The contractile vacuole complex is a membrane-bound osmoregulatory organelle observed in freshwater protozoa and sponges (Jeeps, 1947; Patterson, 1980). The organelle of Paramecium multimicronucleatum consists of a central contractile vacuole (CV) and 5–10 radial arms surrounding the CV. A radial arm consists of (1) an ampulla that connects with the CV, (2) the collecting canal, which is continuous with the ampulla, (3) the smooth spongiome that branches from the collecting canal and (4) the decorated spongiome, which is continuous with the smooth spongiome and ends blindly in the cytosol (Hausmann and Allen, 1977).

It has long been known that the membrane of the contractile vacuole complex of Paramecium exhibits dynamic movements in association with its osmoregulatory activity (Allen and Fok, 1988; Patterson, 1980). It is also known that the organelle’s V-type proton pump activity is involved in the segregation of excess cytosolic water acquired osmotically (Fok et al. 1995; Ishida et al. 1993). As a next step in understanding how the contractile vacuole complex carries out its osmoregulatory function, we measured the electrical properties of the membrane of the contractile vacuole complex. Any change in the organelle’s input capacitance signifies a change in its membrane area, since capacitance is directly correlated with membrane area. Measurement of the organelle’s membrane potential helps to locate the organelle’s electrogenic site(s), and measurement of the organelle’s membrane resistance defines its ion permeability properties.

In the present paper, we relate the electrophysiological parameters of the contractile vacuole complex of P. multimicronucleatum measured using a fine-tipped microelectrode inserted into the CV in vivo, to its osmoregulatory activity. We will then propose a hypothesis for the mechanism of osmoregulation in this freshwater unicellular protozoan.
organism. Some portions of this paper have been presented verbally elsewhere (Naitoh et al. 1997b).

Materials and methods

Cells

Cells of Paramecium multimicronucleatum (syngen 2) (Allen and Fok, 1988) were grown in an axenic culture medium at 24 °C (Fok and Allen, 1979) and were harvested at the mid-logarithmic growth phase. These cells were washed with a saline solution containing 1.0 mmol l\(^{-1}\) KCl, 1.0 mmol l\(^{-1}\) CaCl\(_2\) and 1.0 mmol l\(^{-1}\) Mops–KOH buffer (pH 7.0). The cells were equilibrated in the solution for more than 4 h prior to experimentation (Naitoh et al. 1997a).

Manipulation

A minute amount of the saline solution containing an equilibrated cell was introduced into a silicon oil drop (approximately 3 mm in diameter) in the experimental vessel. As shown in Fig. 1A, the cell was arrested, so that the CV remained in the visual field of the microscope, by inserting a glass microneedle into its central portion. The tip of a microcapillary electrode filled with 3 mol l\(^{-1}\) KCl (e1; approximately 50 MΩ) was then inserted into the cytosol. Additional saline solution was then introduced into the vessel to ensure that the cell was bathed in excess saline. A fine-tipped microcapillary electrode filled with 3 mol l\(^{-1}\) KCl (e2; approximately 100 MΩ) was then inserted into the CV to measure the organelle’s membrane potential (E\(_{cvc}\)). This electrode also served to inject square current pulses (I; −0.3 to +0.3 nA, 50 ms duration, 4 Hz) to determine the organelle’s input resistance and input capacitance. An electrical oscillation generated by a temporary (approximately 20 ms) overcompensation of the stray capacitance of the head amplifier of the constant-current supply system was indispensable for successful penetration of the electrode tip into the CV. The tip of a current drain electrode (e3; a glass capillary containing 3 mol l\(^{-1}\) KCl in 2 % agar, approximately 5 kΩ) was placed into the external saline solution approximately 5 mm away from the impaled microelectrode and grounded. The temperature of the experimental vessel was held at 17 °C.

Electrical signals

The equivalent circuit for a cell with a contractile vacuole complex being subjected to electrophysiological examination is shown in Fig. 1B. This circuit consists of three components connected in series: (a) a component that corresponds to the series resistance (R\(_{0}\)), (b) a component that corresponds to the membrane of the contractile vacuole complex and (c) a component that corresponds to the surface membrane of the cell. Component a consists mostly of the resistance of electrode e2. Component b is regarded as a battery (an electromotive force across the organelle’s membrane; E\(_{cvc}\)) with an internal resistance (the organelle’s input resistance; R\(_{cvc}\)) connected in parallel with a capacitance (the organelle’s input capacitance; C\(_{cvc}\)). Similarly, component c is regarded as a battery (an electromotive force across the cell surface membrane; E\(_{m}\)) with an internal resistance (the cell’s input resistance; R\(_{m}\)) connected in parallel with a capacitance (the cell’s input capacitance; C\(_{m}\)).

A potential shift across each circuit component caused by a square current pulse (I) is schematically shown to the right of each corresponding component circuit in Fig. 1B (ΔE\(_{a}\), ΔE\(_{b}\) and ΔE\(_{c}\) for the component circuits a, b and c, respectively). ΔE\(_{a}\) is an ohmic potential shift with a steady value which equals I×R\(_{0}\). ΔE\(_{b}\) is assumed to be a single-exponential potential shift that reaches a steady potential level equal to I×R\(_{cvc}\) with a time constant of R\(_{cvc}\)×C\(_{cvc}\) after the onset of the pulse. This potential shift is followed by an exponential decrease in the potential shift to the previous level with the same time constant after the pulse has been turned off. It is well known that the cell surface membrane of Paramecium has some voltage-activated ion channels (Naitoh, 1982), so that ΔE\(_{m}\) does not follow a single-exponential time course until it reaches a steady potential level that equals I×R\(_{m}\)+ΔE\(_{m}\). ΔE\(_{m}\) is the change in E\(_{m}\) due to the activation of voltage-sensitive ion channels, and R\(_{m}\) also changes according to the activation of ion channels; see the red trace in Fig. 1Cii) after the onset of the pulse. This potential shift is followed by a non-exponential decrease in the potential to the previous level after the pulse has been turned off. The potential shift caused by an injected pulse detected through electrode e2 (ΔE\(_{2}\)) is the algebraic sum of these three potential shifts (ΔE\(_{2}\) = ΔE\(_{a}\)+ΔE\(_{b}\)+ΔE\(_{c}\)) and is shown to the right of Fig. 1B.

Representative traces of the electrical signals obtained from electrodes e1 (E\(_{1}\); red trace) and e2 (E\(_{2}\); black trace) are shown in Fig. 1Ci. The zero potential level for these traces is equal to the ground potential. Portions of these traces corresponding to the thin blue column are magnified 10 times in time scale and shown in a thicker blue column to the right of the figure. It is clear from these time-expanded traces that potential shifts caused by injected current pulses are seen as a train of potential deflections. Each of the potential deflection in E\(_{1}\) corresponds to ΔE\(_{a}\) and that in E\(_{2}\) corresponds to ΔE\(_{2}\). The direction and degree of each potential deflection depend on the direction and intensity of injected pulse.

The organelle’s transmembrane potential (E\(_{cvc}\)), input resistance (R\(_{cvc}\)) and input capacitance (C\(_{cvc}\)) are extracted from traces E\(_{1}\) and E\(_{2}\) according to the following procedure. (1) Subtraction of E\(_{1}\) from E\(_{2}\) gives the potential trace shown in Fig. 1Cii. The zero potential level for this trace is equal to the cytosolic potential. Each of the potential deflections corresponds to ΔE\(_{a}\)+ΔE\(_{b}\). (2) Removal of the potential trace segments including the potential deflections from the trace. Each potential segment starts from the beginning of the deflection (corresponding to the start of the pulse) and ends 50 ms after the end of the pulse (the duration of the segment is 100 ms). At this time, the deflection has virtually subsided since the largest time constant among those for the component circuits is less than approximately 25 ms (for component c). (3) Interpolation of a line into each vacant portion of the trace caused by the removal of each segment. The resulting
Fig. 1. Schematic representation of the procedures for the electrophysiological examination of the in situ contractile vacuole complex of a *Paramecium multimicronucleatum* cell, the computer-aided control of the experiment and the data analysis. (A) Arrangement of the electrodes and a flow-chart of the computer-aided controls. (B) Equivalent circuit for a cell with a contractile vacuole complex subjected to electrophysiological examination. In this model, the series resistance ($R_s$) is $100\,\text{M}\Omega$, the input resistance ($R_{cvc}$) and the input capacitance ($C_{cvc}$) of the contractile vacuole complex are $30\,\text{M}\Omega$ and $170\,\text{pF}$, respectively, the input resistance ($R_m$) and the input capacitance ($C_m$) of the cell are $20\,\text{M}\Omega$ and $1\,\text{nF}$, respectively, and the intensity of the current pulse ($I$) is $-0.2\,\text{nA}$. (C) Procedures for the analysis of electrical signals obtained from the cell. (i) The red trace is an electrical signal obtained from the electrode inserted into the cytosol ($e_1$ in A) corresponding to $E_1$ in B, and the black trace is the recording obtained from the electrode inserted into the contractile vacuole ($e_2$ in A) corresponding to $E_2$ in B. (ii) The difference between $E_1$ and $E_2$ ($E_2-E_1$). (iii) The contractile vacuole membrane potential with reference to the cytosolic potential ($E_{cvc}$ in B) obtained from Cii by interpolating a line into each potential trace segment of 100 ms duration starting from the beginning of each current pulse ($I$ in A and B). (iv) The membrane potential shifts across the contractile vacuole membrane ($\Delta E_b$ in B) caused by injection of current pulses ($I$). See the text for details. CV1, CV2, contractile vacuoles; n, glass microneedle; $E_m$, the cell’s membrane potential with reference to the external solution; VCR1, VCR2, video recorders; e1-3, electrodes.
smoother trace is regarded as $E_{\text{cvc}}$ and is shown in Fig. 1Ciii, together with its time-expanded portion on the right. (4) Subtraction of $E_{\text{cvc}}$ and $\Delta E_a$ from the trace in Fig. 1Ciii. The resulting trace is shown in Fig. 1Civ. Each potential deflection, which is clearly shown in the time-expanded trace on the right, corresponds to $\Delta E_a$. Each trace for $\Delta E_a$ is fitted to a single-exponential curve to obtain the organelle’s time constant ($\tau_{\text{cvc}}$) and $R_{\text{cvc}}$. $C_{\text{cvc}}$ is then obtained by dividing $\tau_{\text{cvc}}$ by $R_{\text{cvc}}$.

**Computer-aided analysis**

A schematic diagram of the arrangement of the computer-aided data analysis system is shown in Fig. 1A. The electrical signals obtained from the microelectrodes were fed into a computer (Power Macintosh 7600/136, Apple Computer Inc., Cupertino, CA, USA) through an A/D–D/A converter (ITC 16, Instrutech Corp., Great Neck, NY, USA). These electrical signals were continuously backed up by a VCR (VCR1; SLV-495, Sony Corp., Park Ridge, NJ, USA) through a PCM recorder (VR-10B, Instrutech Corp., Great Neck, NY, USA). This computer system was also used to control the sequence of the experiments. Images of the cell obtained using Nomarski optics ($\times$40 objective lens on a Leitz-DMIRB microscope, Leica Mikrosk u. System, GmbH, Wetzlar, Germany) were monitored on a television screen after capture by a video camera (CCD-72, Dage MIT Inc., Michigan City, IN, USA) and continuously video-recorded (VCR2; AG-6300, Panasonic Indust. Co., Secaucus, NJ, USA) at 30 frames s$^{-1}$ during the experiments. The video images of the contractile vacuole complex were fed into the same computer using a frame grabber for simultaneous analysis of the electrical and morphological events in the contractile vacuole complex associated with its exocytotic activity. The contrast of the video images was enhanced using a video-image-enhancing processor built into the camera system and the computer system. The membrane area of the CV was estimated on the basis of its displayed image, which was considered to be an ellipsoid.

Software for feeding the electrical signals into the computer and for generating the various magnitudes and sequences of current pulses required for the experiments was developed on the basis of the IgorPro (WaveMetrics, Inc., Lake Oswego, OR, USA) and PulseControl XOP software packages (Herrington et al. 1995). Software for analyses of the images of the CV was the public domain NIH Image Program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

**Quick-freeze deep-etch electron micrographs of the P-fracture faces of the membranes of the contractile vacuole complex**

Living cells were concentrated, frozen rapidly without using chemical fixatives, fractured, deep-etched and rotary-shadowed as previously reported (Schroeder et al. 1990).

**Results**

We performed 10 series of experiments with 10 different cells. All the data shown in the figures of this paper (except for the electron micrograph in Fig. 4) were obtained from a representative series of experiments from a single cell. No qualitative differences in the data were found between different cells.

**Morphological changes in an impaled contractile vacuole during its exocytotic activity**

An impaled contractile vacuole complex underwent exocytosis at fairly regular intervals (exocytotic cycles). Changes in shape exhibited by an impaled CV during three consecutive exocytotic cycles are shown in Fig. 2, both as video-recorded images (Fig. 2A) and as changes in membrane area (Fig. 2Bi). In the fluid-filling phase (frames 0–6, 9–27, 30–45, 48–59), the contractile vacuole gradually increased in membrane area to a steady value ($2.3\times10^3\mu m^2$ for the first exocytotic cycle, $2.1\times10^3\mu m^2$ for the second exocytotic cycle, $1.9\times10^3\mu m^2$ for the third exocytotic cycle; $1.7\times10^3\pm0.5\times10^3\mu m^2$, mean $\pm$ s.d. obtained from eight different CVs). The CV became rounded (the rounding phase, frames 7, 28, 46), while the membrane area remained more or less unchanged. The CV then suddenly decreased in size and disappeared as the CV pore opened and its fluid was expelled to the cell exterior (the fluid-expulsion phase, frames 8, 29 and 47; expulsion phases $e_1$, $e_2$ and $e_3$, respectively). The CV soon became observable again as its size increased after the pore closed and fluid filling resumed.

**Changes in the electrophysiological parameters of the contractile vacuole complex during exocytotic cycles**

Fig. 2B shows the changes in the organelle’s input capacitance, membrane potential with reference to the cytosolic potential and input resistance during the three consecutive exocytotic cycles shown in Fig. 2A. The input capacitance (Fig. 2Bi) remained unchanged during the fluid-filling phase at approximately 210 pF ($181\pm63$ pF, $N=8$, mean $\pm$ s.d.). It then decreased markedly to a lower value before the start of fluid expulsion (25 pF before $e_1$, 21 pF before $e_2$, 19 pF before $e_3$; 19.2±4.0 pF, $N=7$), during a time when the size of the CV remained more or less unchanged. Capacitance could not be determined during the fluid-expulsion phase because the current pulses passed mostly through the opened pore. The resistance was far lower through the open pore, which was estimated to be approximately 1 M$\Omega$ on the basis of the pore size (Naitoh et al. 1997a) and the specific resistance of the cytosol (assumed to be equivalent to 70 mmol l$^{-1}$ KCl solution; Ishida et al. 1996) than through the CV membrane itself (approximately 200 M$\Omega$, see below). Capacitance was again detectable as the organelle entered the fluid-filling phase and quickly increased to a steady high value, where it remained unchanged at this value throughout the rest of the filling phase. The membrane potential (Fig. 2Bi) remained unchanged at a steady level during the fluid-filling phase (approximately 77 mV in this case; 53.8±13.2 mV, $N=10$). The potential then decreased to its lowest level before the start of fluid expulsion (13.6 mV before $e_1$, 9.7 mV before $e_2$, 7.5 mV before $e_3$; 10.0±3.8 mV, $N=9$). The membrane potential then suddenly decreased to its lowest level before the start of fluid expulsion (13.6 mV before $e_1$, 9.7 mV before $e_2$, 7.5 mV before $e_3$; 10.0±3.8 mV, $N=9$). The membrane potential then suddenly decreased to its lowest level before the start of fluid expulsion (13.6 mV before $e_1$, 9.7 mV before $e_2$, 7.5 mV before $e_3$; 10.0±3.8 mV, $N=9$).
changed to approximately 30 mV in association with the start of fluid expulsion. At this time, the potential corresponds to the surface membrane potential of the cell measured with reference to the cytosolic potential since the CV fluid is now electrically connected to the external solution through the opened CV pore. The membrane potential then dropped to zero or to a slightly negative potential for a few seconds after the pore closed before the start of the next phase of fluid filling (−6.1 mV after e1, −8.0 mV after e2, −5.5 mV after e3; −2.0±4.4 mV, N=8).

The organelle’s input resistance (Fig. 2Biv) was approximately 31 MΩ (31.2±15.0 MΩ, N=9) during the fluid-filling phase. It increased to a higher value during the rounding phase (210 MΩ in the first cycle, 191 MΩ in the second cycle, 203 MΩ in the third cycle; 295±186 MΩ, N=6). It tended to decrease slightly immediately before the start of fluid expulsion. It resumed its low value when fluid filling started after fluid expulsion.

**Current–voltage relationship in the membrane of the contractile vacuole complex**

To examine the conductance properties of the membrane of the contractile vacuole complex, the potential shifts across the organelle’s membrane (ΔV) caused by injecting current pulses of varied strengths (I; −0.3 to +0.3 nA) were determined during the three exocytotic cycles (Fig. 3Ai, immediately before fluid expulsion; Fig. 3Aii, during fluid filling; the maximum CV diameter was 24.6±2.6 µm, six consecutive exocytotic cycles). The values for ΔV were plotted against their corresponding current strengths in Fig. 3B. Straight lines were plotted according to the least-squares method. The slope of the line,
which corresponds to the organelle’s input resistance, was steeper during the rounding phase immediately before fluid expulsion (corresponding to the time when the input capacitance was less than 18 pF and the resistance was $192.3\pm6.5 \text{ M}\Omega$, $N=76$ current pulses applied during this time) than during the fluid-filling phase (corresponding to the time when the input capacitance was more than 80 pF and the resistance was $39.9\pm0.7 \text{ M}\Omega$, $N=458$ current pulses applied during this time).

**Discussion**

The exocytosis-associated morphological changes in the contractile vacuole

The morphological changes observed in an impaled CV during exocytosis (Fig. 2A) were essentially identical to those in the non-impaled CV reported by previous investigators (Allen and Fok, 1988; Patterson, 1980), although the pulsation frequency was slightly lower in the impaled CV than in the non-impaled one.

The relationship between changes in the input capacitance and exocytosis-associated membrane dynamics in the contractile vacuole complex

The input capacitance decreased markedly before the start of fluid expulsion and then quickly resumed its previous value in association with fluid filling (Fig. 2Bii). This suggests that the CV membrane is electrically isolated from the rest of the organelle’s membrane, i.e. the radial arms, before the fluid is expelled and electrically reconnected to the rest of the organelle’s membrane after the fluid is expelled. It also implies that no new membrane is added to the organelle as the CV increases in volume.
The membrane area of the rounded CV immediately before the start of fluid expulsion was estimated by dividing the lowest input capacitance determined before the start of fluid expulsion by the specific membrane capacitance of a conventional biomembrane (1.0 μF cm⁻²) (Cole, 1968). The estimated values were 2.5 × 10⁴ μm² before e₁, 2.1 × 10³ μm² before e₂ and 1.9 × 10³ mm² before e₃ (1.9 ± 0.4 × 10³ mm², N=7, mean ± S.D.). These values are consistent with the CV membrane area estimated from its diameter before the start of fluid expulsion (2.3 × 10³ μm² before e₁, 2.1 × 10³ μm² before e₂, 1.9 × 10³ μm² before e₃; 1.7 ± 0.5 × 10³ μm², N=8) (Fig. 1Bi). The consistency of this value strongly supports the idea that the input capacitance immediately before the start of fluid expulsion is due only to the CV membrane and therefore supports the idea that the CV becomes isolated from the rest of the organelle before the fluid is expelled and is then reconnected after the fluid is expelled. Previous morphological observations suggesting that the collecting canals break away from the CV before fluid expulsion and are then reattached to the CV at the start of fluid filling (Allen and Fok, 1988; Patterson, 1980) were strongly supported by the organelle’s input capacitance measurements.

A constant high input capacitance during the fluid-filling phase (181 ± 63 pF, N=8) corresponds to the capacitance of the organelle’s overall membrane area, which is estimated to be approximately 18 × 10³ μm² by dividing the capacitance by the specific membrane capacitance of 1.0 μF cm⁻² (Cole, 1968). We also estimated the length constant of the spongiome according to the cable equation of Hodgkin and Rushton (1946) to be 59 μm. Values for the parameters used for this calculation are (1) 4.4 kΩ cm² for the specific membrane resistance, which is estimated from the input resistance and the membrane area of the CV in the rounding phase, (2) 140 Ω cm for the resistivity of the internal solution, which is estimated on the basis of the assumption that it is equivalent to a 70 mmol l⁻¹ KCl solution and (3) 45 nm for the diameter of the spongiome (the mean of 40 nm for the smooth spongiome and 50 nm for the decorated spongiome).

Allen et al. (1990) indicated that the width of fluorescence microscope images of the radial canals exposed to a monoclonal antibody raised against the decorated spongiomes is approximately 5 μm. This implies that the distance from the collecting canal, which sits at the centre of the radial arm, to the blind end of the decorated spongiome is approximately 2.5 μm. The length constant of 59 μm is approximately 20 times longer than this length. This implies that the interior of the contractile vacuole complex is virtually isopotential. It can, therefore, be said that the input capacitance of the contractile vacuole complex during the fluid-filling phase (181 pF) corresponds to the total membrane area of the organelle (18 × 10³ μm²). It should be noted here that the total membrane area of a contractile vacuole complex estimated from

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**Fig. 5. Schematic model of processes postulated to be involved in water segregation by the contractile vacuole complex in *P. multimicronucleatum*. CV, contractile vacuole; AP, ampulla; CC, collecting canal; SS, smooth spongiome; DS, decorated spongiome; MT, microtubule bundle. P, contractile vacuole pore region; CM, cell membrane. See the text for details.**
measurements obtained from electron micrographs of the organelle is approximately $14 \times 10^3 \, \mu m^2$ (R. D. Allen, unpublished data).

The electrogenic site of the contractile vacuole complex examined by measuring its membrane potential

Our findings that the positive membrane potential is markedly decreased when the CV membrane is isolated from the rest of the organelle’s membrane and then resumes its previous value when the CV membrane is reconnected to the rest of the membrane of the organelle (Fig. 2Biii) imply that most of the organelle’s positive membrane potential is generated somewhere in the contractile vacuole complex rather than in the CV membrane per se. Previous cytochemical (Allen et al. 1990; Ishida et al. 1993) and electron microscope (Fok et al. 1995) examinations of this organelle have demonstrated that V-type proton pumps are present only in the membranes of the decorated spongiomes. We therefore conclude that the positive potential that we obtained from the CV is the potential generated in the spongiome membrane associated with its water segregation activity. These findings, together with our previous finding (Fok et al. 1995) that the proton pumps in the decorated spongiome membrane of *P. multimicronucleatum* are essentially identical in their immunological characteristics with those of the CV of *Dictyostelium discoideum* (Heuser et al. 1993), support the hypothesis proposed by Giglione and Gross (1995) that the proton pumps in the contractile vacuole membrane are electrogenic. A positive contractile vacuolar potential has also been reported in *P. caudatum* (Yamaguchi, 1960) and *Amoeba proteus* (Josefsson, 1966).

It has been reported that, in vorticellid ciliates, positive perturbations of the normally negative cell surface membrane potential (membrane depolarizations) were observed in association with CV pulsations (Moreton and Amos, 1979; Shiono et al. 1980). Such cell membrane potential perturbations were not seen in *Paramecium*. In *Tetrahymena pyriformis*, it has been reported that the cell always shows a minor jerky motion in its movement in association with its CV pulsations (Cameron and Burton, 1969). The jerky movements are assumed to be caused by short periods of reversed beating of cilia in response to membrane depolarizations associated with its CV pulsations. *Tetrahymena* cilia are known to reverse their beating direction in response to membrane depolarization (Onimaru et al. 1980). On the basis of differences in the structure of the contractile vacuole complex in the vorticellid ciliates and *Tetrahymena* (Patterson, 1980) and in *Paramecium*, disconnection of the CV membrane from the decorated spongiome membrane may not occur in the former two ciliates. Since it is probable that these first two species employ the same fluid segregation mechanism as *Paramecium*, the positive potential generated in the decorated spongiome membrane could spread electrotonically towards the cell surface membrane when the CV membrane is fused with the surface membrane through an open CV pore. This potential change would then be detected by a microelectrode inserted into the cytosol or by ciliary reversal causing jerky movements of the cell.

The residual membrane potential of the contractile vacuole

A small CV potential which is positive (approximately 10 mV) to the cytosolic potential was observed even after the CV had been disconnected from the electrogenic decorated spongiome membrane (Fig. 2Biii). This residual positive potential is assumed to be the diffusion potential of an electrolyte(s) due to its concentration difference between the CV fluid and the cytosol. It is conceivable that the concentration of this electrolyte in the CV may be modified by the external solution immediately before pore closure, so that the potential across the CV membrane immediately after pore closure (before fluid filling starts) would be different from that immediately before the start of fluid expulsion. However, since the CV membrane potential immediately after pore closure was almost zero or slightly negative (Fig. 2Biii; $-2.0 \pm 4.4$ mV, $N=8$), it is more likely that the electrode tip during this time is no longer in the CV but is instead in the cytosol as a result of the tip being pushed out of the tubulated CV membrane following fluid expulsion (Naitoh et al. 1997a). Subsequently, the tip may remain outside the CV until it re-enters the CV as the CV expands.

A hypothesis for the mechanism of fluid segregation in the contractile vacuole complex

The potential generated across the decorated spongiome membrane due to proton pump activity would probably allow counter-ions to move from the cytosol into the lumen of the decorated spongiome using an unknown transport mechanism(s). These anions would then be free to diffuse along the lumen of the smooth spongiomes, which connect the decorated spongiomes to the collecting canals, and along the collecting canal to the CV lumen; they could act as an osmolyte all along the way. Excess cytosolic water could enter the lumen through the membranes of these radial arms, as well as through the CV, in response to the osmotic pressure difference caused by the presence of these anions in the lumen. The fluid content of the CV would then be expelled to the cell exterior during the fluid-expulsion phase. The most likely anion species that could be continuously eliminated by the cell is postulated to be bicarbonate (Giglione and Gross, 1995; Heuser et al. 1993). Other anions such as Cl$^-$ would form acid, and acidification in the lumen of this organelle has never been observed in *Paramecium* during its osmoregulatory activity (A. K. Fok and R. D. Allen, unpublished data; see also Allen and Fok, 1997). This anion may be produced as the final product of the cell’s respiration, CO$_2$, and a viable cell would therefore never have a shortage of this ion. The importance of bicarbonate transport across the cell membrane during cell volume regulation has previously been reported and discussed for vertebrate cells (Baumgarten and Feher, 1995; Hoffmann, 1986; Maren, 1988; Zeuthen, 1992). This idea for the water segregation mechanism in the contractile vacuole complex is shown schematically in Fig. 5.
Implication of aquaporin for water transport through the membrane of the contractile vacuole complex

In connection with the above hypothesis for cytosolic fluid segregation, it is interesting to note that quick-freeze deep-etch electron micrographs of the P-fracture face of the membranes of the CV and of the collecting canals of P. multimicronucleatum clearly show the presence of a uniform population of protein granules of approximately 9 nm in diameter in these membranes (Fig. 4). It is possible that these granules have water-permeable properties, like the aquaporins (Van Os et al. 1994; Verkman et al. 1996). They might allow the passage of a sufficient quantity of water into the contractile vacuole complex to account for its observed fluid output.

It has recently been reported that aquaporins show a rather low ionic conductance (Regan et al. 1997; Yool et al. 1996), although the single aquaporin conductance has not yet been estimated. If the granules observed in the electron micrographs are the only granules responsible for the input resistance of the CV during the rounding phase, we can estimate what the single intramembrane granule conductance would be. This value was estimated to be approximately 0.4 fS from values for the input resistance of the CV during the rounding phase, we can estimate what the single aquaporin conductance has not yet been estimated. If the granules observed in the electron micrographs are the only granules responsible for the input resistance of the CV during the rounding phase, we can estimate what the single intramembrane granule conductance would be. This value was estimated to be approximately 0.4 fS from values for the input resistance of the CV during the rounding phase, we can estimate what the single intramembrane granule conductance would be. This value was estimated to be approximately 0.4 fS from values for the input resistance of the CV during the rounding phase.

Conductance properties of the contractile vacuole membrane

The membrane potential shifts across the contractile vacuole membrane caused by current pulses injected into the CV increased linearly with increasing current intensity (Fig. 3). The linear relationship implies that very few voltage-activated ion channels are present in the membranes and/or that ion channels are not activated in the voltage range modified by the injected current pulses (~60 to +60 mV for the CV membrane, −20 to +20 mV for the organelle’s overall membrane).

The input resistance of the contractile vacuole complex suddenly increased immediately before the start of fluid expulsion, but then resumed its previous lower value (Fig. 2Biv). Thus, an increase followed by a decrease in the CV input resistance corresponds to disconnection and reconnection of the CV membrane to its radial arms.

It should be noted that the input resistance of the CV itself is approximately seven times larger than that of the overall organelle (201 MΩ versus 31 MΩ). However, since the CV membrane area is less than one-tenth of that of the organelle’s overall membrane area (21 pF versus 210 pF), the specific membrane conductance, or the membrane’s ion permeability, is slightly higher in the CV membrane than in the overall organelle membrane.

In conclusion, by inserting fine-tipped microelectrodes into the contractile vacuole complex of a living P. multimicronucleatum cell, we have revealed the electrical behaviour of different segments of this organelle. Our results contribute to a better understanding of the mechanisms underlying the cytosolic fluid segregation required for osmoregulation of this cell. The experimental techniques employed in these experiments should prove useful for numerous studies into the morphological and physiological changes at the level of the subcellular compartment in situ, where organization in relation to specific function is being studied.

This work was supported by NSF Grant MCB 95 05910.

References


