted from 7.3 to 7.8, when the cell is exposed to the light [17,18]. It was reported that 1 unit change in extracellular pH corresponded to about 0.2 unit change in cytoplasmic pH [19]. Furthermore, the plasmalemma exposed to an extremely high pH (higher than 10.5) behaves as a pH electrode [12], showing high permeability to OH^- (or H^+). Therefore, the alkalinity of the extracellular solution around the D-region might increase self-catalytically: it may be possible that the increased OH^- level in the cytoplasm caused by light or high extracellular pH opens the OH^- channel from inside the plasmalemma.

3.4. Effect of the amount of water phase on kinetics

Fig. 7 shows two examples of light-on/off kinetics of the D-region: one concerns 30 μl water phase, the other 300 μl. We can see that the D-region is formed faster in the small amount of water phase, but the recovery to the homogeneous state is slower in this case. This tendency surely agrees with the theoretical result [20].

We define the relaxation times $\tau\uparrow$ and $\tau\downarrow$ as those required for the change of $(1 - e^1)$-times the total change in membrane potential in the D-region according to the on and off transitions, respectively. These correspond to the fast components of relaxation. The dependence of these quantities on the amount of water phase is summarized in fig. 8. Such a dependence is due to the circulating electric current established around the internode; the amount of water directly affects the spatial pattern of electric current.

The time course of the membrane potential in fig. 7 shows a rather periodic variation. Several electric membrane a potential oscillations, including this kind of oscillation, have been analyzed precisely by Hansen [21]. The oscillation suggests a participant role of a positive-feedback origin in the formation of acid/alkali bands. Since a similar oscillation can be observed in the electric spatial pattern emerging in multicellular systems such as bean roots [22], the oscillation can be consid-

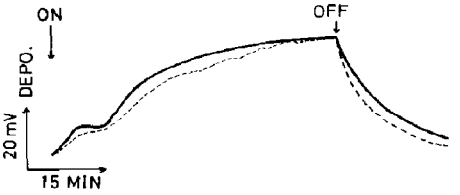

Fig. 7. Relaxation of the membrane potential of the D-region according to the light-on/off transitions. Amount of extracellular water: 30 μl (———), 300 μl (- - - - -). The experiment was performed under CO_2-free conditions.

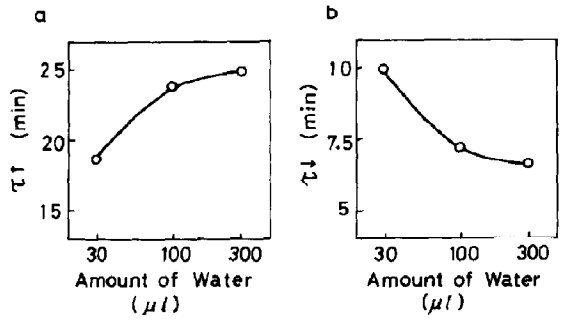

Fig. 8. Dependence of relaxation times of on ($\tau\uparrow$) and off ($\tau\downarrow$) transitions on the amount of water. The data concern the fast component of the kinetic process. This result may be compatible with that of a theoretical analysis of the nonequilibrium band model [20].

ered as being accompanied by the formation of electric homogeneity.

The response of the membrane potential in the D-region to the light-on/off stimulation (fig. 7) did not show the transient hyperpolarization, and differs from the case in fig. 3. This may be due to the presence of ample water phase in this case compared with the original water-film electrode experiment. In fact, the theory [20] predicts an anomalous behavior of H^+ accumulation along the cell surface when the amount of water is very small.

4. Discussion

With the aid of an extracellular microelectrode, Walker and Smith [23] found that large currents circulated between the acid and alkaline regions of Characean internodes in the light; the local current densities were about 7.5 $\mu A/cm^2$. With the Jaffe-Nuccitelli vibrating probe [24], the inward current was estimated as being between 27 and 80 $\mu A/cm^2$ and the outward current in the range 14–40 $\mu A/cm^2$, equivalent to an OH^- efflux of 270–800 $pmol/cm^2$ per s and to HCO_3^- influx of 140–400 $pmol/cm^2$ per s, respectively [25]. In the present technique, on the other hand, the peak inward current density was about 0.5 $\mu A/cm^2$ (corresponding to efflux of OH^- or influx of H^+ of 5.1 $pmol/cm^2$ per s) and the peak outward current was about 0.7 $\mu A/cm^2$ (H^+ efflux or HCO_3^- influx of 7.3 $pmol/cm^2$ per s). It should be noted that the cell was suspended in a moist chamber and therefore the shunt conductance/diffusive pathway external to the plasmalemma was much smaller than in the former two reports [23,25], in which the cells were submerged in APW.

It is obvious from those experiments and the present result that, when the Characean internodes are exposed to the light, alternate acidic and alkaline regions are quite often formed and that large electric currents from acidic to alkaline regions are detected extracellularly. However, it remains an open question as to what kind of ionic species would be responsible for the main electric current carrier in each region.

Recently, Toko et al. [26,27] gave a theoretical explanation of this acid/alkali pattern formation on the basis of nonequilibrium thermodynamics. According to their theory, H^+ (or OH^-) circulation appears spontaneously between acidic and alkaline regions when light beyond some threshold value is imposed on the cell. The appearance of high pH values near alkaline regions originates from spatially separated passive H^+ influx (or OH^- efflux) associated with a local activation of H^+ pumps in acidic regions: this kind of dissipative structure can be stabilized only under the above conditions far from equilibrium. Furthermore, the kinetics of the pattern-formative process is discussed theoretically in the accompanying paper [20] for a simplified model system.

Let us consider the results of N_2, O_2, CO_2-free air to air stimulation in figs. 4 and 5. On longer exposure to the CO_2-free condition, the amplitude of the electric pattern was more effectively reduced by replacement with air. This seems rather mysterious, however, the low level of activity of dissolved inorganic carbon species should be expected in the cytoplasm even in cells exposed to the completely CO_2-free condition, including pure O_2 or pure N_2, because of respiration and of photosynthesis. Furthermore, in several microalgae it has been reported that a periphartic enzyme such as a carbonic anhydrase (CA), which maybe also exists in Chara [28], changed the activity so as to depend upon the extracellular CO_2 concentration significantly: when high-CO_2-adapted cells

are transferred to low-CO_2 conditions, the CA activity may increase. This increase is accompanied by a parallel increase in the rate of photosynthetic CO_2 fixation under CO_2-limiting conditions [29].

But several experiments have shown that the large amount of light-induced electric current continues even in the absence of extracellular DIC [9]. This suggests that the electric current seems to be a process independent of the extracellular DIC species; the current is connected with the flow generated by the intracellularly produced OH^- in photosynthesis, and furthermore depends on the H^+ electrogenic process. This process is generally believed to be more activated in light and maintains the membrane potential at a much deeper level than the diffusion potential [5,6]. If one considers that the increased cytoplasmic OH^- concentration may open the putative OH^- channel, then OH^- produced by photosynthesis can be extruded from the open channel according to the electrochemical gradient which is considered to be generated by the electrogenic H^+ pump. Thus, it would be reasonable that we obtain a pH higher than 10 around the D-region without taking into account a special mechanism for pumping out OH^-.

A straightforward assumption to explain the stabilization of alternating D- and H-regions is that the putative OH^- channels normally closed are uniformly distributed but the threshold level for opening the channels depends upon the position along the cell. Since the membrane potential might be affected by the OH^- concentration difference across the membrane [12], the potential pattern along the cell can be expected to appear. Another assumption may be that the activity of photophosphorylation is not uniform along the length of the cell. Even if these kinds of inhomogeneity are not assumed, however, we can explain the origin of electric inhomogeneity based on the nonequilibrium theory [20,27]: provided that the membrane permeability in some local places happens to be high, then it can accelerate the alkalinization in an atuocatalytic fashion. However, this process cannot proceed over the entire cell length, because the electrogenic H^+ pumps always produce the acidification. As a result, the alkalinization and acidification processes must balance and locate ina spatially separate way with each other.

Finally, we must point out that the high pH values can be easily attained. In the region exposed to an extremely strong intensity of a small spot light ($\sim 100\ \mu m$ in diameter), the chloroplasts are detached from the cell cortex. This region then turned out to be alkaline, as expressed by Kamitsubo's window [30]. In this case we confirmed the extremely low specific resistance of 15 $k\Omega \cdot cm^2$. This means that the alkalinization can occur straightforwardly only if the membrane permeability becomes high as a result of some perturbation. As another example, by attaching a high pH agar block to the surface of a small region of internode, the agar block can increase the pH in the cytosol to open the OH^- channel [1]. These results agree with the discovery [12] that the plasma membrane becomes permeable to OH^- (or alternatively to H^+) at high external pH. Even in these cases, it may be essential to raise the cytoplasmic pH above the threshold level to open the OH^- (or H^+) channels in the initial stages.

Recently, Bisson [31] reported that in darkness the membrane did not behave as a pH electrode even at $pH > 10.5$. No photophosphorylation is not expected in this case and hence the activity of the electrogenic H^+ pump might be partially decreased. Thus, the cytoplasm should be acidified by darkness, e.g., to pH 7.3, and maybe more. To open the OH^- (or H^+) channels in the dark-adapted cell, therefore, a much higher extracellular pH would be required if it were physiologically possible. Actually the magnitudes of membrane potential and resistance decreased remarkably when the cell was perfused at $pH > 7.5$ in both the light and dark [13]. In addition, the electric membrane characteristics of Characean internodes obtained by a voltage or a current clamping method [11,31] may be very close to those in the H-regions studied here. Thus, it seems that the depolarization state of the D-region becomes unstabilized or inactivated and then has characteristics analogous to those of the H-region. To study this apparent homogeneity realized by the voltage clamping method is a future task.

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