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Functional Characterization of Contractile Vacuole Isolated from *Amoeba proteus*

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ABSTRACT. **Contractile vacuoles (CVs) released from cells of *Amoeba proteus* were used to analyze its function *in vitro*. When CV was transferred to a hypertonic medium, its volume decreased within 10 sec. When it was subsequently returned to its original medium, it quickly started swelling. However, it ruptured before recovering its initial volume. These results suggested that the CV membrane is semi-permeable and that the fluid is collected by the osmotic gradient *in vivo*. The water permeability of membrane of isolated CV was calculated from the rate of osmotic volume change to be 0.94 μm/sec · OsM. This high value suggested that CV membrane is equipped with water channel.**

CV contracted (or burst) quickly upon addition of 1 mM ATP. Contraction was induced by ATP, but not by other nucleotides, GTP, ITP, ADP, or the analogues of ATP, AMP-PNP and ATPγS. It was suggested that the contraction of isolated CV was caused by increase in the tension of its membrane by ATP.

Key words: contractile vacuole/*Amoeba proteus*/osmolality/water permeability/ATP

Introduction

In higher animals forming multicellular systems, cells and body fluids are isotonic. For protozoa living in fresh water, since the osmolality of cytoplasm is higher than that of the extracellular medium, water always enters the cell along the osmotic gradient across the plasma membrane. However, the fact that protozoan cells can maintain their morphology and volume is strictly dependent on the function of their contractile vacuole (CV), by which water is extruded.

The process of water extrusion by CV is composed of three steps: diastole (filling); systole (vacuolar contraction); and expulsion (the release of vacuolar fluid from CV to cell exterior) (Patterson, 1980). The CV periodically repeats diastole and systole, slowly expanding to fill fluid from cytoplasm during diastole, and then quickly contracting to release fluid to cell exterior at systole.

The function of CVs has been extensively studied in *Paramecium* (Allen, 2000), in which CV apparatus is highly differentiated and the intracellular position of CV is fixed. However, the situation is significantly different in *Amoeba* cells, the form of which is always changing such that the position of CV is not fixed in the cell. To the best of our knowledge, studies on CV of *Amoeba* are few.

The association of various proteins with the CV complex has been reported in amoeboid cells of *Dictyostelium discoideum* (cited in Gerish *et al*., 2002). However, the mechanism of CV function to extrude water still remains unsolved. This material is not always suitable for analysis at cellular level, because of its very small cell size. Under such situations, we revived studies using the large amoeba, *A. proteus*, in hopes that information accumulated in materials suitable for molecular biological studies will open new aspects of approach in the present material.

The present study aims at elucidating the molecular mechanism of CV function. To establish a system for *in vitro* analysis, the isolation of intact CV is essential. In the present study, we succeeded in isolating CV having semi-permeability and revealed some of the membrane characteristics of the isolated CVs.

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Abbreviations: AMP-PNP, 5'-adenylylimido diphosphate; ATPγS, adenosine 5'-(3-O-thio)triphosphate; CV, contractile vacuole; EGTA, ethyleneglycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; GTP, guanosine triphosphate; ITP, inosine triphosphate; EMP, 5 mM EGTA, 6 mM $MgCl_2$, 30 mM PIPES, and 67 mM KOH; EMH, 5 mM EGTA, 6 mM $MgCl_2$, 30 mM HEPES, and 27 mM KOH.

Table I. COMPOSITION OF MEDIA (mM)

Medium	EMP	EMH	Hypertonic medium	ATP medium
EGTA	5	5	5	5
$MgCl_2$	6	6	6	6
PIPES	30	–	30	–
HEPES	–	30	–	30
KOH	67	27	67	27
Sorbitol	100	145	500	145
ATP	–	–	–	1
Osmolality (mOsM)	200	200	600	200
pH	7.0	7.5	7.0	7.5

PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); HEPES, 2-[4-(2-hydroxyethyl)-1-piperaziny]-ethanesulfonic acid

Materials and Methods

Cell culture

Amoeba proteus was cultured in KCM medium (0.7 mg KCl, 0.8 mg $CaCl_2$, 0.8 mg $MgSO_4$-$7H_2O$/1 l) in a plastic box (30×22×5 cm) at 25°C and fed on *Tetrahymena pyriformis* washed with KCM medium, according to the method of Griffin (1960). *Amoeba* cells were starved for at least two days before use in order to decrease food vacuoles.

Measurement of intracellular osmolality

Amoeba cells (about 0.2 g) were collected by centrifugation at 2,000×g for 30 sec at room temperature, and washed twice with distilled water. After removing distilled water, cells were frozen at –20°C overnight, and then thawed at room temperature. After centrifugation at 10,000×g for 10 min, the osmolality of the supernatant was measured by vapor pressure osmometer (model 5520, Wescor Inc., Logan, UT USA).

Isolation of contractile vacuole

Amoeba cells (about 0.1 g) were washed twice with either EMP medium or EMH medium (Table I) (Shimmen and MacRobbie, 1987). Cells were resuspended in the same medium and kept for 20 min at room temperature. A drop of EMP or EMH medium containing cells was put on a slide glass and covered with a cover glass. When the medium between the slide glass and the cover glass was soaked up with a piece of filter paper, cells were pressed between two glasses. Cells became flattened and finally CVs were released into the bathing medium.

Exchange of bathing medium of isolated CV

The bathing medium was exchanged by adding a drop of solution to one side of cover glass and soaked at opposite side using a piece of filter paper. Table I shows composition of media used.

Results and Discussion

To examine the physiological function of CV *in vitro*, analysis must be carried out in a medium isotonic to intracellular osmolality. Therefore, we first measured the osmolality of cells. Osmolality of *A. proteus* was found to be 241±3.61 mOsM (average from 6 samples). When cells were incubated in EMP medium, CVs stopped contracting (data not shown), indicating that the osmolality of this medium (200 mOsM) is close to intracellular osmolality. CV could be effectively isolated, when cells were ruptured in EMP medium. Hereafter, we will simply call EMP medium as an isotonic medium.

Fig. 1 shows the process of CV release. When cells were incubated in EMP medium, they became spherical. Many pseudopod-like structures were formed around the cell (Fig. 1Ab). In this cell, CV was localized to the periphery of the cell (arrowhead). CV was released by bursting such cells. Fig. 1B shows two typical examples (cell 1: a–c, cell 2: d–f). When the cell was pressed flat by soaking up the bathing medium with a piece of filter paper, the membrane of the cell became smooth. In cell 1, CV was localized in the hyaline area lacking granular cytoplasm (Fig. 1Ba, arrowhead). During pressing, CV was gradually released into the bathing medium (b, c). In cell 2, CV was first localized in the area filled with granular cytoplasm (Fig. 1Bd, arrowhead). It was released into the bathing medium together with the cytoplasmic granules (Fig. 1Be, f).

Exact ionic composition of amoeba cells has not been reported to the best of our knowledge. Two kinds of medium mimicking cytoplasmic composition of plant cells, EMP and EMH, have been developed by Shimmen and MacRobbie (1987). Therefore, we examined both media in isolating CV from amoeba cells. Since fundamentally the same results were obtained for both media, we used either EMP or EMH in the following experiments. EGTA contained in both media is important, since it is generally known that cytoplasmic free Ca^{2+} is very low at the resting state (Bush, 1993). Taylor *et al*. (1973) used EGTA in studies on amoeboid movement. Although EGTA binds Ca^{2+} strongly, it binds Mg^{2+} weakly (Portzehl *et al.*, 1964). This characteristic of EGTA is also important in studying intracellular activities, since enzymes such as ATPase need Mg^{2+} as a cofactor.

First, we examined the osmotic properties of the isolated CV. A drop of a medium was placed at one end of the cover glass and the space between two glasses was perfused by soaking the medium at the other side of the cover glass. By the perfusion, small granules around the CV were swept away, indicating that the bathing medium around the CV was replaced. However, the CV stayed in its original place during the perfusion. When perfusion was carried out with an isotonic medium, CV did not show appreciable change of volume (data not shown), indicating that CV did not respond to simple mechanical (or flow) agitation. However,

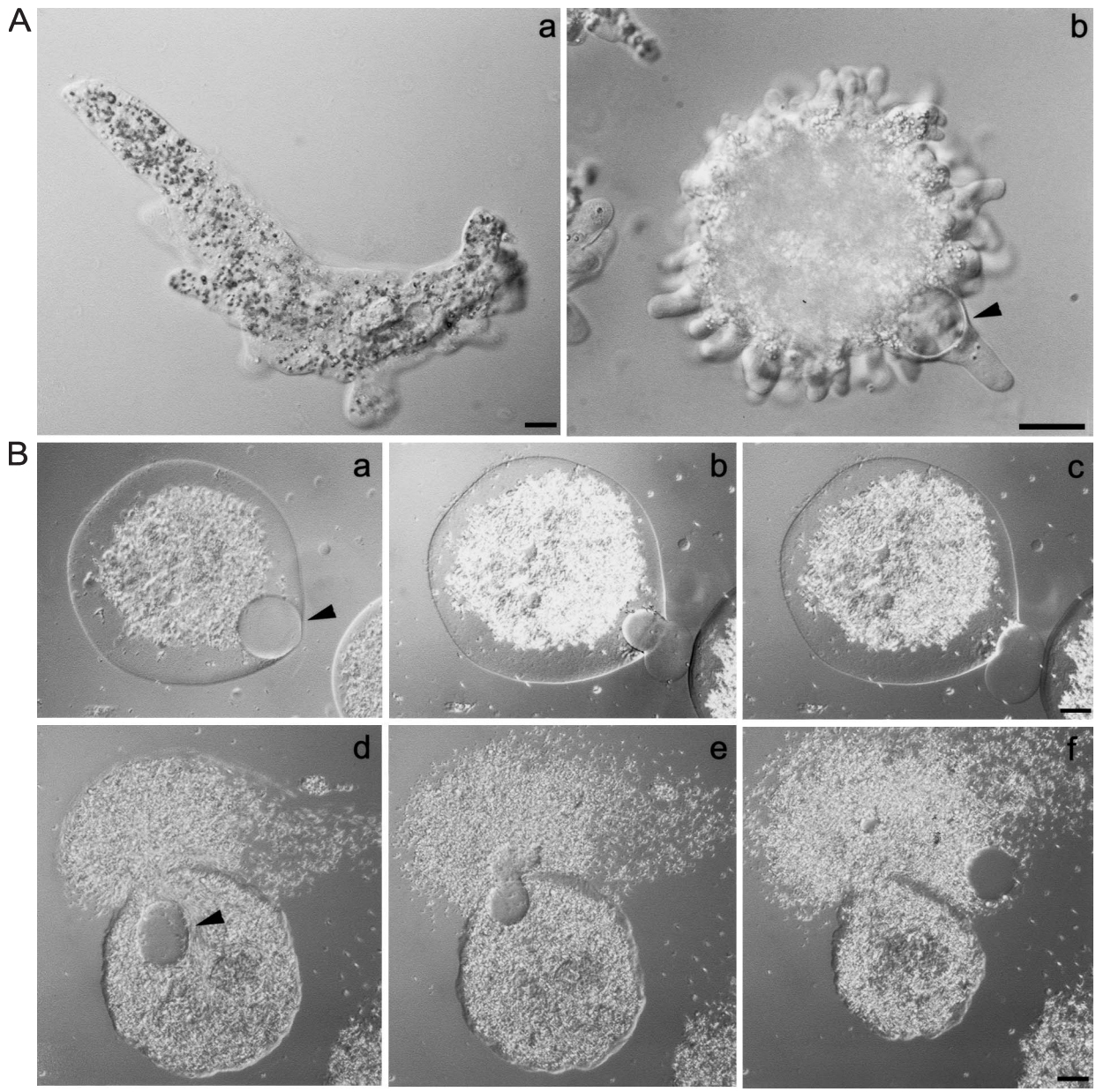

Fig. 1. Release of CV from cell. A: Cell in the culture medium (a) and that incubated in EMP medium (b). B: Process of CV release. Two cells are shown, cell 1 (a–c) and cell 2 (d–f). Arrowheads show CV. For further explanation, see the text. (Bar=20 μm)

change of osmolality of the external medium induced significant and sudden change of CV volume. Fig. 2 shows a typical example. Before osmotic stimulation, the diameter of the CV was 41.0 μm (Fig. 2A; –1s). When osmolality was increased to 0.6 OsM by perfusion, the CV started to decrease its size within 10 sec and continued shrinking (up to 18.4 μm (Fig. 2A; 12s–24s)). When the external medium was returned to EMP medium, the CV started swelling (Fig. 2B; 12s–25s). It ruptured before recovering its initial size (Fig. 2B; 26s–29s). Thus, it was found that the membrane of CV is semi-permeable. To the best of our knowledge, this is the first observation of osmotic volume change of CV isolated from *A. proteus*.

CV does not burst during diastole in the cell. It is suggested that membrane components are supplied to cover the increase in surface area during expansion *in vivo* (Pappas and Brandt, 1958).

Volume change of the CV of Fig. 2 was analyzed as shown in Fig. 3. The water permeability of the CV membrane was calculated by analyzing the shrinking phase in Fig. 3. It seemed that the volume change was composed of two steps, the first small decrease and the following linear

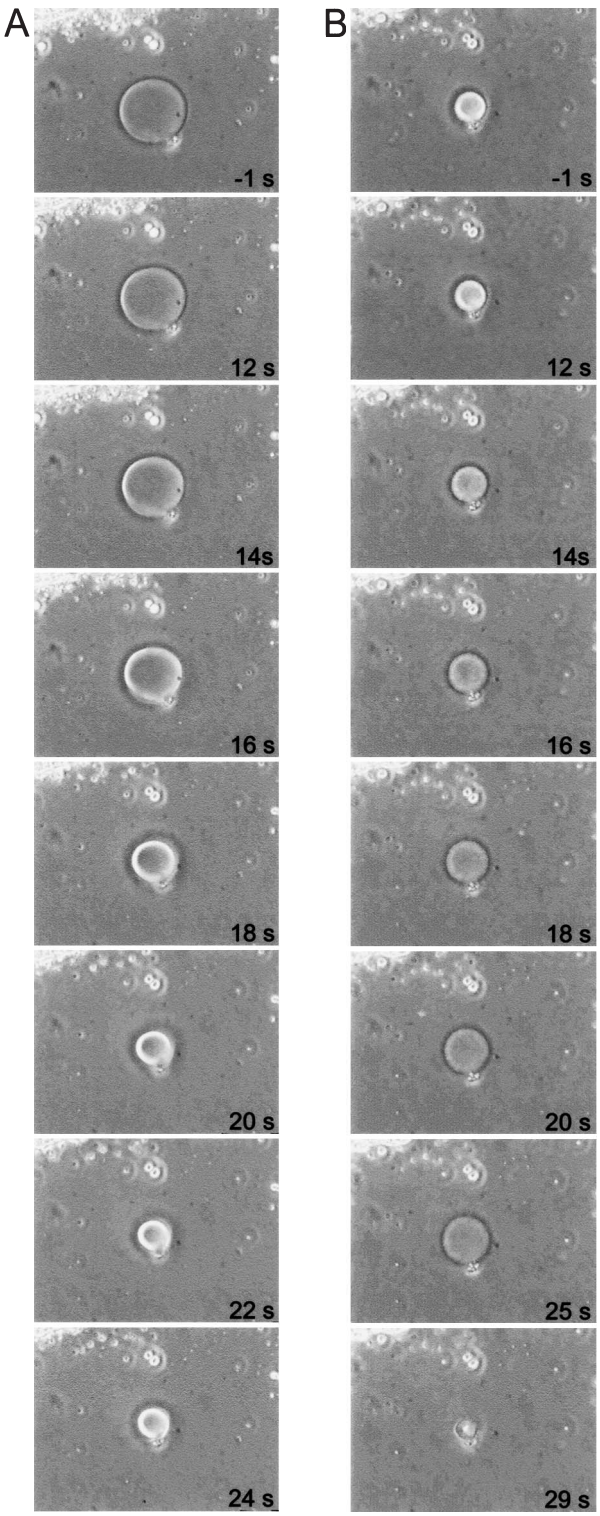

Fig. 2. Osmotic volume change of isolated CV. A: Shrinkage induced by hypertonic treatment. B: Expansion induced by returning to the original (isotonic) medium. Before recovering the original volume, the CV burst (26–29s). Numbers in pictures represent time (sec) after start perfusion. (Bar=20 μm)

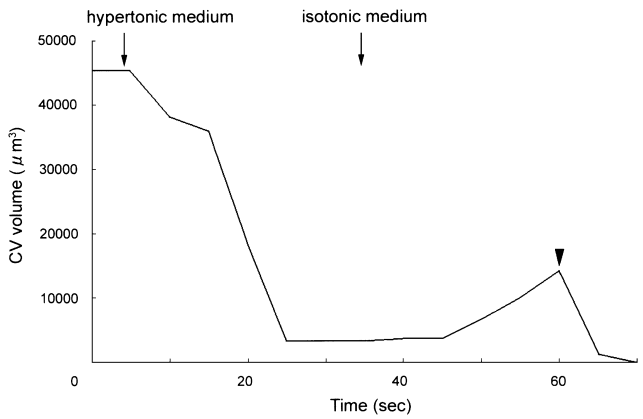

Fig. 3. Time course of CV volume change induced by hypertonic treatment. This curve was obtained by measuring the change of diameter of the CV in Fig. 2. First and second arrow indicate start of perfusion with hypertonic medium and isotonic (original) medium, respectively.

phase. We suspected that the initial small change involved not only decrease in the CV volume but also an artifact. Since CVs are released from the cell by severely decreasing the gap between the cover glass and the slide glass, CV might be slightly flattened. By adding the medium, the gap might be increased, resulting in decrease in the CV diameter measured in upper view of the CV. Therefore, the calculation was carried out by analyzing the linear part of the record (15s–25s), and the value was found to be 1.06 μm/sec · OsM. The same analysis was carried out for 12 CVs and the average water permeability was 0.94±0.36 μm/sec · OsM. This value is close to those reported for the plasma membrane of frog egg or human red blood cell (Tazawa and Shimmen, 1981). Because expression of the water channel in these cells has been reported (Preston *et al.*, 1992; Virkki *et al.*, 2002), it is likely that CV membrane of *A. proteus* is also equipped with water channel. To examine this possibility, the effect of mercuric chloride was studied. However, water permeabilities of the CV membrane did not change in the presence of 0.1 mM $HgCl_2$ (data not shown). This result does not exclude the possibility of equipment of the CV membrane with water channel, since it has been reported that some *aquaporins* were not inhibited by $HgCl_2$ (Ishibashi *et al*., 1997; Nicchia *et al*., 2000).

We examined the effect of ATP on isolated CVs. CVs were released into EMH medium lacking ATP, and ATP was added by the perfusion. No appreciable change was observed for 2–3 min after application of ATP. Judging from Fig. 2 and Fig. 3, sufficient amounts of ATP would have reached the surface of the CV during this time. CV suddenly contracted (shrunk, or burst) after a delay of 2–3 min. Since it was so quick, we could not record the process. Therefore, Fig. 4 shows CV before addition of ATP (left) and that after contraction (right). After the contraction, an amorphous structure supposed to be CV membrane remained. ATP-induced contraction was observed in 60%

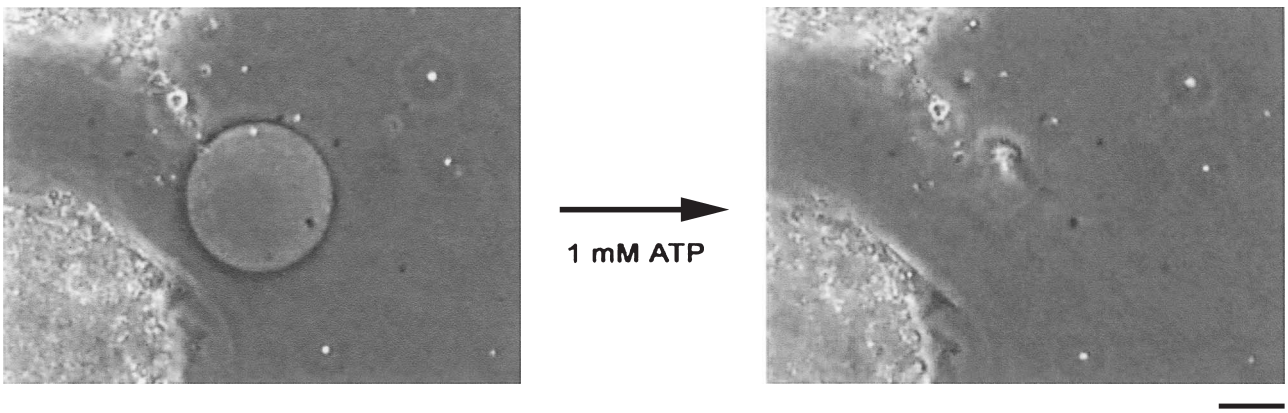

Fig. 4. Rapid contraction of isolated CV induced by 1 mM ATP. Left panel: before addition of ATP. Right panel: 138 sec after addition of 1 mM ATP. (Bar=20 μm)

(9 out of 15) among CVs examined.

The effect of other nucleotides or ATP analogues was examined. At least 10 CVs were examined for each chemical. None of the chemical examined, GTP ITP, ADP, AMP-PNP and ATPγS, induced contraction at 1 mM (Fig. 5). Since ATPγS was ineffective, it is suggested that protein phosphorylation is not involved in this phenomenon. The ineffectiveness of AMP-PNP, an unhydrolyzable analogue of ATP, suggested that hydrolysis of ATP is necessary. At present, the target of ATP hydrolysis is not evident.

The present study unequivocally showed that CV membrane of *A. proteus* is semi-permeable and that water flux into CV occurs along the osmotic gradient across the CV membrane. It has been reported that the activities of K^+ and Cl^- in CV were 1.5 times higher than those of cytosol in *Paramecium* (Stock *et al*., 2002a, 2002b). The situation might be also the case for *A. proteus*. High water permeability of CV membrane demonstrated in the present study suggested that it is equipped with water channel, which will facilitate osmosis along the osmotic gradient. We are studying whether the CV membrane of *A. proteus* is equipped with aquaporin.

Sudden contraction (shrinkage or burst) of CV was induced by perfusion with ATP. This response was specific to ATP. Moreover, this contraction was very quick. Prusch and Dunham (1970) reported that the CV isolated from *A. proteus* contracted by 5 mM ATP. However, their results are significantly different from ours. 1) The extent of contraction was very small. 2) Morphology was very different. Our isolated CV was spherical and did not contain appreciable structure inside. However, their CV was not spherical and contained some remarkable structures which are not observed in CV *in vivo*. We prepared the isolation medium whose chemical composition was close to the cytosolic one. 1) Free Ca^{2+} was decreased by adding EGTA. 2) We added Mg^{2+}, which is necessary for intracellular enzymatic activity,

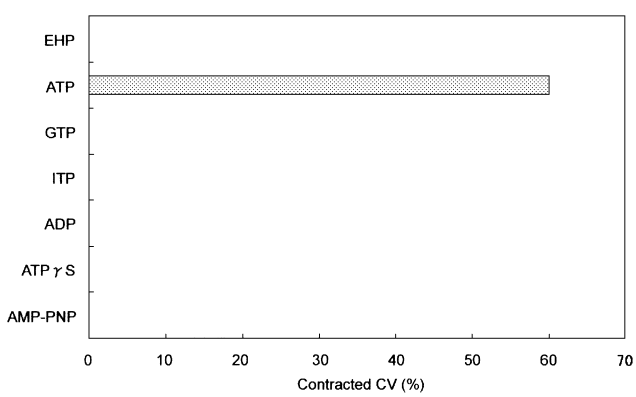

Fig. 5. Effect of nucleotides and ATP analogues on CV contraction. For further explanation, see the text.

such as ATPase. 3) Osmolality of the medium was adjusted close to intracellular osmolarity. Tani *et al*. (2000a, 2000b) reported that periodic tension developed in CVs isolated from *Paramecium*. They suggested that the rounding of the isolated CVs corresponds to the increased membrane tension before fluid expulsion *in vivo*. However, fluid expulsion did not occur in isolated CV. It was suggested that isolated CV lacked a mechanism to form a pore. The tension development in CV isolated from *Paramecium* needed ATP (Tani *et al*., 2000b). ATP-dependent contraction (or bursting) observed in CV isolated from *A. proteus* (Fig. 4) might be caused by increased membrane tension. Namely, this phenomenon may be related to the systole *in vivo*.

The present study showed that the CV membrane is semi-permeable and highly permeable to water. These results suggested that CV membrane is equipped with aquaporin, and that water is collected via osmosis. Thus, mechanism to increase the intra-CV osmolality must operate. Association of H^+-ATPase with CV membrane has been reported in

amoeboid cells of cellular slime mold (Heuser *et al*., 1993; Fok *et al*., 1993), suggesting possible involvement of electrochemical gradient for H^+ in concentration of osmotica into the CV. Thus, the next targets of our research are aquaporin and H^+-ATPase.

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