

## CYCLIC CHANGES IN THE TENSION OF THE CONTRACTILE VACUOLE COMPLEX MEMBRANE CONTROL ITS EXOCYTOTIC CYCLE

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### Summary

The freshwater protozoan *Paramecium multimicronucleatum* maintains a constant cytosolic osmolarity through the exocytotic activity of its contractile vacuole complex. The contractile vacuole (CV) expels the excess cytosolic water, acquired osmotically, to the exterior of the cell at fairly regular intervals. In a single exocytotic cycle, the CV swells as the cytosolic water enters the vacuole, rounds up, and then rapidly shrinks as the fluid is expelled through the pore of the CV. The exocytotic activity of the CV complex can sometimes be slowed and distorted by microelectrode impalement. Using a CV with exocytotic activity slowed in this way makes it possible to determine more precisely the time course of changes in the exocytotic-activity-related membrane capacitance and membrane potential of the organelle. We have clearly demonstrated that the radial arms of the CV were always severed after the CV had exhibited rounding. Microelectrode impalement sometimes caused a failure of the CV pore to open after rounding up, so that the CV entered the next fluid-filling phase without expelling its fluid. The radial arms remained severed from the CV during such prolonged rounding phases and then rejoined the CV at the start of the next fluid-filling phase. The rounding of the CV corresponds to an increase in the tension of the CV membrane. This suggested that the periodic development of increased tension in the CV membrane might be the primary event leading to periodic severing of the radial

arms and the opening of the pore. We then observed that the CV and its radial arms sometimes became fragmented into vesicles when the cell had been mechanically ruptured in a salt solution. Many of the resulting *in vitro* vesicles showed periodic rounding and slackening which occurred at different times so that they were out of phase one with the other. This indicates that the membranes of the CV and the radial arms maintain their ability to develop a periodic increase in tension even after the cell has been ruptured. We propose the hypothesis that the CV membrane (together with its associated cytoskeletal structures) possesses a mechanism by which its tension is periodically increased. Such a periodic change in membrane tension may govern the exocytotic cycle of the contractile vacuole complex by increasing the tension to a point that the radial arms sever from the CV and the pore opens. Conversely, a decrease in the tension causes closure of the pore and rejoining of the radial arms to the CV. Transformation of the CV membrane into 40 nm tubules is assumed to be responsible for the development of tension in the planar CV membrane. The causes of this periodic tubulation must now be sought.

Key words: contractile vacuole complex, exocytotic cycle, membrane tension, membrane capacitance, membrane resistance, membrane tubulation, *Paramecium multimicronucleatum*.

### Introduction

The contractile vacuole complex of the freshwater protozoan *Paramecium multimicronucleatum* expels excess cytosolic water, acquired osmotically, to the exterior of the cell to maintain a constant cytosolic osmolarity. The contractile vacuole (CV), the central exocytotic vesicle of this organelle, undergoes a relatively slow swelling process as cytosolic water enters the vacuole (the fluid-filling phase), followed by a rapid shrinkage of the vacuole as the fluid is expelled to the exterior through a fixed pore (the fluid-expulsion phase). The period of

the exocytotic cycle shortens when the cell is exposed to a more hypotonic solution and a larger amount of water acquired osmotically has to be expelled to the exterior. The exocytotic activity stops altogether when the cell is exposed to an isotonic or hypertonic solution and net osmotic water flow stops (Ishida *et al.* 1996; Kitching, 1956, 1967; Patterson, 1980).

In our previous paper (Tominaga *et al.* 1998), we examined the membrane dynamics of the contractile vacuole complex as well as its exocytotic activity using electrophysiological

techniques. We demonstrated that the radial arms that surround the CV were severed from the CV immediately before fluid expulsion. We also found that the V-type proton pumps, which reside in the membrane of the terminal tubules of the radial arms called the decorated spongione (Hausmann and Allen, 1977), generate an electrochemical potential across the membrane of the organelle. This potential was assumed to energize the transport of osmolytes from the cytosol into the lumen of the organelle so that excess cytosolic water will flow osmotically into the lumen through water channels in the organelle membrane.

It has been known for some time that the CV becomes rounded just before fluid expulsion (the rounding phase) (Patterson, 1980; Patterson and Sleight, 1976). We have demonstrated previously (Tominaga *et al.* 1998) that rounding up takes place in association with severing of the radial arms from the CV. We then postulated that rounding of the CV corresponds to an increase in the tension in the CV membrane. It can, therefore, be anticipated that this tension must be generated in the CV membrane at least once in each exocytotic cycle. We demonstrated earlier (Naitoh *et al.* 1997a) the presence of tension in the CV membrane just before fluid expulsion. This tension determined the effective diameter of the CV pore when it opened at the end of the rounding phase.

Biophysical studies on the fusion of phospholipid bilayers and vesicles have demonstrated that an increase in the tension in the bilayer membrane is a key factor for promoting fusion (Chernomordik *et al.* 1995). It has also been reported that tension in the secretory granule membrane is the critical stress factor for bringing about exocytosis of the granule, i.e. fusion of the vesicle membrane with the plasma membrane (Monck and Fernandez, 1992; Monck *et al.* 1990). It is, therefore, interesting and important for us to examine the relationship between the membrane tension and the membrane dynamics of the contractile vacuole complex during its exocytotic activity. This may allow us to understand the mechanism by which the exocytotic cycle is controlled.

The primary objective of the present paper is to determine whether the development of membrane tension causes severing of the radial arms from the CV or whether this severing somehow brings about the increase in tension. Microelectrode impalement sometimes resulted in the failure of the CV pore to open after becoming rounded so that the size of the CV increased and the exocytotic cycle was prolonged. Prolongation of the cycle made it possible to compare the precise time course between the development of membrane tension, which was exhibited as a change in the 'roundness' of the CV, and the membrane dynamics, measured as changes in the electrophysiological parameters of the organelle, i.e. the input capacitance, input resistance and membrane potential.

We also examined periodic changes in the roundness of the CV *in vitro*, i.e. in the membrane-bound vesicles derived from the CV, and in the radial arms of the contractile vacuole complex. We now propose the hypothesis that the periodic development of tension in the contractile vacuole complex membrane governs the exocytotic cycle of the organelle. Some

of this work has been presented elsewhere in abstract form (Allen *et al.* 1997).

### Materials and methods

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 (final concentration in  $\text{mmol l}^{-1}$ ) 1.0 KCl, 1.0  $\text{CaCl}_2$  and 1.0 Mops-KOH buffer (pH 7.0). The cells were equilibrated in this solution for more than 4 h prior to experimentation (Naitoh *et al.* 1997b).

To examine the *in vivo* contractile vacuole complex electrophysiologically, an equilibrated cell was arrested by inserting a glass microneedle into the central portion of the cell, so that the CV remained in the field of view of the microscope. The tip of a KCl-filled ( $3 \text{ mol l}^{-1}$ ) microcapillary electrode (resistance approximately 50 M $\Omega$ ) was then inserted into the cytosol. A fine-tipped microcapillary electrode (resistance approximately 100 M $\Omega$ ), also filled with  $3 \text{ mol l}^{-1}$  KCl, was inserted into the CV to measure the membrane potential of the organelle. This electrode was also used to inject square current pulses (0.3 nA, 50 ms duration, 4 Hz) into the CV to determine the time constant of the organelle, from which its input resistance and input capacitance were estimated (Tominaga *et al.* 1998). The tip of a current drain electrode (a glass capillary containing  $3 \text{ mol l}^{-1}$  KCl/2% agar, approximately 5 k $\Omega$ ) was placed into the external saline solution approximately 5 mm away from the impaled microelectrode and was grounded.

Electrical signals obtained from the electrodes were displayed on a computer monitor. Images of the CV, obtained using Nomarski optics, were concurrently displayed on a television screen through a video camera. The electrical signals and the video signals were continuously recorded on video tape for subsequent and simultaneous analysis of the electrical and morphological events in the contractile vacuole complex that accompanied its exocytotic activity. The hardware and software for controlling the sequence of the experiments, applying current pulses to the CV, grabbing the video frames, feeding the electrical signals and the video signals into the computer and analyzing the data were essentially the same as those described in our previous paper (Tominaga *et al.* 1998).

To examine the CV morphologically *in vitro*, a cell equilibrated in a standard saline solution was placed in a small droplet of the saline solution under silicone oil on a glass slide. The saline was pipetted out of the droplet so that the cell was squeezed by the saline/silicone oil interphase until it ruptured. The cell rupture process was continuously monitored on a television screen and video-recorded. The shapes of the CV and of fragmented membrane-bound vesicles derived from the CV and/or the radial arms were analyzed with the aid of a computer. The temperature of the experimental vessel was electronically maintained at 17 °C.

Results are presented as means  $\pm$  S.D. (*N*).

## Results

### Morphological changes in the contractile vacuole impaled by a microelectrode

The contractile vacuole impaled by a microelectrode showed regular exocytosis, but sometimes made aborted attempts at fluid expulsion. The morphological changes of an impaled CV demonstrating such irregular exocytosis are shown in Fig. 1A. To quantify the morphological changes, the CV volume,  $V_{CV}$ , and the index of the roundness of the CV,  $R_{CV}$ , were estimated from the CV profile (Fig. 1A) on the assumption that the shape

of the CV approximates an ellipsoid.  $R_{CV}$  was defined as the ratio of the CV volume to the volume of a sphere with a surface area equal to that of the CV. This ratio can be formulated as:

$$R_{CV} = V_{CV} / \frac{\pi}{6} \left( \frac{A_{CV}}{\pi} \right)^{3/2}, \quad (1)$$

where  $A_{CV}$  is the surface area of the CV. The ratio is maximal (equal to 1) when the CV is spherical.  $V_{CV}$  is shown in Fig. 1B and  $R_{CV}$  in Fig. 1C.

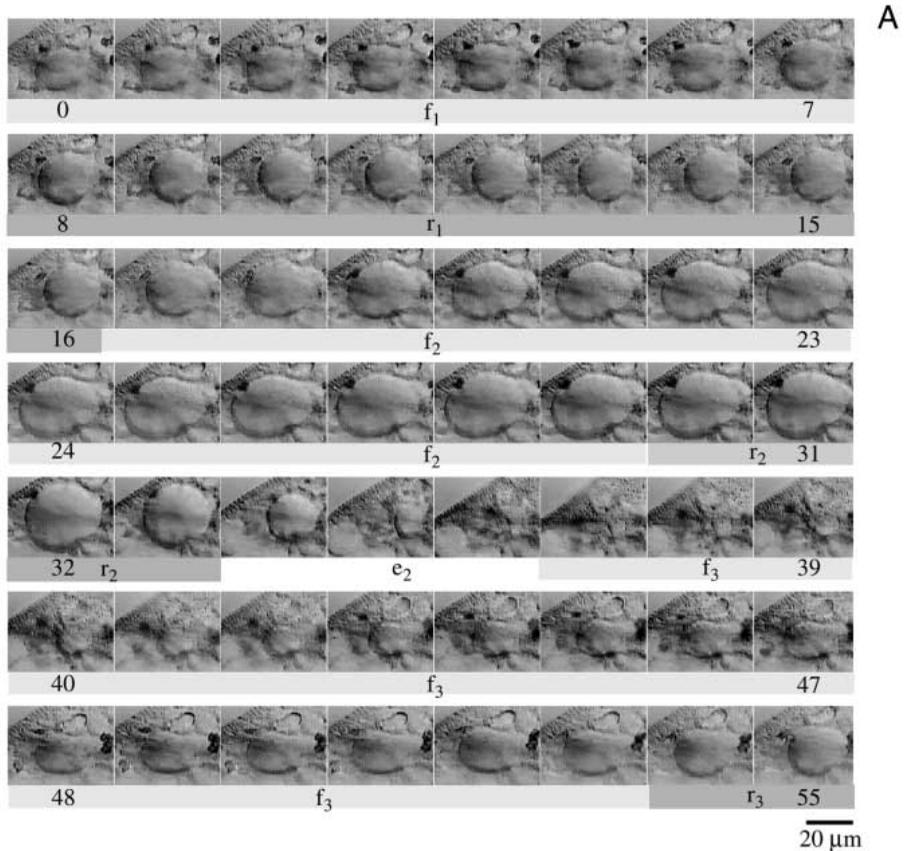
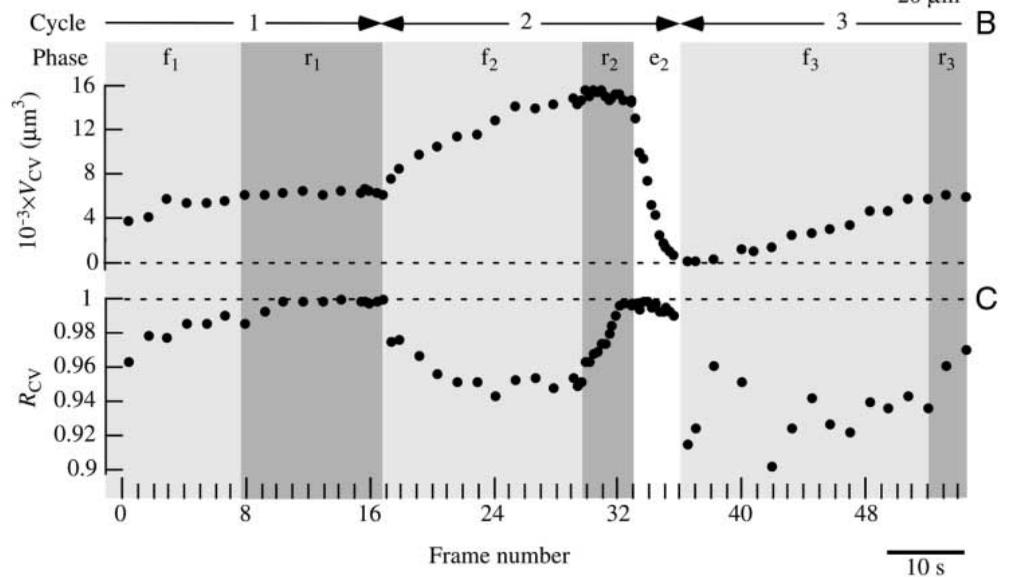


Fig. 1. Morphological changes in an impaled contractile vacuole (CV) of a cell of *Paramecium multimicronucleatum* taken during three actual or attempted exocytotic cycles (cycles 1, 2 and 3). (A) A series of 56 consecutive images (numbered 0–55) of the CV profile. f, the fluid-filling phase; r, the rounding phase; e, the fluid-expulsion phase. The numbers 1, 2 or 3 beside each letter correspond to cycles 1, 2 or 3. (B) Changes in the volume of the CV ( $V_{CV}$ ) during the three exocytotic cycles. (C) Changes in the degree of roundness ( $R_{CV}$ ) of the CV during the three exocytotic cycles. Frame numbers refer to the image frames in A. Rounding phases are also indicated by shading in A, B and C.



In the first exocytotic cycle (cycle 1 in Fig. 1),  $V_{CV}$  gradually increased (the fluid-filling phase,  $f_1$ ) until the CV rounded up as  $R_{CV}$  reached a maximum. The start of the rounding phase ( $r_1$ ) was not clearly defined in this cycle. The value of  $R_{CV}$  remained maximal (equal to 1) for approximately 20 s (during  $r_1$ ), while  $V_{CV}$  remained unchanged. The CV did not show fluid expulsion as would normally have been seen following rounding, but instead entered the fluid-filling phase ( $f_2$ ) of a second exocytotic cycle (cycle 2).  $V_{CV}$  then increased to an unusually large steady value during  $f_2$ , and the profile of the CV again became elliptical as  $R_{CV}$  decreased. The CV again rounded up as  $R_{CV}$  returned to 1 ( $r_2$ ), while  $V_{CV}$  remained unchanged. A few seconds after  $R_{CV}$  had again reached a maximum, fluid expulsion started, as shown by a rapid decrease in  $V_{CV}$  towards zero (the fluid-expulsion phase of cycle 2,  $e_2$ ). The CV then entered its third fluid-filling phase (cycle 3,  $f_3$ ) immediately after the end of fluid expulsion, as indicated by an increase in  $V_{CV}$ . The profile of the CV was again elliptical during  $f_3$ . A new steady value for  $V_{CV}$  was reached and this remained unchanged during the following rounding phase ( $r_3$ ) until another fluid expulsion started (not shown).

#### *Changes in the membrane area of the CV and the input capacitance of the contractile vacuole complex*

Changes in the membrane area of the CV,  $A_{CV}$ , and the input capacitance of the organelle during the three consecutive exocytosis events presented in Fig. 1A are shown in Fig. 2A,B, respectively, displayed on the same time scale as in Fig. 1B,C. The input capacitance remained unchanged at approximately 147 pF, while  $A_{CV}$  gradually increased to a steady value of approximately  $1.7 \times 10^3 \mu\text{m}^2$  during  $f_1$ . The mean input capacitance of this CV measured for eight consecutive fluid-filling phases was  $181.2 \pm 62.7$  pF ( $N=8$ ). The input capacitance began to decrease to approximately 18 pF immediately after the start of  $r_1$  and remained unchanged at this low level for approximately 10 s, while  $A_{CV}$  remained unchanged at the steady value. The mean input capacitance of this CV measured during seven consecutive rounding phases was  $15.2 \pm 6.8$  pF ( $N=7$ ).

The input capacitance then began to increase towards a steady value of approximately 171 pF towards the end of  $r_1$ , although  $A_{CV}$  remained unchanged at its steady  $r_1$  value. Approximately 5 s after the start of the increase in capacitance,  $A_{CV}$  began to increase towards a new higher steady value of approximately  $3.1 \times 10^3 \mu\text{m}^2$ , i.e. the fluid-filling phase of cycle 2 ( $f_2$ ) had begun. The input capacitance increased rapidly to reach its higher steady value at the beginning of  $f_2$  and remained unchanged at this level for most of  $f_2$ . At the same time,  $A_{CV}$  increased gradually to reach a plateau much later than the start of the capacitance plateau.

The input capacitance began to decrease again to a value of approximately 27 pF a few seconds after  $A_{CV}$  had reached a steady value at the start of  $r_2$ .  $A_{CV}$  decreased slightly during  $r_2$ . The CV then entered a fluid-expulsion phase ( $e_2$ ). The measurable membrane area decreased to zero as the CV

expelled its fluid to the exterior of the cell. Capacitance could not be determined during  $e_2$  since the current pulses for measuring the input capacitance escaped through the opened pore. Capacitance was again detectable as the organelle entered the fluid-filling phase ( $f_3$ ) and, as before, it increased quickly from its lowest value of approximately 0.7 pF (not shown in Fig. 2B because it is off the scale) to a steady value of approximately 148 pF. The input capacitance remained unchanged at this value, while  $A_{CV}$  gradually increased to a steady value of approximately  $1.7 \times 10^3 \mu\text{m}^2$  during  $f_3$ . The CV then entered its third rounding phase ( $r_3$ ).

#### *Changes in the membrane potential of the contractile vacuole complex*

The changes in the membrane potential with reference to the cytosolic potential during three consecutive exocytosis events (shown in Fig. 1A) are shown in Fig. 2C. The membrane potential of the CV remained unchanged at a steady level of approximately 63 mV ( $53.8 \pm 13.2$  mV,  $N=10$ ) during  $f_1$ , while the input capacitance remained unchanged at a high steady value (147 pF). The potential then decreased to a low level of approximately 10 mV ( $10.0 \pm 3.8$  mV,  $N=9$ ) immediately after the start of  $r_1$  as the input capacitance decreased. The membrane potential started to recover its former high level during late  $r_1$  and the early part of  $f_2$  as the input capacitance started to resume its previous high value. Both the membrane potential and the input capacitance remained unchanged at their respective high levels during the remainder of  $f_2$ .

The membrane potential began to decrease again immediately after the start of  $r_2$  as the input capacitance decreased. The membrane potential then suddenly changed to approximately 30 mV in conjunction with the start of fluid expulsion ( $e_2$ ). This potential corresponds to the surface membrane potential of the cell with reference to the cytosolic potential, since the CV fluid, where the tip of the microelectrode is located, is electrically connected to the external solution through the opened CV pore. The membrane potential then dropped to almost zero for a few seconds when the pore closed. It returned to its previous positive value as the input capacitance increased and fluid filling started ( $f_3$ ). The potential remained unchanged at this high level during  $f_3$  and early  $r_3$ , while the input capacitance remained unchanged at a higher steady level.

#### *Changes in the input resistance of the contractile vacuole complex*

Changes in the input resistance of the organelle (shown in Fig. 1A) are shown in Fig. 2D, and the corresponding specific input resistance during three consecutive exocytosis events is shown in Fig. 2E. The specific input resistance was determined by dividing the time constant of the electrical circuit equivalent to the organelle,  $\tau$ , by the specific membrane capacitance of a conventional biomembrane ( $1.0 \mu\text{F cm}^{-2}$ ) (Cole, 1968).

The input resistance (Fig. 2D) of approximately  $48 \text{ M}\Omega$  ( $31.2 \pm 15.0 \text{ M}\Omega$ ,  $N=9$ ) during  $f_1$  began to increase to a higher value immediately after the start of  $r_1$ , reaching a maximum of

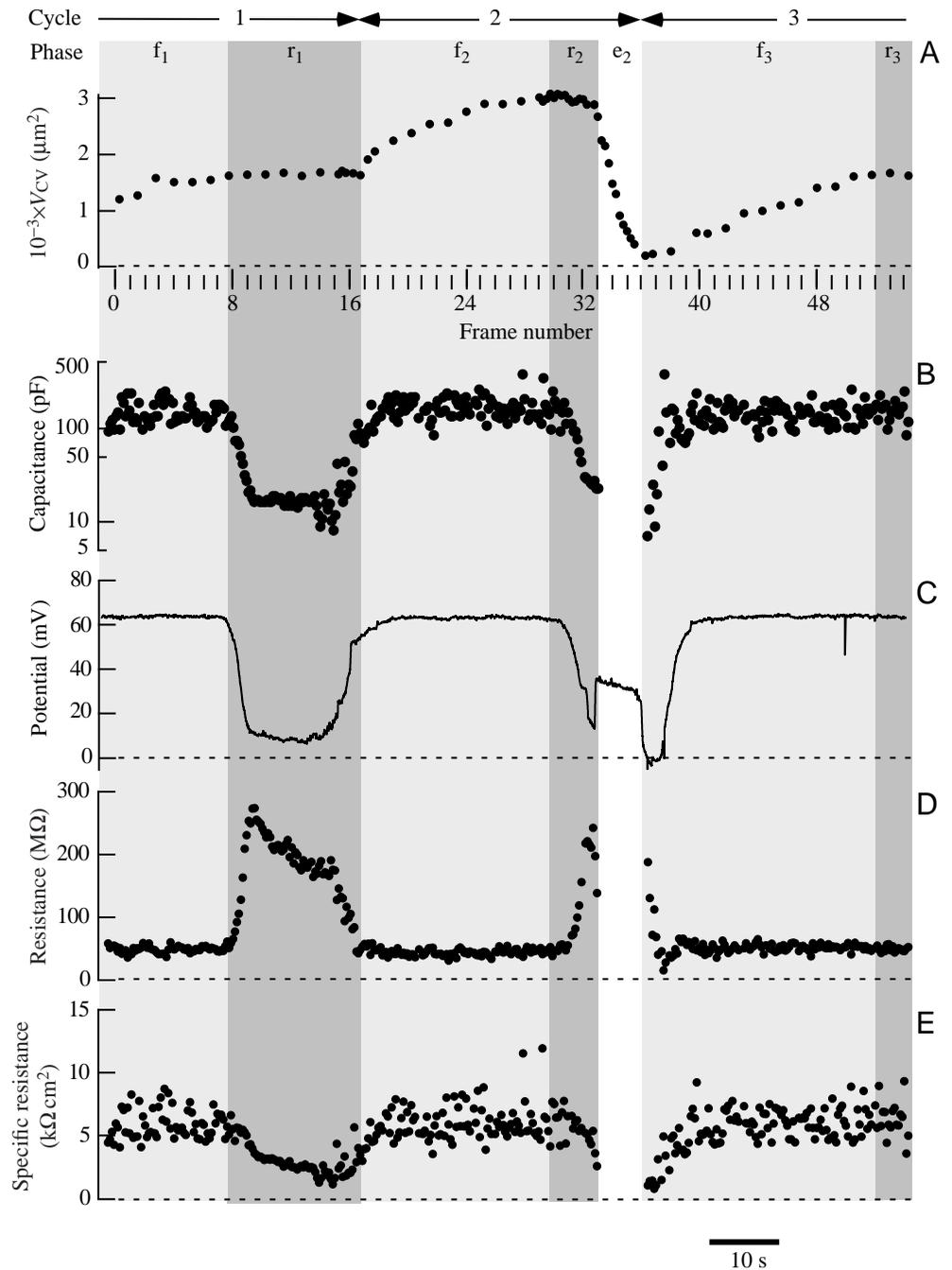


Fig. 2. Changes in the area of the contractile vacuole (CV) membrane ( $A_{CV}$ ; A), the input capacitance (B), the membrane potential (C), the input resistance (D) and the specific input resistance (E) in the same contractile vacuole complex of *Paramecium multimicronucleatum* as shown in Fig. 1 during three actual or attempted exocytotic cycles (cycles 1, 2 and 3). The frame numbers in A correspond to those in Fig. 1A (numbered 0–55), and the lettering, numbering and shading are as described in Fig. 1.

approximately  $276 \text{ M}\Omega$  ( $295 \pm 186 \text{ M}\Omega$ ,  $N=6$ ) in parallel with a decrease in the input capacitance (Fig. 2B). The input resistance gradually decreased to a lower value of approximately  $190 \text{ M}\Omega$ , while the input capacitance of the CV remained unchanged at its lower value for approximately 11 s. The input resistance then returned to its previous lower value in late  $r_1$  in parallel with increasing input capacitance. Both the input resistance and the input capacitance remained unchanged at their respective low and high values during  $f_2$ . The input resistance then increased again to its higher value immediately after the start of  $r_2$  in parallel with decreasing input capacitance. The input resistance then began to return to a lower value during  $r_2$  as it had during

$r_1$  but, since  $r_2$  was soon followed by  $e_2$ , the input resistance could not be determined after  $r_2$ . The input resistance was again measurable as  $f_3$  started. During  $f_3$ , resistance quickly returned to its low value mirroring the input capacitance, which remained unchanged at its high value.

The specific input resistance of the organelle (Fig. 2E) was approximately  $6.0 \text{ k}\Omega \text{ cm}^2$  during the fluid-filling phases. It decreased to approximately  $3.0 \text{ k}\Omega \text{ cm}^2$  as the input capacitance of the CV (Fig. 2B) decreased to its lowest value during the rounding phase. The specific resistance then gradually decreased to a lower level of approximately  $1.7 \text{ k}\Omega \text{ cm}^2$ , while the input capacitance remained unchanged

at its lowest value during the rounding phase. This was best observed during  $r_1$ , since the rounding phase was prolonged and was not followed by fluid expulsion.

*Morphological changes in the vesicles derived from the contractile vacuole complex in mechanically ruptured cells*

The membranes of the contractile vacuole and the radial

canals were fragmented and became membrane-bound vesicles of varying sizes when the cell was mechanically ruptured. A series of consecutive images of the vesicles in a ruptured cell is shown in Fig. 3A. The shape of the vesicle changed as it escaped from the cell through its ruptured portion.

To determine more easily the period of the cyclic changes in the roundness of these vesicles *in vitro*, we employed an

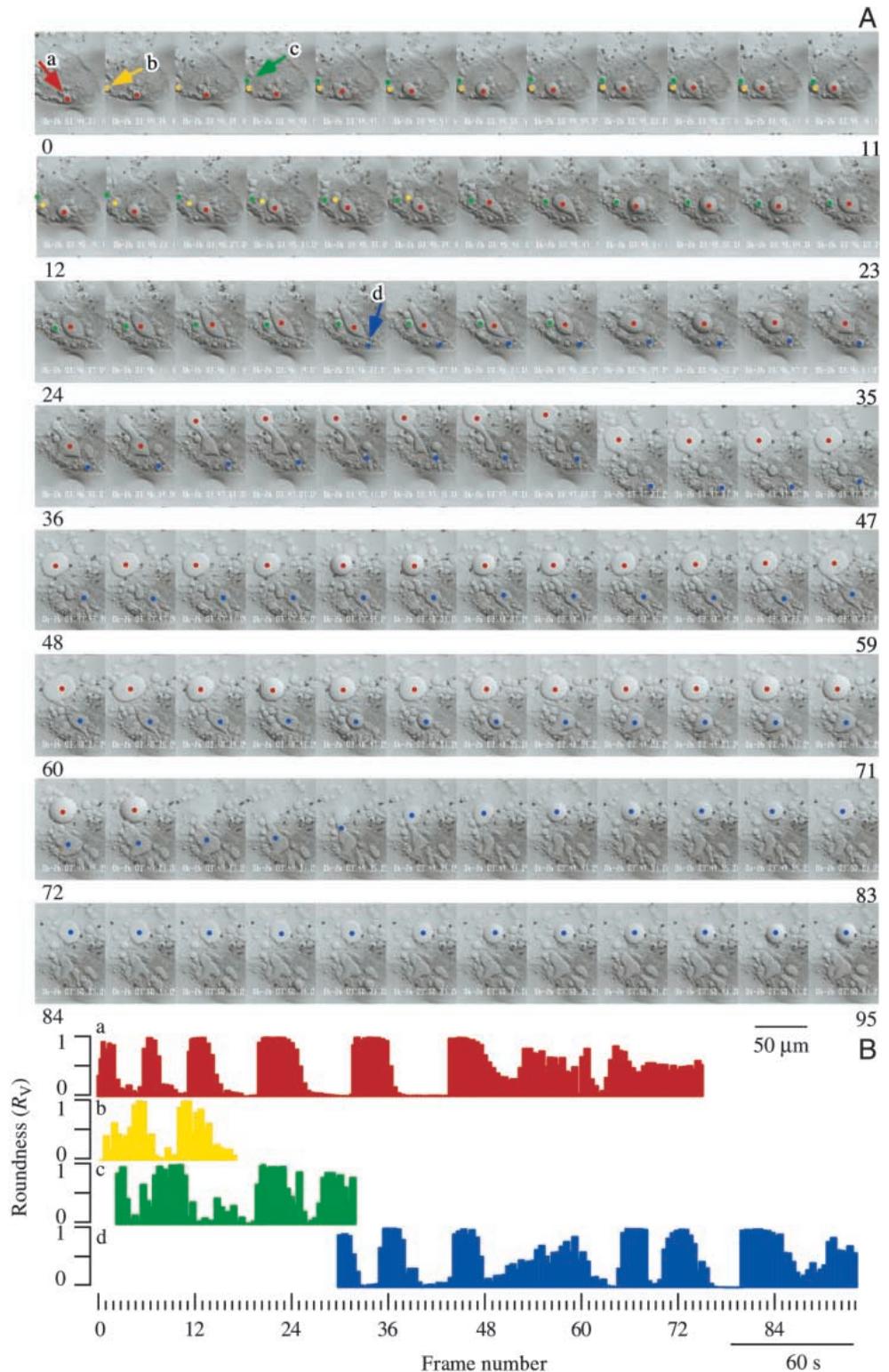


Fig. 3. A series of 96 consecutive images (numbered 0–95) showing morphological changes in the membrane-bound vesicles derived from the contractile vacuole complex after a cell of *Paramecium multimicronucleatum* was mechanically ruptured (A). The images were taken every 4 s. Four different vesicles (a, b, c, d), dotted in four different colours, were followed to determine changes in vesicle roundness ( $R_v$ ). Changes in the roundness of each vesicle can be followed by its corresponding colour (B). Each arrow with a corresponding colour points to a vesicle at the start of the roundness measurement. See text for details.

exaggerated new index of roundness,  $R_V$ , which is different from  $R_{CV}$  employed for the CV *in vivo* in the previous section (see the Appendix for the determination of  $R_V$ ).  $R_V$  is maximal (equal to 1) when the vesicle is spherical, while it is minimal (equal to 0) when the vesicle is elongated. The time course of change in  $R_V$  in four different vesicles (a–d) is shown in Fig. 3B.

Vesicle a, which is a remnant of the CV and is dotted red, rounded up as  $R_V$  (Fig. 3Ba) increased towards 1 (frame 2). The vesicle increased in size as it fused with the surrounding vesicles and elongated as  $R_V$  decreased towards zero (frames 3–4). The vesicle soon became rounded again (frames 5–6) as  $R_V$  became 1. The vesicle repeated this cycle of elongation (frames 7–10, 13–18) and rounding (frames 11–12, 19–22). The vesicle, which seemed at first to be attached to the cell surface membrane at the pore region of the CV, later became detached from the cell membrane as it became rounded (frame 20). The vesicle repeated the cycle of elongation (frames 23–30 and frames 34–40) and rounding (frames 31–33 and frames 41–45) even after it had become detached from the cell surface membrane and had moved away from the ruptured cytoplasm of the cell. The vesicle fused with surrounding vesicles at a time when it was elongated (frames 4, 18, 29, 60), whereas it left a portion of its membrane behind as it became rounded (frame 43). The vesicle was finally released from the cytoplasm into the dilute saline solution together with other cellular debris (frames 44–73). This *in vitro* vesicle also showed repeated cycles of rounding (frames 52, 64, 73) and slackening, although it did not always show elongation between two consecutive roundings. Instead, the vesicle underwent flattening due to compression by the silicone oil/water interface, so that it appeared to become larger in diameter during these periods. The vesicle finally disintegrated immediately after it showed a final rounding (frame 74). The intervals between each of the eight consecutive cycles of rounding were approximately 20, 25, 40, 50, 45, 55 and 55 s.

Vesicle b, which is dotted yellow, showed rounding twice (frames 4, 11) and then fused with vesicle a (frame 18). The period between these two roundings was approximately 23 s.

Vesicle c, which is dotted green, showed four consecutive roundings (frames 3, 11, 25, 31) before it fused with vesicle a (frame 32). The periods between these roundings were approximately 28, 50 and 35 s.

Vesicle d, which is dotted blue, exhibited eight consecutive roundings (frames 28, 33, 43, 58, 65, 71, 80, 95) until it disintegrated after it moved out of the cell into the standard saline solution (disintegration is not shown in Fig. 3). Changes in the shape and size of this vesicle were essentially identical to those exhibited by the remnant CV (vesicle a), with the difference that the vesicle was never attached to the cell membrane. The vesicle increased in size as it fused with other vesicles between the rounding phases. It left behind a portion of its membrane as it became rounded (frame 78). The intervals between seven successive roundings were approximately 25, 38, 48, 35, 25, 43 and 38 s.

It is important to note that fusions between vesicles from the

anterior contractile vacuole complex and the posterior contractile vacuole complex were frequently observed. However, fusion between vesicles derived from the contractile vacuole complex and food vacuoles was never observed.

## Discussion

### *Tension development in the CV membrane triggers severing of the radial arms from the CV*

Insertion of a microelectrode into the CV caused prolongation of the rounding phase and occasional abortion of fluid expulsion. Contractile vacuole complexes with such distorted exocytotic activity made it possible to determine the relationship between electrical events across the membrane of the organelle and its exocytotic-activity-related membrane dynamics more precisely than was possible from an organelle with normal exocytotic activity.

The volume of the CV increased while the input capacitance and the membrane potential of the organelle remained constant at their respective higher values during the first fluid-filling phase ( $f_1$ ; Figs 1B, 2B,C). In our previous paper (Tominaga *et al.* 1998), we demonstrated that the higher value for membrane capacitance corresponded to the overall membrane area of the whole organelle and that the higher value for the membrane potential probably corresponded mostly to the potential generated across the decorated spongiome membrane by V-type proton pumps. We therefore concluded that the radial arms are connected to the CV during the fluid-filling phase.

The CV then became rounded (frame 8; Fig. 1A,C) and remained rounded without showing fluid expulsion until it entered the second fluid-filling phase ( $f_2$ ) (frame 18; Fig. 1). In the regular exocytotic cycle, fluid expulsion takes place immediately after the CV shows rounding (Patterson, 1977; Tominaga *et al.* 1998). The input capacitance and the membrane potential decreased to their respective lower values immediately after the CV became rounded, and both remained low throughout the remainder of rounding ( $r_1$ ; Fig. 2B,C). According to our previous study (Tominaga *et al.* 1998), decreases in the capacitance and the potential correspond to severing of the radial arms from the CV, i.e. to isolation of the membrane of the CV from the membrane associated with the rest of the organelle. The radial arms then remain disconnected from the CV during the rounding phase. In a regular exocytotic cycle, severance is followed by opening of the pore of the CV. Rounding without fluid expulsion was observed quite frequently in an impaled CV, but fluid expulsion without rounding was never observed. On the basis of these observations, it is conceivable that the periodic rounding of the CV is one, if not the primary, event in the exocytotic cycle of the contractile vacuole complex.

During the second fluid-filling phase ( $f_2$ ), the CV slackened as its roundness,  $R_{CV}$ , decreased and the volume of the CV began to increase again until it had become more than twice as large as it had been at the end of  $r_1$ . The radial arms had rejoined with the CV during  $f_2$ , as indicated by the higher values for both input capacitance and membrane potential (Fig.

2B,C). The CV then entered into the next rounding phase ( $r_2$ ). It was clearly demonstrated in the rounding phase of this unusually large CV that decreases in both input capacitance and membrane potential were preceded by rounding of the CV (the start of rounding corresponds to frame 30, while the start of the decrease in input capacitance or membrane potential corresponds to frame 31). In other words, severing of the radial arms from the CV occurred after the CV had started rounding.

We postulate that rounding of the CV may correspond to an increase in the membrane tension of the CV. It is therefore highly probable that an increase in the tension of the CV membrane contributes to the severing of the radial arms from the CV and the subsequent opening of the pore. Conversely, a decrease in membrane tension triggered by slackening of the CV membrane due to fluid expulsion may lead both to the closing of the pore and to the fusion of the collapsed CV membrane with the radial arms. The failure of CV pore opening during  $r_1$  could mean that the increased tension in the CV membrane was not large enough to trigger CV pore opening or that the pore was blocked by some other means.

*Is the tension generated in the CV membrane large enough to cause severing of the radial arms from the CV?*

By employing a compression method (Cole, 1932), early workers demonstrated periodic changes in the tension of the plasma membrane accompanied by cell divisions in fertilized eggs of marine invertebrates, such as sea urchin and starfish (Dan, 1963; Hiramoto, 1963; Mitchison and Swann, 1954; Wolpert, 1966; Yoneda and Dan, 1972). The tension in the egg membrane changed in an approximate range from  $10^{-4}$  to  $10^{-3} \text{ N m}^{-1}$ . Tension became maximal immediately before the start of cleavage and reached a minimum after cleavage. Blastomeres, therefore, showed periodic rounding and slackening in association with cycles of cell division.

More recently, using a laser tweezer technique to measure tether force, Dai and Sheetz (1995) estimated that the tension in the plasma membrane of a blood cell varies between  $10^{-5}$  and  $10^{-4} \text{ N m}^{-1}$ . They pointed out that an increase in the tension in the plasma membrane enhances exocytosis and inhibits endocytosis. The tension of the plasma membrane is also deemed to be an important factor in controlling the shape of the cell and its motility (Sheetz and Dai, 1996).

Monck *et al.* (1990) and Monck and Fernandez (1992) found that a high membrane tension in the secretory granule of mast cells was the critical stress that brings about exocytotic fusion of granules. On the basis of their biophysical studies on the fusion of phospholipid bilayers and vesicles, Chernomordik *et al.* (1995) proposed that an increase in bilayer tension was a key factor for promoting fusion.

We estimated the tension in the CV membrane at the end of the rounding phase to be approximately  $2.6 \times 10^{-4} \text{ N m}^{-1}$  on the basis of the rate of fluid expulsion through the CV pore in a ruptured cell (see the Appendix). This value approximates that for the maximum membrane tension involved in cell division and for the tension that causes exocytotic membrane fusion. This coincidence between the magnitudes of these membrane

tensions supports the idea that the tension developed in the CV membrane is large enough to cause the severing of the radial arms from the CV and subsequent pore opening. In this connection, we have previously demonstrated that the tension in the CV membrane immediately before fluid expulsion, i.e. at the end of the rounding phase, determines the pore size of the CV (Naitoh *et al.* 1997a). The mechanism(s) by which tension leads to these exocytosis-associated membrane interaction remains unknown.

*The tension development mechanism(s) resides in the CV membrane*

When the *Paramecium multimicronucleatum* cell was mechanically ruptured, the smooth membrane of the contractile vacuole complex (membranes from the CV and the radial arms) became membrane-bound vesicles of differing sizes (Fig. 3A). These *in vitro* vesicles showed a periodic rounding and slackening at more or less regular intervals. This was true not only of vesicles in the cytoplasm but also of those in a saline solution after they had been extruded from ruptured cells (Fig. 3B). These findings strongly support the idea that the periodic development of tension is an intrinsic characteristic of the smooth spongiome membrane (together with any necessary associated cytoskeletal structure) and exists apart from the fluid segregation activity of the decorated spongiome. The fact that the different vesicles showed independent rounding cycles, even when free of the cytoplasm, suggests that water-soluble factors in the cytosol may not be directly involved in regulating the cycle.

A vesicle will sometimes show fission when it is more or less rounded, i.e. when tension in the vesicle membrane is presumably high. In contrast, vesicles sometimes show fusion when their membranes are slack, i.e. when their tension is presumably low (Fig. 3A). These fission and fusion events in or between *in vitro* vesicles seem to correspond to severing and joining of the CV with the radial arms, as occurs in the *in vivo* contractile vacuole complex.

*Mechanism for the development of tension in the CV membrane*

It is likely that membrane is recruited to the CV from the contractile vacuole complex itself when the CV swells during the fluid-filling phase. If this membrane supply is somehow disrupted, the tension in the existing CV membrane would be expected to increase as the CV swells. This increased tension would cause rounding of the CV.

As noted above, we found that the rounding of the CV started before the CV membrane became isolated from the rest of the organelle membrane. This implies that the supply of membrane to the CV stops before the isolation of the CV from its arms. This was clearly demonstrated during rounding phase  $r_2$ , in which decreases in input capacitance and membrane potential were preceded by an increase in the roundness of the CV,  $R_{CV}$  (Figs 1C, 2B,C). At first, it was assumed that fluid filling continued after the supply of membrane had stopped and, therefore, the CV became

rounded. However, we then observed that the roundness increased even though the volume of the CV remained unchanged at its maximum level during  $r_2$  (Fig. 1B,C). This implies that the tension increase during  $r_2$  is not caused by fluid filling. Furthermore, the membrane area of the CV decreased slightly in the later phase of  $r_2$ , especially after the CV membrane had become isolated from the membrane of the rest of the organelle (Fig. 2A,B). These facts strongly support the idea that the CV itself possesses the mechanism(s) by which the amount of more or less planar CV membrane (the apparent surface area of the CV) is controlled, i.e. can stop the supply of planar membrane to the CV and retrieve excess planar membrane from the CV. This idea is consistent with our previous conclusion that one means of tension development resides in the CV membrane.

In two previous papers (Naitoh *et al.* 1997a,b), we demonstrated the presence of tension in the CV membrane. We also demonstrated in electron micrographs of the CV taken during the normal fluid-expulsion phase that the CV membrane was transformed into 40 nm tubules, and that the tubule membrane was continuous with the CV membrane (Naitoh *et al.* 1997a). Furthermore, we were able to estimate the bending energy that came to be stored in the more or less planar CV membrane as the 40 nm tubules were expanded into the planar CV membrane (Naitoh *et al.* 1997b). The calculated stored bending energy corresponded well with the work subsequently done by the *in vitro* CV during fluid expulsion occurring in the absence of cytosolic pressure, which could be effectively eliminated by cell rupturing. It should be noted that the cytosolic pressure is the main cause of the much faster fluid expulsion from the CV *in vivo*. We therefore proposed the hypothesis that the transfer of bending energy from the membrane being transformed into 40 nm tubules to the planar membrane of the rounded CV leads to an increase in tension in the remaining planar membrane during the rounding phase.

Here, we propose the overall hypothesis that a periodic change in the tendency of the CV membrane to transform into 40 nm tubules (tubulation activity) is responsible for the periodicity of tension development in the CV membrane and, therefore, for the exocytotic cycle of the CV. That is, tubulation activity begins to increase at the beginning of the rounding phase so that the CV membrane starts to transform into 40 nm tubules. This results in a decrease in the ratio of the effective surface area of the CV to its volume. The tension in the membrane increases as the surface area to volume ratio decreases, and it reaches a maximum as this ratio becomes minimal, i.e. as the CV becomes spherical ( $R_{CV}=1$ ). As previously mentioned, this increase in membrane tension leads to severing of the radial arms from the CV and the subsequent opening of the pore.

Fluid expulsion leads to slackening of the CV membrane, i.e. a decrease in membrane tension and the transformation of the planar membrane into 40 nm tubules. This is true of the normal expulsion process, in which the rate of tubulation is slower than the rate of fluid expulsion (Naitoh *et al.* 1997b). A

decrease in membrane tension causes closure of the pore and subsequent reattachment of the radial arms to the CV. The CV then enters a new fluid-filling phase. At this time, the tubulation activity is somehow decreased, so that the CV can again receive fluid from the swollen ampullae. The ampullae typically fill as cytosolic water continues to cross their membrane while the CV is in its expulsion phase (Patterson, 1980).

When the CV pore fails to open, as in  $r_1$ , the tubulation activity is somehow decreased at the end of rounding so that any 40 nm tubules associated with the CV membrane can again be used to form additional planar membrane. This decreased tubulation activity also allows reattachment of the radial arms to the CV and allows the CV to enter a new fluid-filling phase. The CV can then swell as additional fluid enters from the ampullae and as additional membrane is supplied from any excess CV-associated tubules. This implies that tubulation activity decreases at the end of the rounding phase and that CV membrane tension decreases independently of fluid expulsion, supporting the idea that the tubulation activity cycle primarily governs the exocytotic cycle.

The mechanism by which tubulation activity is periodically controlled remains unknown. One possibility is that a cytoskeletal factor or factors may be involved. Studies on the close association between the CV membrane and microtubular ribbons (Naitoh *et al.* 1997a) may lead to an understanding of how such an association could be involved in a periodic change in tubulation activity in both *in vivo* vacuoles and *in vitro* vesicles.

#### *Estimation of the difference in osmolarity between the CV fluid and the cytosol*

In our most recent paper (Tominaga *et al.* 1998), we proposed a hypothesis for the water segregation mechanism in the contractile vacuole complex of *Paramecium multimicronucleatum*. First, the V-type proton pumps in the decorated spongiome membrane generate an electrochemical potential gradient across the organelle membrane. This potential allows the flow of counter-anions from the cytosol into the lumen of the complex. The anions then act as an osmolyte to pull cytosolic water into the lumen through water channels postulated to be present throughout the smooth membrane of the complex.

The observation that the CV ceased to increase in size during the early part of the rounding phase, even though the radial arms were still connected to it (Fig. 2;  $r_1$ ,  $r_2$ ), suggests that entry of water from the radial canals may be prevented by an increase in internal pressure in the CV due to an increase in membrane tension. After severing of the radial arms from the CV, its volume remained unchanged for a short time before the start of fluid expulsion, when roundness was at a maximum (equal to 1) (Fig. 2;  $r_2$ ). In this period of the exocytotic cycle, therefore, the internal pressure in the CV caused by membrane tension is equal to the osmotic pressure difference between the CV fluid and the cytosol. Therefore, the difference in osmolarity between the CV fluid and the cytosol,  $\Delta C$ , can be

estimated from the internal pressure of the CV,  $P_{CV}$ , according to van't Hoff's formula for a dilute solution:

$$\Delta C = \frac{P_{CV}}{RT}, \quad (2)$$

where  $R$  is the gas constant ( $8.3 \text{ N m mol}^{-1} \text{ K}^{-1}$ ) and  $T$  is absolute temperature,  $290 \text{ K}$  ( $17^\circ \text{C}$ ). The osmolyte concentration difference calculated by introducing  $46 \text{ N m}^{-2}$  for  $P_{CV}$  (see the Appendix) is approximately  $1.9 \times 10^{-2} \text{ mosmol l}^{-1}$ . This implies that the CV fluid is only slightly hypertonic, or virtually isotonic, to the cytosol immediately before it is expelled to the exterior of the cell.

Patterson (1977) reported that the volume of the CV decreased as it became rounded immediately before the start of fluid expulsion. If the tension developed in the CV membrane is relatively large, the internal pressure generated might be strong enough to push water from the CV back into the cytosol (superfiltration), or into the radial canals if they are still connected to the canals, causing a decrease in the volume of the CV. However, we did not observe a measurable decrease in the volume of the CV at the end of the rounding phase.

#### Conductance properties of the CV membrane

We have demonstrated a marked decrease in the specific input resistance of the organelle (to approximately half its initial value) in association with the severing of the CV membrane from that of the rest of the organelle (Fig. 2E,  $r_1$ ). This implies that the specific conductance of the CV membrane is higher than that of the rest of the organelle membrane (approximately  $3.3 \times 10^{-4} \text{ S cm}^{-2}$  for the isolated CV compared with  $1.7 \times 10^{-4} \text{ S cm}^{-2}$  for the whole organelle). The membranes of the collecting canals and the smooth spongiomes are similar to the CV membrane in their electron microscopic appearance (R. D. Allen, unpublished observation). These membranes are termed the 'smooth spongiome' membrane, because they do not have 'pegs', which correspond to the V-type proton pumps (Fok *et al.* 1995). According to electron microscope examinations of the contractile vacuole complex, the smooth membrane area is approximately one-quarter of the total membrane area of the organelle (R. D. Allen, unpublished data). Thus, the membrane area of the decorated spongiome constitutes approximately three-quarters of the total membrane area of the contractile vacuole complex. The specific conductance of the decorated spongiome membrane can, therefore, be estimated to be approximately  $1.1 \times 10^{-4} \text{ S cm}^{-2}$  (see the Appendix). The decorated spongiome membrane possesses so many pegs that it would probably not be possible for this membrane to possess all the protein complexes corresponding to the ion channels and/or water channels that would appear to be needed for the high conductance expected of the whole organelle. However, in our previous study (Tominaga *et al.* 1998), we demonstrated the presence of what might be water channels in an electron micrograph of the smooth membrane of the contractile vacuole complex. It has

recently been reported that aquaporin, a water channel, shows an ionic conductance, although it is rather low and the single aquaporin conductance has not yet been estimated (Regan *et al.* 1997; Yool *et al.* 1996).

We also found that the specific input resistance of the CV during the rounding phase (as is clearly shown in  $r_1$ ) decreased with time, while the membrane potential remained essentially unchanged. At present, we do not know whether the stretching of this membrane sufficiently to generate a membrane tension of  $2.6 \times 10^{-4} \text{ N m}^{-1}$  (see the Appendix) could increase the conductance of water channels or of some other ion channels in the CV membrane. It is interesting to note here that the threshold membrane tension needed to activate mechanosensitive ion channels was reported to be  $5 \times 10^{-4}$  to  $10^{-3} \text{ N m}^{-1}$  in the vertebrate hair cell membrane (Howard *et al.* 1988) and  $4 \times 10^{-3}$  to  $5 \times 10^{-3} \text{ N m}^{-1}$  in the yeast plasma membrane (Gustin *et al.* 1988).

Electrophysiological examinations of cellular membranes in relation to tension generation, such as that in the contractile vacuole complex, are needed for a more complete understanding of the biological significance of membrane tension in the control of organelle activities.

#### Appendix

##### Determining the index of roundness of a membrane-bound vesicle

The index of roundness of the vesicle,  $R_V$ , is obtained as follows. The ratio of the width to the length of a given vesicle ( $r_0$ ) is determined every 2 s from video images grabbed into a computer and plotted against time (Fig. 4A). This time course of changes in the ratio is smoothed by Gaussian filtration ( $r_s$ ; Fig. 4B). Subtraction of  $r_s$  from  $r_0$  gives the time course of changes in the difference,  $x$  ( $r_0 - r_s$ ), which is positive when the ratio increases and negative when the ratio decreases (Fig. 4C). To exaggerate an increase in the roundness,  $x$  is translated into  $R_V$  according to a sigmoid function:

$$R_V = \frac{1}{1 + \exp(-x/a)}, \quad (\text{A1})$$

where  $a$  is a constant. The value for  $a$  is arbitrarily chosen so that  $R_V$  becomes 1 when the ratio increases or the vesicle tends to round up. It becomes zero when the ratio decreases or the vesicle tends to elongate. The value employed was 0.5.  $R_V$  obtained in this manner is shown in Fig. 4D.

##### Estimation of the tension in the CV membrane

In a previous paper (Naitoh *et al.* 1997b), we determined the rate of fluid expulsion from a contractile vacuole,  $dV_{CV}/dt$  (where  $V_{CV}$  is the volume of the CV and  $t$  is time), in a mechanically ruptured cell of *Paramecium multimicronucleatum* to be approximately  $4.5 \times 10^3 \mu\text{m}^3 \text{ s}^{-1}$ . Since the cytosolic (turgor) pressure is effectively eliminated by rupturing the cell, the CV fluid is driven out through the CV pore only by the pressure difference between the interior and

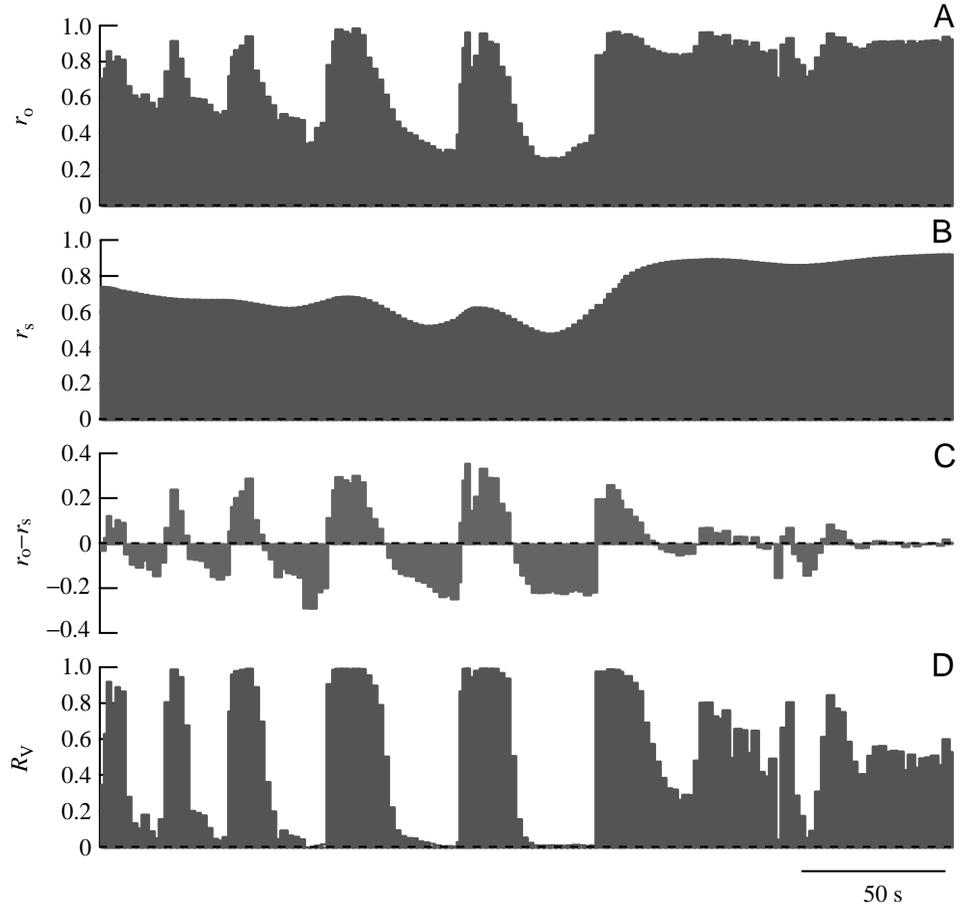


Fig. 4. Procedures for determining the index of roundness,  $R_V$ , of an *in vitro* membrane-bound vesicle derived from the contractile vacuole or the ampullae of *Paramecium multimicronucleatum*. The data used in this figure are the same as those used for Fig. 3Ba. See text for details.  $r_0$ , ratio of the width to the length of a vesicle;  $r_s$ , smoothed ratio.

the exterior of the CV (the internal pressure of the CV,  $P_{CV}$ ) generated by the tension in the CV membrane ( $T_{CV}$ ) (Harvey, 1954). The relationship between  $P_{CV}$  and the rate of fluid expulsion can be formulated according to the law of Hagen and Poiseuille as:

$$\frac{dV_{CV}}{dt} = \frac{\pi D_P^4 P_{CV}}{128 \eta L_P}, \quad (\text{A2})$$

where  $\eta$  is the viscosity of the fluid,  $D_P$  is the diameter of the cross section of the CV pore and  $L_P$  is the length of the pore.  $\eta$  is assumed to be equal to the viscosity of water,  $10^{-3} \text{ N m}^{-2} \text{ s}$ . Values for  $D_P$  and  $L_P$  are  $1.7 \mu\text{m}$  and  $2.1 \mu\text{m}$ , respectively, determined from electron micrographs of CVs obtained from cells equilibrated in a dilute saline solution (Naitoh *et al.* 1997a). The value for  $P_{CV}$ , obtained by introducing values for the appropriate variables into equation A2, was found to be approximately  $46 \text{ N m}^{-2}$ .

The relationship between the tension in the membrane,  $T_{CV}$ , and the internal pressure,  $P_{CV}$ , in a spherical contractile vacuole is represented by the Laplace formula:

$$P_{CV} = \frac{4T_{CV}}{D_0}, \quad (\text{A3})$$

where  $D_0$  is the diameter of the CV immediately before fluid expulsion. The tension, calculated by introducing  $46 \text{ N m}^{-2}$  for

$P_{CV}$  and  $23 \mu\text{m}$  for  $D_0$  (Naitoh *et al.* 1997b) into equation A3, was found to be approximately  $2.6 \times 10^{-4} \text{ N m}^{-1}$ .

#### Estimation of the specific conductance of the decorated spongiome membrane

The conductance of the overall contractile vacuole complex is the sum of all conductances and can be written as;

$$gA = g_s A_s + g_d A_d, \quad (\text{A4})$$

where  $g$  is the specific conductance of the total membrane of the organelle,  $g_s$  is the specific conductance of the smooth spongiome membrane,  $g_d$  is the specific conductance of the decorated spongiome membrane,  $A$  is the membrane area of the overall organelle,  $A_s$  is the membrane area of the smooth spongiome membrane and  $A_d$  is the membrane area of the decorated spongiome membrane. The total membrane area,  $A$ , can be written as:

$$A = A_s + A_d. \quad (\text{A5})$$

According to electron microscope examinations of the contractile vacuole complex (R. D. Allen, unpublished data), the smooth spongiome membrane area is approximately one-quarter of the total membrane area of the organelle.  $A_s$  can therefore be written as:

$$A_s = A/4. \quad (\text{A6})$$

By introducing equations A5 and A6 into equation A4,  $g_d$  can be written as:

$$g_d = \frac{4}{3} g - \frac{1}{3} g_s. \quad (\text{A7})$$

The value of  $g_s$ , approximately  $3.3 \times 10^{-4} \text{ S cm}^{-2}$ , is the reciprocal of the specific input resistance of the CV when it is isolated from the radial arms during  $r_1$  (Fig. 2E), and the value of  $g$ , approximately  $1.7 \times 10^{-4} \text{ S cm}^{-2}$ , is the reciprocal of the specific input resistance of the contractile vacuole complex when the CV is connected to the radial arms during  $f_1$ ,  $f_2$  and  $f_3$ . The value for  $g_d$  is, therefore, approximately  $1.1 \times 10^{-4} \text{ S cm}^{-2}$ .

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