

PERIODIC TENSION DEVELOPMENT IN THE MEMBRANE OF THE *IN VITRO* CONTRACTILE VACUOLE OF *PARAMECIUM MULTIMICRONUCLEATUM*: MODIFICATION BY BISECTION, FUSION AND SUCTION

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Summary

The contractile vacuole of the freshwater protozoan *Paramecium multimicronucleatum* is a membrane-bound exocytotic vesicle that expels excess cytosolic water. The *in vitro* contractile vacuole isolated from *P. multimicronucleatum* along with a small amount of cytosol and confined under mineral oil showed periodic rounding and slackening at fairly regular intervals. Activity lasted for over 30 min at room temperature (24–27 °C). The rounding of the *in vitro* contractile vacuole corresponded to the increased membrane tension of the *in vivo* contractile vacuole that occurs immediately before fluid expulsion. Unlike the *in vivo* contractile vacuole, the *in vitro* contractile vacuole did not expel fluid, since it lacked a mechanism to form a pore. The subsequent slackening of the *in vitro* contractile vacuole corresponded to the fluid-filling phase of the *in vivo* contractile vacuole that occurs at decreased membrane tension. Fluid filling occurred in the *in vitro* contractile vacuole only when it was isolated together with its radial arms. *In vitro* membrane-bound vesicles obtained by ‘bisecting’ (although the two parts were not always identical in size) an *in vitro* contractile vacuole established their own independent rounding–slackening cycles. *In vitro* contractile vacuole vesicles could fuse again when the vesicles slackened. The fused vesicle then showed a rounding–slackening cycle with a period closer to that of the vesicle that exhibited the shorter cycle period. An additional rounding phase of the *in vitro*

contractile vacuole could be induced by applying suction to a portion of its membrane with a micropipette when the contractile vacuole was in its slackened phase. This suggests that maximum tension development in the contractile vacuole membrane can be triggered when tension is increased in any part of the contractile vacuole membrane. The time from the start of an extra rounding phase to the next spontaneous rounding and for subsequent rounding–slackening cycles was nearly the same as that before the extra rounding phase. This implies that there is no master pacemaker to control the rounding–slackening cycle in the contractile vacuole membrane. Severed radial arms also became vesiculated and, like contractile vacuole membranes, these *in vitro* vesicles showed independent rounding–slackening cycles and vesicle–vesicle fusions. Thus, membrane derived from the radial arm seems to be identical in its tension-developing properties with the contractile vacuole membrane. ATP was found to be required for contractile vacuole rounding but inhibitors of actin or tubulin polymerization, such as cytochalasin B and Nocodazole, had no effect on the *in vitro* contractile vacuole’s rounding–slackening cycle.

Key words: contractile vacuole, rounding–slackening cycle, membrane, tension, membrane fusion, suction, tension-induced tension development, *Paramecium multimicronucleatum*.

Introduction

The contractile vacuole complex is a membrane-bound osmoregulatory organelle of *Paramecium* through which excess cytosolic water, acquired osmotically, is expelled from the cell in order to maintain a constant cytosolic osmolarity. The organelle is composed of a central contractile vacuole surrounded by 5–10 radial arms. Each radial arm consists of an ampulla adjacent to the contractile vacuole, a collecting canal continuous with the ampulla, a smooth spongiome that branches from the collecting canal and a decorated spongiome

that is continuous with the smooth spongiome at its inner periphery and ends blindly in the cytosol at its outer periphery (Hausmann and Allen, 1977).

It has been observed that the contractile vacuole rounds up just before fluid expulsion (rounding phase) (Patterson, 1980; Patterson and Sleight, 1976). By using electrophysiological techniques, we were previously able to demonstrate (Tominaga et al., 1998a) that rounding of the contractile vacuole began immediately before severing of the radial arms from the

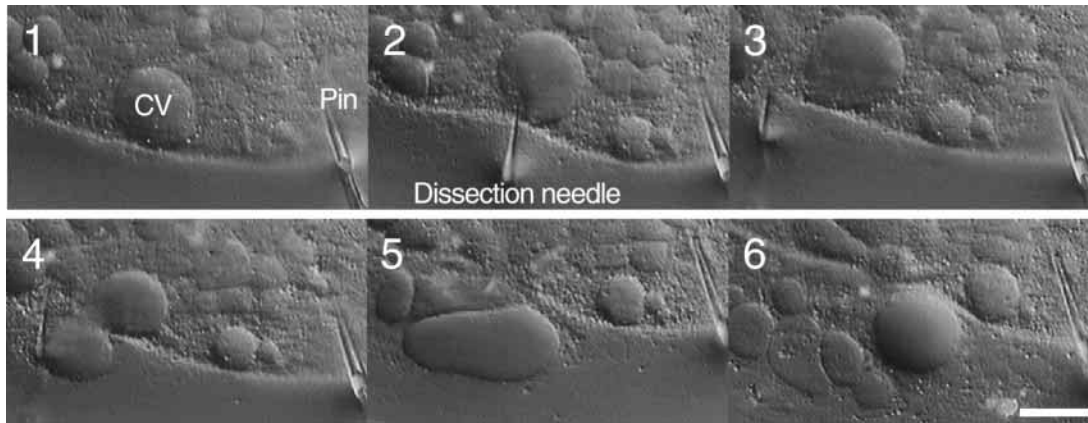


Fig. 1. Dissection procedures for the isolation of the contractile vacuole and its surrounding radial arms from *Paramecium multimicronucleatum*. Frame 1, a portion of a compressed cell, in which a contractile vacuole (CV) is seen to attach to the cell membrane. Pin, the microneedle used to hold the cell onto the glass slide. Frames 2, 3, tearing the cell membrane with the tip of a dissection needle. Frame 4, the contractile vacuole being extruded from the cell through the torn portion of the cell membrane. Frame 5, the isolated contractile vacuole in its slackening phase. Frame 6, the isolated contractile vacuole is rounded. Scale bar, 20 μm .

contractile vacuole, which could be detected as a marked decrease in the input capacitance of the contractile vacuole complex. Rounding of the contractile vacuole was maximal immediately before fluid expulsion. Since rounding of the contractile vacuole corresponds to an increased tension in the contractile vacuole membrane, it is assumed that the membrane tension of the contractile vacuole plays an important role in severing of the radial arms and in the subsequent opening of the pore of the contractile vacuole. This periodic membrane tension development seems to control the membrane dynamics associated with the exocytotic activity of the contractile vacuole complex.

Tominaga et al. (1998b) also found that membrane-bound vesicles derived from the contractile vacuole complex underwent independent rounding–slackening cycles when isolated from the ruptured cell. This suggests that the membrane of the contractile vacuole complex possesses an inherent mechanism by which its tension can be periodically controlled.

To understand the mechanism(s) by which the contractile vacuole membrane tension is periodically controlled, we examined the rounding–slackening cycles of vesicles derived from the contractile vacuole and its surrounding radial arms, which had been microdissected from *Paramecium multimicronucleatum* into a small amount of surrounding cytosol and confined under mineral oil. Special attention was given to the following: (1) the relationship between the phases of the rounding–slackening cycles of the two membrane-bound vesicles obtained by ‘bisecting’ one isolated *in vitro* contractile vacuole; (2) how a cycle is re-established after the two vesicles of the bisected contractile vacuole were allowed to fuse again into one vesicle; (3) the effects of a localized increase in the tension of the membrane of the contractile vacuole, achieved by applying suction to the membrane through a micropipette, on the rounding–slackening cycle; (4) the effects of some inhibitors of actin or tubulin polymerization on the rounding–slackening

cycle, to determine the possible involvement of actin filaments and/or microtubules in rounding of the contractile vacuole; and (5) the ATP requirement for rounding of the contractile vacuole.

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 harvested at the mid-logarithmic growth phase. These cells were washed with standard saline solution containing (final concentration in mmol l^{-1}) 4.0 KCl, 1.0 CaCl_2 and 20 Mops-KOH buffer (pH 7.0). The cells were equilibrated in the solution for more than 4 h prior to experimentation (Naitoh et al., 1997).

Isolation of the contractile vacuole and radial arms from the cell

Procedures for isolating the contractile vacuole and radial arms from the cell are shown in Fig. 1. An equilibrated cell was placed into a small droplet of saline solution under mineral oil on a glass slide. Excess saline was pipetted out of the droplet so that the cell was compressed by the saline–oil boundary (frame 1). The cell was then ruptured by tearing the cell membrane near the contractile vacuole pore region with the tip of a fine dissection microneedle (frames 2, 3). The contractile vacuole and radial arms were then squeezed out of the cell (frames 4–6). The remaining portions of the ruptured cell were removed from the droplet using the microneedle. The contractile vacuole remained in a small droplet of the cytosol under mineral oil together with some radial arms and other organelles such as digestive vacuoles. *In vitro* contractile vacuoles showed rounding and slackening cycles for more than 30 min when held at room temperature (24–27 °C).

Recording and analysis of the images of the *in vitro* contractile vacuole

To examine changes in shape of the *in vitro* contractile vacuole and/or radial arms, images obtained using differential interference contrast (DIC) optics (Leica Fluotar $\times 63$, numerical aperture 0.7 objective lens on a model DMIRB inverted microscope, Leica Inc. Deerfield, IL, USA) were continuously recorded on VHS videotape using a video cassette recorder (VCR; AG 6300, Panasonic Industrial Co., Secaucus, NJ, USA) after capture by a CCD camera (CCD-72, Dage MTI Inc., Michigan City, IN, USA). Morphological changes in the *in vitro* contractile vacuole and radial arms were analyzed using the software packages NIH Image 1.60 (<http://rsb.info.nih.gov/nih-image/>) and Canvas 5.0 (Deneva Software, Miami, FL, USA) in a computer (Power Macintosh 7600/132, Apple Computer Inc., Cupertino, CA, USA).

To display the rounding and slackening cycles graphically, the apparent area of each vesicle, as seen from above, was measured and plotted against each corresponding time. The area was larger when the vesicle slackened, since the tension in the vesicle membrane was low, allowing the vesicle to be compressed by the saline–oil boundary tension and, thus, to spread out. Conversely, the area was smaller when the vesicle rounded, since the tension in the vesicle membrane was high, permitting the vesicle to become rounded against the saline–oil boundary tension.

Suction of the *in vitro* contractile vacuole membrane and photoelectric detection of the start of rounding

A minute portion of the membrane of the *in vitro* contractile vacuole was sucked into a microcapillary suction pipette (the tip is approximately $1\ \mu\text{m}$ in inner diameter, heat-polished and filled with the standard saline solution) by lowering the internal pressure of the capillary by sucking air out of the capillary by mouth through Teflon tubing. The moment of membrane suction was detected by monitoring an increase in the electrical resistance of the capillary as it becomes plugged with membrane. The increase in resistance was displayed as an increase in the output voltage of a constant current stimulator, from which $1\ \text{nA}$ was constantly supplied to the pipette.

To monitor the start of rounding of the *in vitro* contractile vacuole, a position-sensitive photodetector (Kamimura, 1987) was placed at the edge of the image of the contractile vacuole on an image plane in the vertical cylinder of the microscope triocular attachment. The output voltage of the detector changed as the edge of the contractile vacuole moved in association with rounding and was recorded simultaneously with the electrical signal corresponding to the moment of membrane suction. These signals were used to examine the time relationship between membrane suction and the start of suction-induced rounding.

The electrical signals were fed into the computer through an AD/DA converter (ITC-16; Instrutech Corp. Great Neck, NY, USA) and Igor Pro (WaveMetrics, Inc., Lake Oswego, OP, USA) and PulseControl XOP software packages (Herrington et al., 1995). Images of the contractile vacuole were video-

recorded together with the electric signals to make it possible to correlate the morphological changes of the contractile vacuole with the electrical signals.

Application of chemicals to the *in vitro* contractile vacuole

Saline solution containing a test chemical was introduced into a micropipette with an inner tip diameter of approximately $1\ \mu\text{m}$. The tip was placed approximately $5\ \mu\text{m}$ away from the *in vitro* contractile vacuole, and approximately $20\ \text{pl}$ of the chemical-containing solution was squirted against the contractile vacuole. The chemicals employed were EGTA, Nocodazole {methyl-[5-(2-thienylcarbonyl)-1-H-benzimidazol-2-yl] carbamate}, Colcemid (demecolcine; *N*-deacetyl-*N*-methylcolchicine), colchicine, cytochalasin B and concanamycin B. All the chemicals except concanamycin B were obtained from Sigma (St Louis, MO, USA). Concanamycin B was obtained from Dr Miwa of Ajinomoto Co. Ltd, Shizuoka, Japan.

Cell perfusion

To perfuse the *Paramecium multimicronucleatum* cell with a saline solution, the tip of a micropipette (approximately $1\ \mu\text{m}$ in inner diameter) filled with perfusion solution was inserted into the cell, and the solution was injected into the cell using a microsyringe (Hamilton Co., Reno, NV, USA). Injection of the solution caused the cell to swell and rupture. After rupture, the perfusion solution passed throughout the cell as it was being injected. The total amount of solution that passed through the cell was more than $2\ \text{nl}$ (10 times as much as the cell volume). The perfusion solution consisted of (final concentration in mmol l^{-1}) 10 EGTA, 30 KCl, 1 GTP (Sigma, St Louis, MO, USA), 0.04 Taxol (paclitaxel; Sigma, St Louis, MO, USA) and 20 Mops-KOH (pH 7.0).

Results

Rounding–slackening cycles of the *in vitro* contractile vacuole and radial arms

Fig. 2 shows 20 consecutive images of a representative *in vitro* contractile vacuole and its radial arm taken every 1.6 s to show morphological changes over time. The *in vitro* contractile vacuole (CV in frame 1) continued rounding (frames 1–10) and slackening (frames 11–20) after it had been isolated from the cell. Three consecutive rounding–slackening cycles of this *in vitro* contractile vacuole are shown as changes in its apparent area in Fig. 3. The period of each cycle was approximately 30 s.

As shown in Fig. 2, an *in vitro* radial arm became visible as it swelled, while the contractile vacuole was rounding (RA in frame 5). The radial arm continued to swell to its maximum degree (frame 17) until it fused with the contractile vacuole (frame 18), at a time when the contractile vacuole was slackening after it had attained its maximum rounding (frame 10). The radial arm then became less visible as its fluid entered the contractile vacuole after it had fused with the contractile vacuole (frames 18–20).

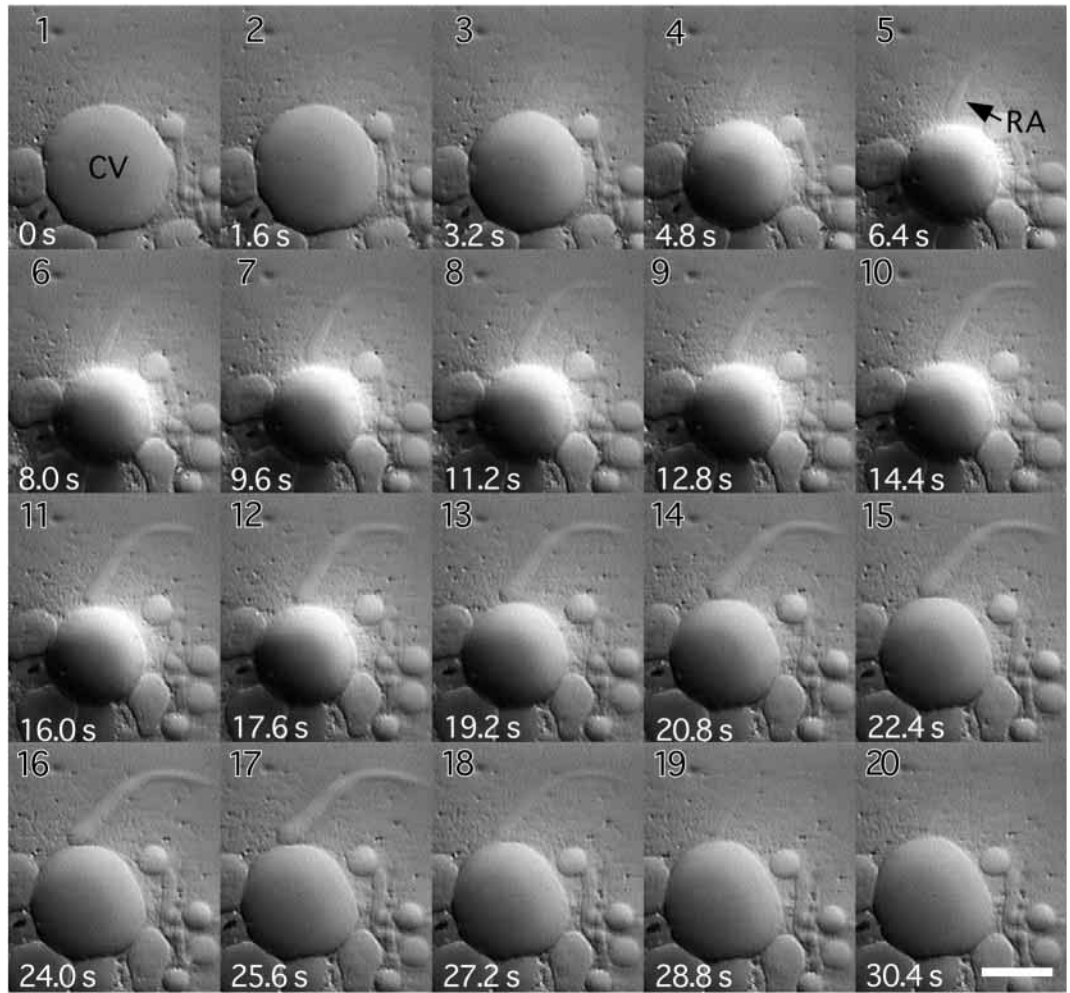


Fig. 2. Consecutive video-recorded images of an isolated *in vitro* contractile vacuole (CV) and its radial arms (RA). The *in vitro* contractile vacuole rounds (frames 1–10) and then slackens (frames 11–20). The *in vitro* radial arm swells (frames 4–17) and thins (frames 18–20). Most of the smaller vesicles are digestive vacuoles; however, some of them are vesicles derived from the radial arm. Scale bar, 20 μm .

The *in vitro* radial arm, which showed swelling–thinning cycles, ultimately failed to fuse with the contractile vacuole and disintegrated into several small vesicles. The vesicles of

the radial arm continued to round and slacken along with the contractile vacuole. A series of VCR images of the radial-arm-derived vesicles showing rounding–slackening cycles is shown

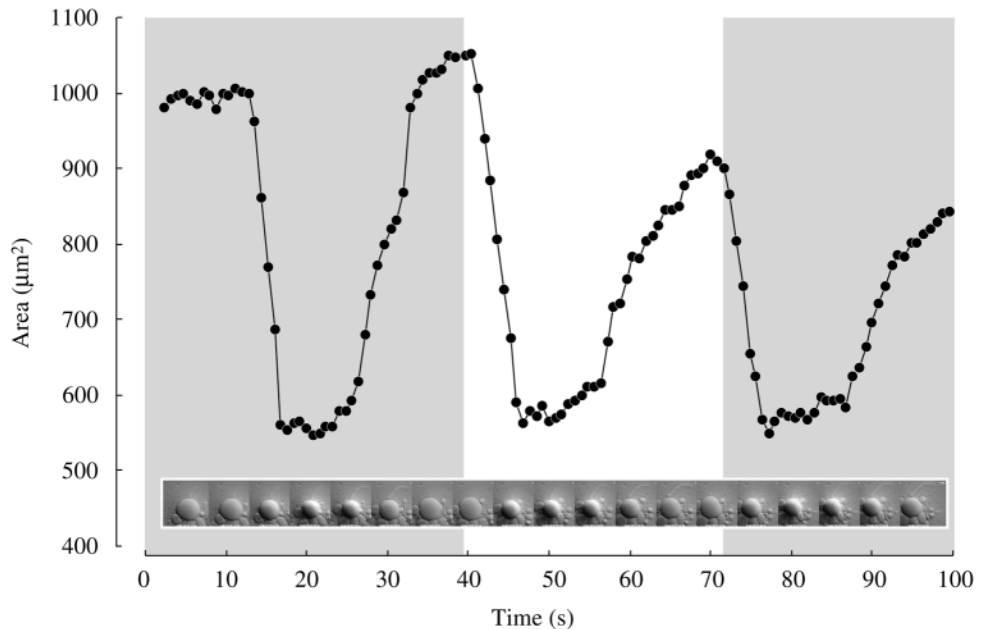


Fig. 3. Three consecutive rounding–slackening cycles of an isolated *in vitro* contractile vacuole. This *in vitro* contractile vacuole is the same as that shown in Fig. 2. Rounding–slackening is shown as a change in the apparent area of the contractile vacuole as seen from above. The second cycle (in the central white column) corresponds to the cycle shown in Fig. 2. Time zero was chosen arbitrarily. Consecutive video-recorded images of the contractile vacuole corresponding to these three rounding–slackening cycles are shown in the lower portion of this figure. The center of each frame corresponds to the time when the frame was taken on the time scale (abscissa).

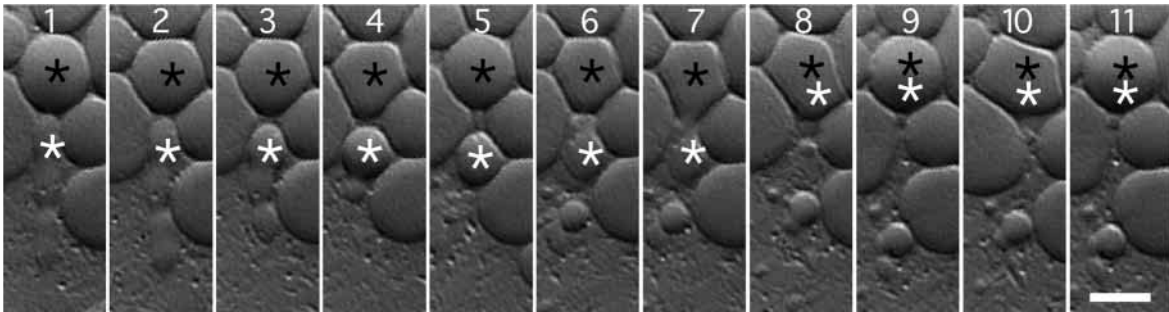


Fig. 4. Consecutive video-recorded images of the vesicles derived from an isolated *in vitro* radial arm. Frames 1–6, the vesicle marked by a black asterisk is derived from the proximal portion of the radial arm (ampulla) and the smaller vesicle marked by a white asterisk is derived from the distal portion of the radial arm. Frames 7–11, these two vesicles fuse; the resulting vesicle is marked with both a white and a black asterisk. Most of the other vesicles are digestive vesicles that do not show rounding–slackening cycles. Scale bar, 10 μm .

in Fig. 4. The proximal portion of an *in vitro* radial arm (the ampulla; marked by black asterisks) showed rounding (frames 1, 5) and slackening (frames 2–4, 6, 7) after its distal portion (marked by white asterisks) had been severed. The distal portion also became a vesicle and showed rounding–slackening, although it was not clearly observed because of its small size (rounding is seen in frame 4). In frame 7, the distal vesicle fused with the proximal vesicle at a time when both had slackened. The fused vesicle (marked by black and white asterisks) showed rounding (frames 9, 11) and slackening (frames 8, 10). The radial-arm-derived vesicle also sometimes fused with the contractile vacuole when they were both slackened (data not shown). However, the contractile-vacuole-complex-derived vesicles were never seen to fuse with either digestive vacuoles or the cell membrane.

Rounding–slackening cycles of an in vitro contractile vacuole before and after its bisection into two vesicles

An *in vitro* contractile vacuole was ‘bisected’ into two vesicles (the left vesicle is slightly larger than that the right in Fig. 5) by tearing the contractile vacuole with the tip of a fine microneedle. The needle was kept between the two vesicles to prevent them from re-fusing after bisection. Special attention was given to the rounding–slackening cycles of the two vesicles and to the cycles of the original contractile vacuole. Fig. 5 shows consecutive video-recorded images (Fig. 5A) and rounding–slackening cycles (Fig. 5B) obtained from a representative *in vitro* contractile vacuole.

As mentioned previously, the *in vitro* contractile vacuole exhibited rounding and slackening cycles of fairly regular duration (Fig. 5A; frames 1–5, Fig. 5B; filled diamonds). The period of the cycle was 30.3 ± 5.0 s (mean \pm s.d. for the four cycles preceding bisection). After bisection (Fig. 5A; frames 6–12), both vesicles showed independent rounding–slackening cycles, i.e. their cycles were out of phase. This is especially clear for the cycles corresponding to frames 9–11, in which the two cycles were almost 180° out of phase. The period of the rounding–slackening cycle was 47.7 ± 2.9 s for the vesicle to the left of the microneedle in Fig. 5A (filled circles in Fig. 5B) and 38.6 ± 3.2 s for the vesicle to the right of the microneedle in

Fig. 5A (open circles in Fig. 5B). These are mean values each obtained from three successive cycles newly established after bisection (obtained after frame 8 in Fig. 5A).

Rounding–slackening cycles of an in vitro vesicle obtained by fusion of two vesicles

Fig. 6 shows consecutive video-recorded images (Fig. 6A) and rounding–slackening cycles (Fig. 6B) of two *in vitro* vesicles, obtained by bisecting an *in vitro* contractile vacuole (the same vacuole and vesicles as in Fig. 5), before (frames 1–6) and after (frames 7–10) they were allowed to fuse with each other. The period of the cycles was 42.0 ± 5.3 s (mean \pm s.d. for four successive cycles before fusion) for the vesicle to the left of the microneedle in Fig. 6A (filled circles in Fig. 6B) and 57.0 ± 19.3 s (mean \pm s.d. for three successive cycles before fusion) for the vesicle to the right of the microneedle in Fig. 6A (open circles in Fig. 6B).

The two vesicles fused when both vesicles were in the slackening phase after the microneedle, which had kept the vesicles apart, was withdrawn (frame 7). The fused vesicle showed rounding–slackening cycles (Fig. 6B; filled diamonds) with a period of 39.0 ± 4.6 s (mean \pm s.d. for four successive cycles after fusion). This value is closer to the value for the vesicle to the left of the microneedle that exhibited the shorter cycle period (42.0 s) than to that for the vesicle to the right of the microneedle that exhibited the longer period (57.0 s) before fusion (Fig. 6A; frames 1–6).

Extra rounding of the in vitro contractile vacuole caused by suction of its membrane

The *in vitro* contractile vacuole showed an extra rounding phase when a minute portion of its membrane was sucked into a micropipette through its heat-polished tip (approximately 1 μm in inner diameter) at a time that the contractile vacuole was slackened. It should be noted that no specific membrane region of the contractile vacuole was more effective than other regions in causing this extra rounding. A series of representative results is shown in Fig. 7, in which rounding–slackening cycles are shown as periodic changes in the output voltage of the photodetector placed over the image

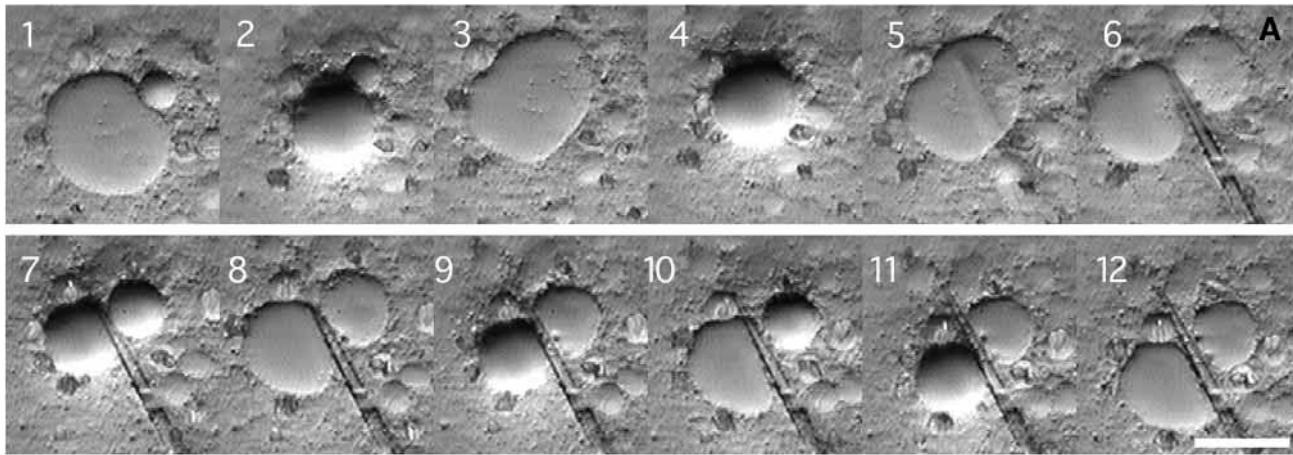
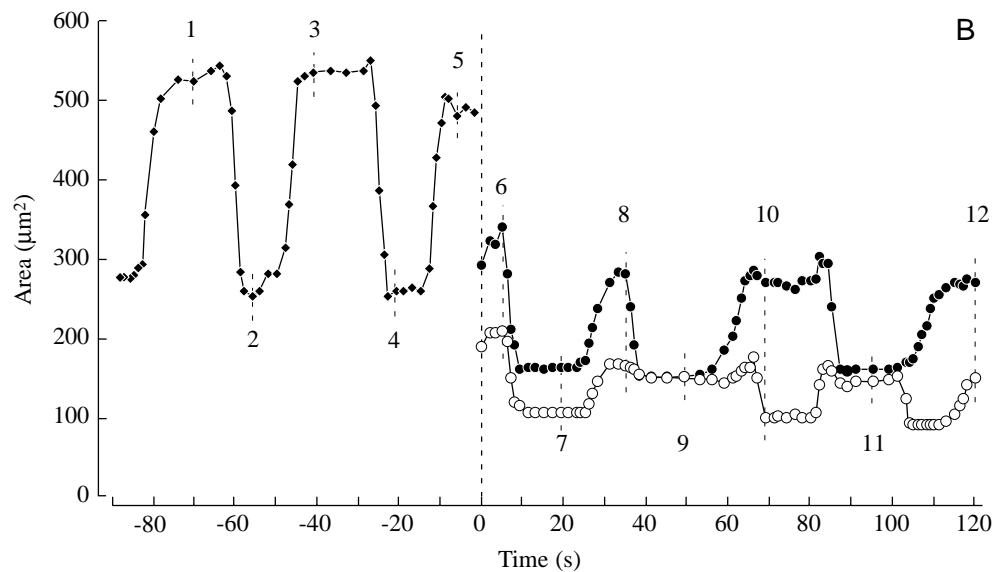


Fig. 5. Rounding–slackening cycles of an isolated *in vitro* contractile vacuole before and after ‘bisection’ into two vesicles. (A) A series of 12 consecutive video-recorded images of the contractile vacuole (frames 1–5) and its bisected products (frames 6–12). The bisecting microneedle can be seen in frames 6–12. Scale bar, 20 μm . (B) Apparent areas of the *in vitro* contractile vacuole and the vesicles formed by bisection plotted against time. These apparent areas correspond to the degree of rounding. Time zero corresponds to the moment when the *in vitro* contractile vacuole was bisected. Filled diamonds, the contractile vacuole before bisection. Filled circles, the vesicle to the left of the bisecting microneedle. Open circles, the vesicle to the right of the bisecting microneedle. Numbers beside the thin vertical dotted lines corresponds to the frame number in A. Each thin vertical dotted line corresponds to the time when that frame was taken.



of the contractile vacuole and the moment of membrane suction is indicated as an increase in the electrical resistance of the suction pipette due to its being plugged by membrane.

Fig. 7Ai shows a series of rounding–slackening cycles of an *in vitro* contractile vacuole in which two extra roundings (arrows) caused by membrane suction can be seen. The resistance of the micropipette (R) was as high as 10 G Ω when the tip of the pipette was in mineral oil and as low as 0.3 G Ω when the tip was in the isolated cytosol. The moment of membrane suction was recognizable as a small spike-like upward deflection corresponding to an increase in R .

Fig. 7Bi,ii shows magnified traces of the output voltage of the photodetector and the resistance of the suction micropipette (R), respectively, corresponding to the second extra rounding phase shown in Fig. 7Ai (surrounded by a broken rectangle). These are presented to demonstrate the precise time relationship between membrane suction and the start of the extra rounding phase. To determine the exact correlation

between the photodetector signal and the actual morphological changes in the contractile vacuole, changes in the apparent area of the images of the contractile vacuole, as an index of its roundness, are shown in Fig. 7Biii on the same time scale as for Fig. 7Bi,ii. Some corresponding VCR images of the contractile vacuole during the second extra rounding phase are shown in Fig. 7Biv.

It is clear from Fig. 7B that the output voltage of the photodetector (Fig. 7Bi) changed over time in parallel with the change in the apparent area of the image of the contractile vacuole (Fig. 7Biii). This implies that changes in the output voltage correspond well with changes in the roundness of the contractile vacuole. The more-or-less steady higher level of output voltage corresponds to the slackened state of the contractile vacuole, the initial downward deflection to the rounding process, the more-or-less steady lower level to the rounding state and the later upward deflection to the slackening process.

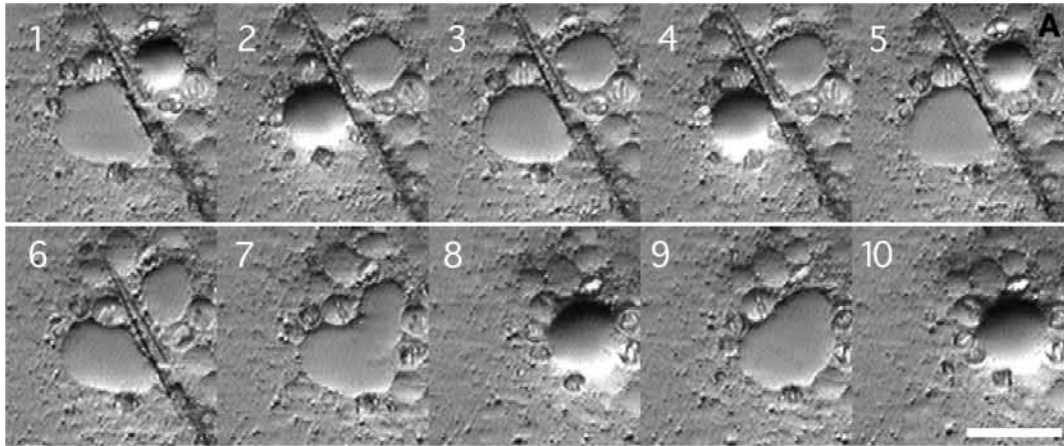
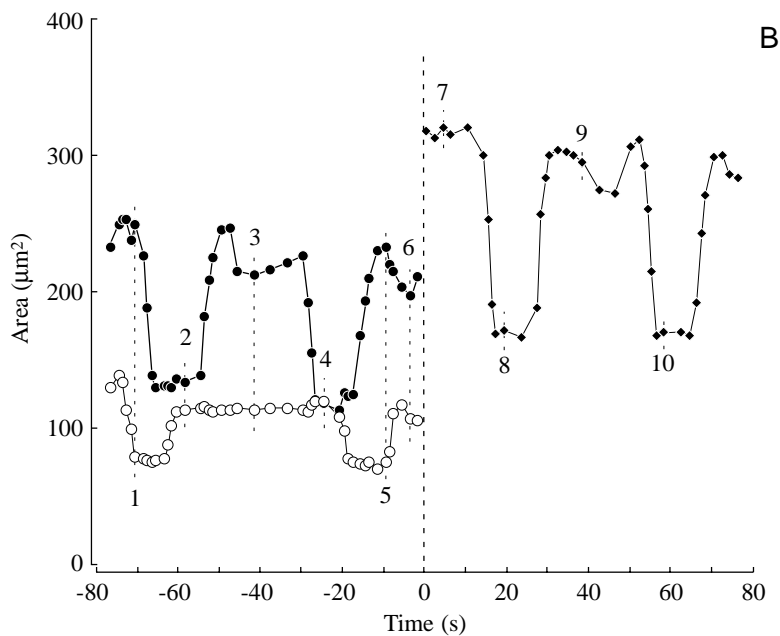


Fig. 6. Rounding–slackening cycles of two vesicles, obtained by ‘bisecting’ an isolated *in vitro* contractile vacuole, before and after they have fused. (A) A series of 10 consecutive video-recorded images of the vesicles (frames 1–6) and of the fused vesicle (frames 7–10). The bisecting microneedle can be seen in frames 1–6. Scale bar, 20 μm . (B) Apparent area of the vesicles, which corresponds to the degree of vesicle rounding, plotted against time. Time zero corresponds to the moment when the two vesicles fused. Filled circles, the vesicle to the left of the bisecting microneedle. Open circles, the vesicle to the right of the bisecting microneedle. Filled diamonds, the vesicle after fusion. Each number beside each thin vertical dotted line corresponds to the frame number in A. Each thin vertical dotted line corresponds to the time when each frame was taken.



It should be noted that the extra rounding phase occurred after cessation of membrane suction (the width of the gray column in Fig. 7B corresponds to the duration of membrane suction). The time from the end of suction (sharp downward deflection in Fig. 7Bii) to the start of an extra rounding phase (downward deflection in Fig. 7Bi) was approximately 2.4 s.

The periods of the rounding–slackening cycles before and after an extra rounding phase were almost identical, although the period tended to increase slightly over time. That is, the periods before and after the first extra rounding phase were 58 s and 64 s, respectively, and those before and after the second extra rounding phase were 71 s and 67 s, respectively.

To examine the refractoriness for the suction-induced extra rounding phase of the contractile vacuole, the contractile vacuole membrane was subjected to suction at different times during its slackening process. Fig. 8 shows a series of normal rounding–slackening cycles and suction-induced extra rounding phases as downward deflections of the output voltage of the photodetector (Fig. 8Ai), and the moment of membrane

suction is indicated by an increase in electrical resistance of the suction pipette (upward spike-like deflection in Fig. 8Aii). Extra rounding phases labeled 1'–6' in Fig. 8Ai were caused by membrane suction labeled 1–6 in Fig. 8Aii, respectively, which were applied at various phases of slackening. It is clear from the figure that extra rounding phases resulted whenever the contractile vacuole had been subjected to suction and that the phase of slackening had no effect on this extra rounding phase.

To study the moment of membrane suction more precisely, extra rounding phases 1' and 2' together with the two preceding spontaneous roundings shown in Fig. 8Ai are presented in Fig. 8Bi on an enlarged time scale (2.7 \times); rounding–slackening cycles are shown as changes in the apparent area of the contractile vacuole. Fig. 8Bii shows a series of consecutive VCR images of the *in vitro* contractile vacuole to demonstrate the changes in the apparent area of the contractile vacuole recorded in Fig. 8Bi. Suction 1 was given to the contractile vacuole at a later phase of slackening (vertical line labeled 1),

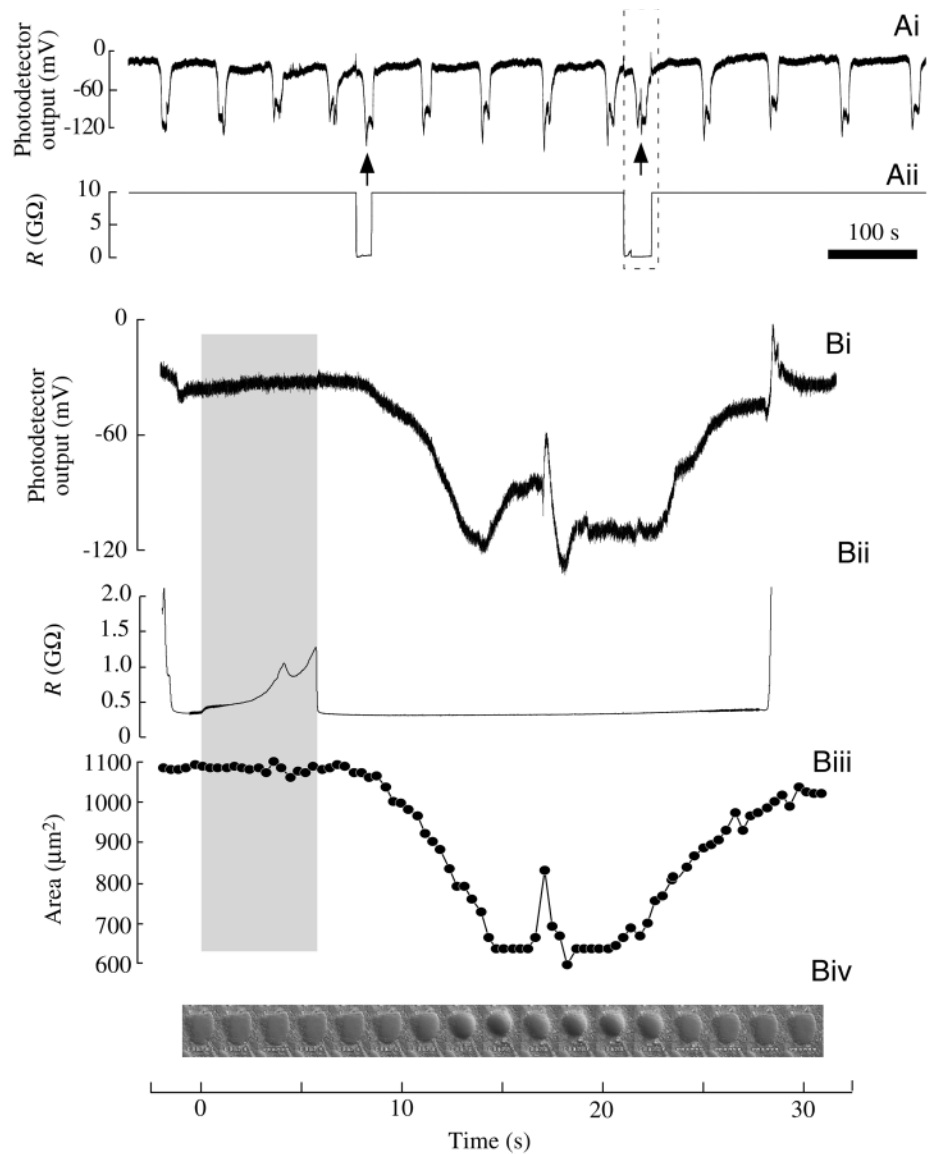


Fig. 7. Extra rounding phases of an isolated *in vitro* contractile vacuole caused by suction of its membrane into a micropipette. (Ai) A series of 14 consecutive rounding-slackening cycles as indicated by the output voltage of a photodetector placed at the image plane of the microscope. The two roundings indicated by arrows were extra rounding phases caused by membrane suction. (Aii) Electrical resistance (R) of the suction micropipette. (Bi) A magnified trace corresponding to the second extra rounding phase shown in Ai (surrounded by a broken rectangle). (Bii) A magnified trace showing the electrical resistance of the suction micropipette corresponding to the second extra rounding phase. (Biii) Apparent area of the contractile vacuole corresponding to the second extra rounding phase. (Biv) The video-recorded images of the contractile vacuole corresponding to the second extra rounding phase (Ai) from which the area of the contractile vacuole (Biii) was estimated. Each image is placed so that its center corresponds to the time when it was taken. The gray column in Bi–Biii corresponds to the duration of membrane suction.

while suction 2 was given at an earlier phase of slackening (vertical line labeled 2). It is clear from the figure that each suction causes maximum rounding.

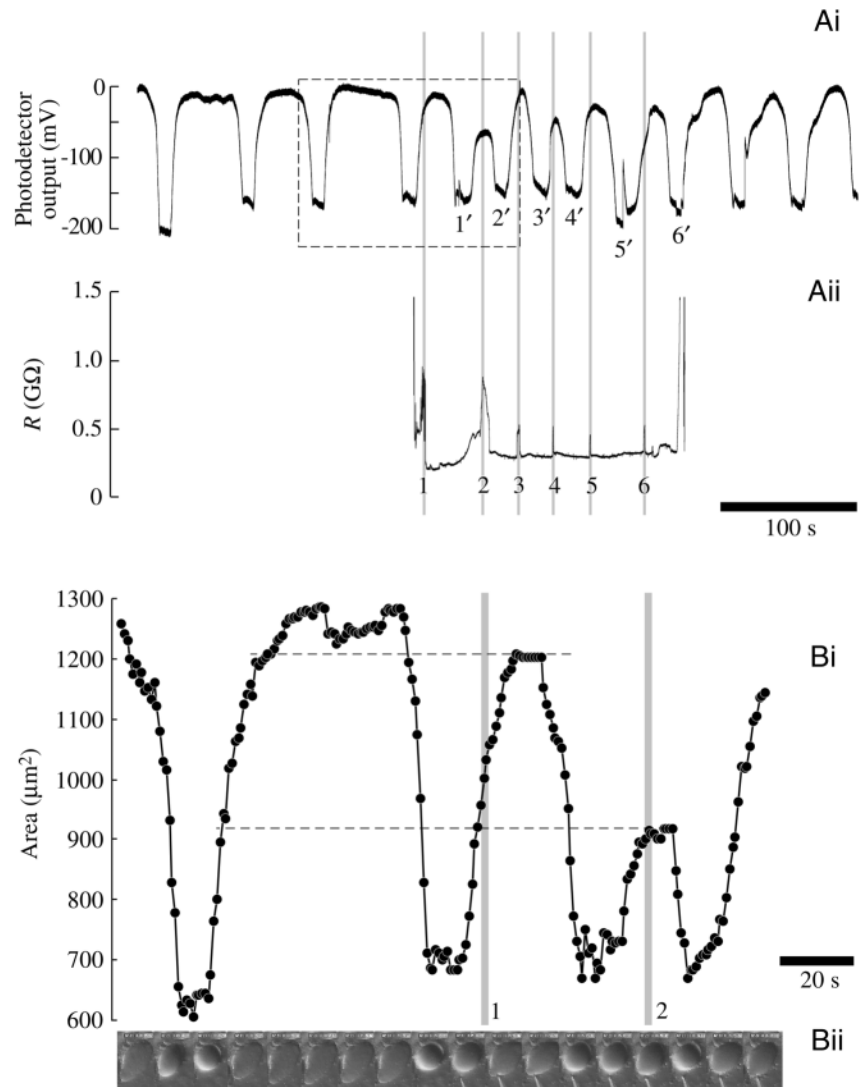
Effects of chemicals on the rounding–slackening cycle of the contractile vacuole

To examine the possible involvement of some cytoskeletal structures, such as actin filaments, spasmin (centrin) filaments and/or microtubules, in the rounding–slackening of the contractile vacuole, the effects of chemicals on the *in vitro* contractile vacuole's rounding–slackening cycle were examined. The chemicals employed were (i) EGTA to reduce the free $[Ca^{2+}]$ in the solution surrounding the contractile vacuole [Ca^{2+} is a cofactor for activation of myosin–actin interaction in skeletal muscles (Murray and Weber, 1974) and for molecular conformational changes in spasmin (Amos, 1975)], (ii) Nocodazole, Colcemid and colchicine to inhibit tubulin polymerization (De Brabander et al., 1976), and (iii)

cytochalasin B, an inhibitor for actin polymerization (Cooper, 1987). In addition to these chemicals, concanamycin B, a potent inhibitor of V-type proton pumps, was used to determine whether the proton pump activity in the decorated spongione (Allen et al., 1990) is somehow involved in the *in vitro* rounding–slackening cycles of the contractile vacuole.

The *in vitro* contractile vacuole continued its periodic rounding–slackening following treatment with a Ca^{2+} -deprived solution that contained (final concentration in $mmol\ l^{-1}$) 10 EGTA, 30 KCl, 1 $MgCl_2$ and 20 Mops buffer (pH 7.0). After an inhibitor had been dissolved in this Ca^{2+} -deprived solution, the solution was squirted against the *in vitro* contractile vacuole. The *in vitro* contractile vacuole continued to show rounding–slackening after treatment with $0.2\ mmol\ l^{-1}$ Nocodazole (in 1% dimethylsulfoxide, DMSO), $2\ mmol\ l^{-1}$ Colcemid (in 1% DMSO), $20\ mmol\ l^{-1}$ colchicine, $0.5\ mmol\ l^{-1}$ cytochalasin B (in 1.6% DMSO) or $0.2\ mmol\ l^{-1}$ concanamycin B (in 2% DMSO).

Fig. 8. Extra rounding phases of an isolated *in vitro* contractile vacuole caused by suction applied to its membrane at different phases of slackening. (Ai) Changes in the output voltage of the photodetector, which was placed at the edge of the image of the contractile vacuole. Downward deflection corresponds to rounding, while upward deflection represents slackening. Numbered downward deflections correspond to extra rounding phases. (Aii) The moment of membrane suction of the contractile vacuole is indicated by an increase in the electrical resistance (R) of the suction pipette. Suctions 1–6 caused extra rounding phase 1'–6', respectively, in Ai. (Bi) Rounding–slackening cycles corresponding to those indicated by the broken rectangle in Ai on an enlarged time scale. They are shown as changes in the apparent area of images of the contractile vacuole. The two horizontal broken lines show the levels of slackening of the contractile vacuole, one (upper line) corresponds to the level from which an extra rounding phase started in response to suction 1 and the other (lower line) corresponds to the level from which an extra rounding phase started in response to suction 2. These levels may be compared with the slackening level of the normal rounding–slackening cycle (the cycle to the left of Bi). (Bii) Video-recorded images of the contractile vacuole corresponding to the rounding–slackening cycles shown in Bi.



ATP-requirement for rounding–slackening of the contractile vacuole

To determine whether ATP is required for rounding and slackening of the contractile vacuole, the ruptured cell of *Paramecium multimicronucleatum* was perfused with an ATP-free Ca^{2+} -deprived solution, which was the same as that used for the examination of the above chemicals but in addition contained 1 mmol l^{-1} GTP and 40 mol l^{-1} Taxol to prevent deterioration of the microtubule ribbons that anchor the contractile vacuole to its pore structure and to the rest of the contractile vacuole complex membrane (Hausmann and Allen, 1977). The cell was perfused until its oral cilia ceased beating. Approximately 20 μl of an ATP-containing solution (1 mmol l^{-1} ATP dissolved in the Ca^{2+} -deprived solution) was then squirted against the contractile vacuole through the tip of another micropipette placed approximately $5 \mu\text{m}$ from the contractile vacuole. A representative experiment is shown in Fig. 9.

As clearly shown in this figure, the contractile vacuole ceased its rounding–slackening cycle and stayed slackened

after perfusion in the ATP-free solution (0 s). The contractile vacuole started to show rounding immediately after the application of ATP-containing solution (10 s), and rounding was completed in approximately 120 s. Beating of the oral cilia was reactivated immediately after the application of ATP, and the cilia continued to beat for the remainder of the experiment (data not shown). It should be noted that the perfused contractile vacuole failed to show slackening once it had rounded maximally in the presence of ATP. Squinting a saline solution that lacked ATP against the perfused contractile vacuole did not cause rounding (data not shown).

Discussion

The contractile vacuole and radial canal membranes possess a mechanism(s) by which their tension is periodically increased

We have demonstrated that the contractile vacuole undergoes rounding–slackening cycles after it has been isolated from the *Paramecium multimicronucleatum* cell and

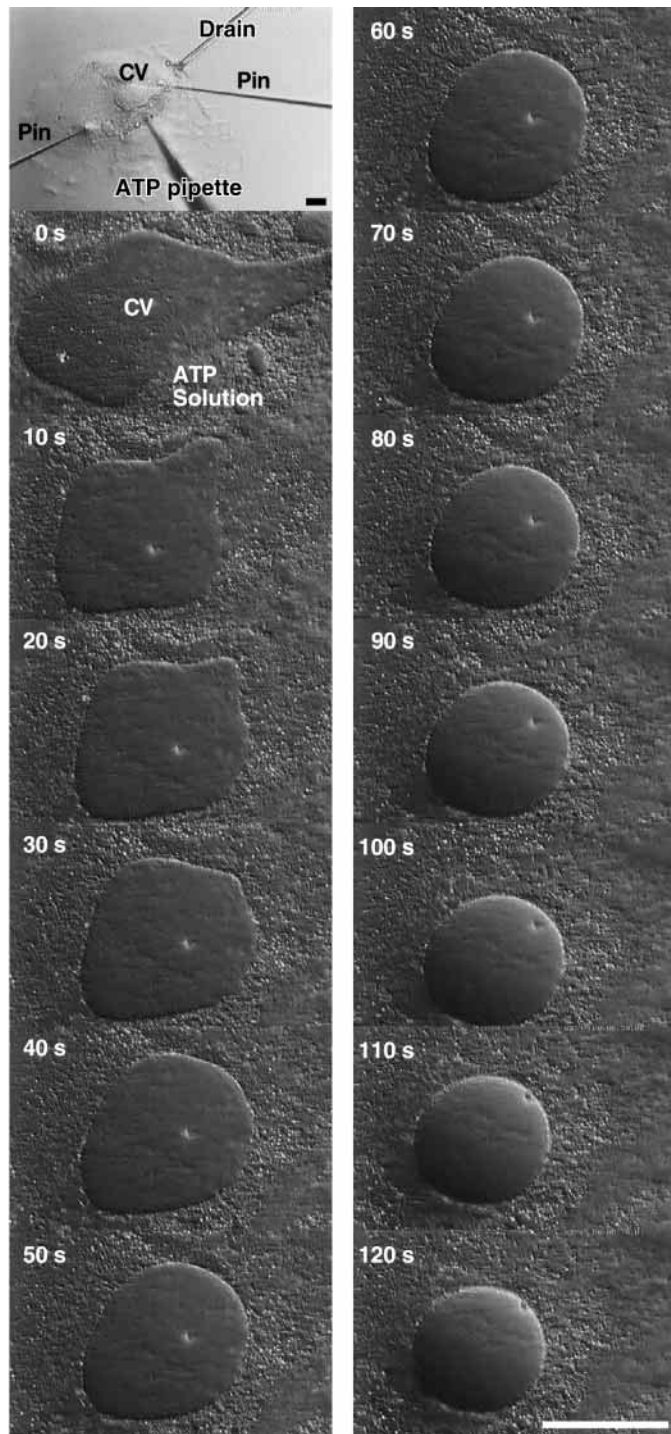


Fig. 9. Consecutive images of the contractile vacuole (CV) in a perfused *Paramecium multimicronucleatum* cell showing rounding in response to the application of ATP. Top frame of the left-hand column, arrangement of pipettes and needles around the contractile vacuole. Pin, needles used to stabilize the cell. ATP pipette, a micropipette for squirting an ATP solution against the contractile vacuole. Drain, a suction pipette for draining cytosolic solution. Scale bar, 50 μ m. ATP was applied at 0 s. In the 0 s frame, the ATP solution can be seen as a small mass of solution with an opacity different from that of the surrounding solution. Complete rounding was accomplished by 120 s. Scale bar, 50 μ m.

is suspended in the cytosol (Figs 2–4). Rounding and slackening of the contractile vacuole corresponds to an increase and decrease, respectively, in the tension of the contractile vacuole membrane. This observation implies that the contractile vacuole membrane possesses a mechanism(s) by which its tension is periodically increased and decreased.

The radial arms become vesiculated after they have been isolated from the *Paramecium* cell. These radial-arm-derived vesicles showed rounding–slackening cycles independent of each other (Fig. 4). Vesicles fused with each other or with the *in vitro* contractile vacuole when they were in the slackening phase. The radial arm membrane, therefore, seems to be essentially identical in its tension-developing properties to the contractile vacuole membrane. This idea is consistent with our electron microscopical observations (Tominaga et al., 1999) that the radial arm membrane is utilized to increase the size of the contractile vacuole after pore opening has been inhibited or during regeneration of the contractile vacuole after it has been ruptured or fragmented.

Rounding of the *in vitro* contractile vacuole corresponds to the rounding that occurs *in vivo* immediately before fluid expulsion (Patterson, 1980; Patterson and Sleight, 1976). *In vitro*, it is assumed that the contractile vacuole is detached from the fixed pore structure in the plasma membrane when it is squeezed out of the cell (Fig. 1). Thus, fusion of the contractile vacuole membrane with the pore and the fluid expulsion that would follow this fusion is no longer possible. Slackening of the contractile vacuole *in vitro* corresponds to the slackening *in vivo* seen during the fluid-filling stage, although in the *in vitro* contractile vacuole additional fluid filling cannot be achieved if the radial arms have become permanently detached from the contractile vacuole and have undergone vesiculation (Fig. 4).

The hypothetical tension-developing mechanism is present throughout the contractile vacuole membrane

The membrane-bound vesicles obtained by bisecting an *in vitro* contractile vacuole showed periodic rounding and slackening, but these cycles become out of phase, even though both vesicles are separated only by a fine microneedle (Fig. 5). This implies that water-soluble factors in the cytosol are not directly determining the timing of the periodic development of membrane tension. It also indicates that any area of the contractile vacuole membrane can possess the tension-developing mechanism so that this mechanism becomes independent in vesicles that are derived from the same membrane. Because of their independence, it was of interest to determine whether there was any relationship between the membrane area (size) of the vesicle and the period of its rounding–slackening cycles. As far as we have been able to determine, the period fluctuates independently of the size of the vesicle. However, this needs to be studied in greater detail.

Tension development in the contractile vacuole membrane is triggered by an increase in membrane tension

Fusion of two *in vitro* vesicles with different periods of

rounding–slackening occurred when both vesicles were in their slackening phases (Fig. 6). It should be noted that the time when the first rounding occurred after the fusion approximated the time that the vesicle with the shorter period would have rounded if it had not fused with the other vesicle with the longer period. Moreover, the period of rounding–slackening cycles after fusion was close to that of the fusing vesicle that had the shorter period (Fig. 6). These results imply that periodic tension development in the membrane of the fused vesicle comes under the control of the periodic tension increase mechanism of the vesicle with the previously shorter period. This suggests the presence of a mechanism of tension development in the contractile vacuole membrane that is activated by an increase in the membrane tension, because the new rounding–slackening cycle is established by the membrane contributed by the vesicle with the faster cycle and thus the one that tends to develop tension sooner.

The presence of this ‘tension-induced tension development’ mechanism in the contractile vacuole membrane was investigated by using suction applied to a small portion of the *in vitro* membrane of the contractile vacuole by a micropipette. This suction caused an extra rounding phase (Fig. 7). Membrane suction is assumed to cause stretching of the membrane, i.e. an increase in the tension in a localized area of the membrane. The presence of a ‘tension-induced maximum tension development mechanism’ in the contractile vacuole membrane implies that the tension development process in the contractile vacuole membrane, visualized as the process of rounding, is regenerative, i.e. the membrane tension increases to its maximum value whenever tension is somehow triggered to increase. Membrane suction always caused an extra rounding phase of the contractile vacuole to its maximum extent whenever suction was applied to the membrane during the slackening phase (Fig. 8). In other words, the contractile vacuole membrane is always ready to respond to a localized increase in its membrane tension by developing maximum membrane tension when the vesicle enters the slackening phase after rounding. This seems to indicate the absence of a refractoriness to a localized increase in the membrane tension in the tension-induced tension development mechanism. However, a more quantitative control of the suction stimulus is needed before the presence or absence of refractoriness can be established with certainty.

No definite region in the contractile vacuole membrane acts as a pacemaker for the rounding–slackening cycle

The period of the rounding–slackening cycle after an extra rounding phase was the same as that before the extra rounding phase (Fig. 7). In other words, membrane suction resets the rounding–slackening cycle of the contractile vacuole. In addition, an extra rounding phase could be induced by applying suction to any portion of the membrane of the slackened contractile vacuole (data not shown). This implies that there is no special region in the contractile vacuole membrane that acts as a pacemaker for the rounding–slackening cycles.

Is ATP required for rounding of the contractile vacuole?

We have demonstrated that perfusion of the *Paramecium multimicronucleatum* cell with an ATP-free solution brought about cessation of both the rounding–slackening cycle of the contractile vacuole and the beating of all the cilia on the cell surface. The ATP concentration in the cell is assumed to be reduced by perfusion. The subsequent squirting of an ATP-containing solution against the inactive contractile vacuole brought about rounding of the contractile vacuole (Fig. 9) as well as reactivation of beating of those cilia close to the contractile vacuole. This implies that ATP is required for rounding of the contractile vacuole.

However, the perfused contractile vacuole did not show slackening after it rounded following the application of ATP. This suggests that a hypothetical chemical/mechanism for relaxing–rounding of the contractile vacuole state, i.e. the contractile vacuole’s state with high membrane tension, has been washed out or disrupted during perfusion.

Is a cytoskeletal motile system involved in rounding–slackening of the contractile vacuole?

We found that the *in vitro* contractile vacuole continued to show rounding–slackening in the presence of 10 mmol l^{-1} EGTA in the surrounding solution. This argues against the possibility that a Ca^{2+} -mediated contractile protein such as spasmin (centrin) is involved in the contractile vacuole’s tension-developing mechanism, although a centrin-positive protein is known to be present in *Paramecium* on the basis of immunofluorescent and immunogold studies (Allen et al., 1998; Garreau de Loubresse et al., 1988).

Administration of 0.5 mmol l^{-1} cytochalasin B did not inhibit the rounding–slackening cycle of the *in vitro* contractile vacuole. This concentration is known to be high enough to inhibit other actin-based phenomena in *Paramecium*, such as pinching-off of the digestive vacuoles from the cytopharynx and closure of the cytoproct after discharge of a spent digestive vacuole (Allen et al., 1995; Allen and Fok, 1985; Fok et al., 1985). It is, therefore, probable that the actin/myosin-based system is not involved in the tension-developing mechanism in the contractile vacuole of *Paramecium*, although myosin-I has been shown to be involved in the water expulsion activity of the contractile vacuole in *Acanthamoeba* (Doberstein et al., 1993).

None of the inhibitors of tubulin polymerization examined (0.2 mmol l^{-1} Nocodazole, 2 mmol l^{-1} Colcemid, 20 mmol l^{-1} colchicine) affected the rounding–slackening cycle of the *in vitro* contractile vacuole. This, however, does not exclude the possibility that microtubules surrounding the contractile vacuole are somehow involved in tension development in the contractile vacuole, since the tolerance for these depolymerizing chemicals is found to be higher in some arrays of microtubules, such as microtubule ribbons and bundles, than in single microtubules (Cohen and Beisson, 1988; Torres and Delgado, 1989).

Is V-type proton pump activity involved in rounding–slackening of the contractile vacuole?

We demonstrated that *in vitro* radial arms showed swelling

and thinning for a while after they had been isolated from the cell (Fig. 2). This implies that the water-segregating function, which resides in the V-type proton pump of the decorated spongiome (Fok et al., 1995), remains active in the *in vitro* radial arm. The *in vitro* radial arm is ultimately transformed into membrane-bound vesicles and sometimes fuses with the *in vitro* contractile vacuole. It is, therefore, possible that the V-type proton pump activity is somehow involved in the rounding–slackening cycles of the *in vitro* contractile vacuole. However, the presence of 0.2 mmol l^{-1} concanamycin B in the surrounding solution had no effects on rounding–slackening of the *in vitro* contractile vacuole. Concanamycin B (at a concentration as low as $1 \text{ } \mu\text{mol l}^{-1}$) was found to inhibit V-type proton pump activity in the decorated spongiome, so that water segregation in the contractile vacuole complex of *Paramecium* was completely blocked (Fok et al., 1995). This implies that the water-segregation activity of V-type proton pumps is not involved in the rounding–slackening mechanism of the *in vitro* contractile vacuole.

Membrane tubulation and tension development in the contractile vacuole membrane

Previously (Allen and Fok, 1988; Naitoh et al., 1997), we have demonstrated by electron microscopy that the contractile vacuole membrane shows a tendency to become transformed into a network of 40 nm tubules during fluid expulsion. We proposed the hypothesis, partly on the basis of these observations, that the apparent retrieval of the contractile vacuole membrane around a constant volume of fluid is accomplished by tubulation and that this might be the primary cause for an increase in the contractile vacuole membrane tension and the resultant rounding of the contractile vacuole (Tominaga et al., 1998b). We were recently able to estimate the membrane tension at the surface of the *in vitro* contractile vacuole in its rounding phase from the degree of its compression by a force due to cytosol–mineral oil boundary tension according to Cole (1932). The tension was comparable to that of the cytosol–mineral oil boundary (Naitoh et al., 1999). If the boundary tension is the same as that for the water–mineral oil boundary (approximately 50 mN m^{-1} ; Israelachvili, 1988), the tension at the surface of the contractile vacuole is a few orders of magnitude higher than the membrane tension of conventional biological membranes (10^{-5} to 10^{-3} N m^{-1}) (Dai and Sheetz, 1995; Hiramoto, 1963; Sheetz and Dai, 1996; Yoneda and Dan, 1972). Normal membranes would be expected to rupture or disintegrate if placed under such a high tension. It is natural, therefore, to consider the presence of a membrane-associated cytoskeletal structure(s) surrounding the contractile vacuole to support the contractile vacuole membrane when it is subjected to such extreme tension. Nevertheless, no ultrastructural or immunological evidence for the presence of such a fibrous meshwork system around the contractile vacuole has been observed in *Paramecium* (Allen and Fok, 1988; Cohen et al., 1984). Whatever causes the membrane to tubulate may also reinforce the membrane during periods of high tension, and this mechanism or these molecules

may also trigger the generation of tension at the surface of the contractile vacuole itself. To protect the contractile vacuole from disintegration during periods of high tension, the system must cover both the planar contractile vacuole membrane and its 40 nm tubules.

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