Biochimica et Biophysica Acta 815 (1985) 410–416
Elsevier

BBA 72611

Ca^{2+} transport studied with arsenazo III in *Tetrahymena* microsomes. Effects of calcium ionophore A23187 and trifluoperazine

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(Received January 2nd, 1985)

Key words: Ca^{2+} transport; (Ca^{2+} + Mg^{2+})-ATPase; Ionophore A23187; Trifluoperazine; (*Tetrahymena* microsome)

Transport of Ca^{2+} in microsomal membrane vesicles of the *Tetrahymena* has been investigated using arsenazo III as a Ca^{2+} indicator. The microsomes previously shown to carry a Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase (Muto, Y. and Nozawa, Y. (1984) Biochim. Biophys. Acta 777, 67–74) accumulated calcium upon addition of ATP and Ca^{2+} sequestered into microsomal vesicles was rapidly discharged by the Ca^{2+} ionophore A23187. Kinetic studies indicated that the apparent K_m for free Ca^{2+} and ATP are 0.4 and 59 μM, respectively. The V_{max} was about 40 nmol/mg protein per min at 37°C. The calcium accumulated during ATP-dependent uptake was released after depletion of ATP in the incubation medium. Furthermore, addition of trifluoperazine which inhibited both (Ca^{2+} + Mg^{2+})-ATPase and ATP-dependent Ca^{2+} uptake rapidly released the calcium accumulated in the microsomal vesicles. These observations suggest that *Tetrahymena* microsome contains both abilities to take up and to release calcium and may act as a Ca^{2+}-regulating site in this organism.

Introduction

The free-swimming protozoa, *Tetrahymena* and *Paramecium* respond to a variety of chemical stimuli by showing a characteristic negative chemotactic response, observed as a temporary reversal of ciliary motion [1,2]. Such locomotive responses are correlated with a transient increase in the intracellular concentration of ionized calcium [3]. Electrophysiological studies have shown that the increase of Ca^{2+} is mediated by a rapid regenerative Ca^{2+} inward current [4,5] through voltage-sensitive Ca^{2+} channels in the ciliary membrane [6,7]. Although entry of external calcium through these channels seems to be the primary signal for ciliary reversal [1], the location and properties of the mechanisms which extrude calcium and maintain the low cytoplasmic Ca^{2+} concentration in *Tetrahymena* have remained uncertain. The ATP-dependent Ca^{2+} pump present in higher organisms in plasma membrane and intracellular organelles such as endoplasmic reticulum plays a critical role in lowering the free intracellular Ca^{2+} concentration [8]. Moreover, the (Ca^{2+} + Mg^{2+})-ATPase in various membrane systems has been studied in considerable detail and is now generally accepted as the enzymatic basis of the Ca^{2+} pump which uses ATP directly as the energy source [9].

In a previous paper from this laboratory [10], it has been demonstrated that the activities of Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase and ATP-dependent Ca^{2+} uptake are present in *Tetrahymena* microsomes and both are apparently bound to the endoplasmic reticulum of this organism. These

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mops, 4-morpholinepropanesulfonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

findings have suggested the possible involvement of the microsomal Ca^{2+} transport system in the regulation of the cytoplasmic free Ca^{2+} concentration which in turn affect various cellular functions such as ciliary reversal in *Tetrahymena*. Furthermore, evidence is now accumulating which indicates that endoplasmic reticulum of noncontractile cells has the ability not only to take up but also release Ca^{2+} [11,12] following cell stimulation as clearly observed in the sarcoplasmic reticulum of muscle cells [13]. It was therefore of interest to investigate the precise properties of the Ca^{2+} transport system in *Tetrahymena* microsomes in the hope of gaining further insight into the role of microsomes as a Ca^{2+}-regulating site in this organism.

In the present report, we have performed a kinetic analysis of the Ca^{2+} transport in *Tetrahymena* microsomes using arsenazo III as a Ca^{2+} indicator. In addition, data are presented which indicate that trifluoperazine is able to release Ca^{2+} from the microsomes and this finding is also discussed in terms of ciliary reversal motion.

Materials and Methods

Materials

Nucleotides (Tris salt) and pure grade arsenazo III were purchased from Sigma Chemical Co., St. Louis, MO. $^{45}CaCl_2$ was obtained from the Radiochemical Centre, Amersham, ionophore A23187 was from Calbiochem and trifluoperazine was supplied by the Yoshitomi Pharmaceutical Co., Osaka, Japan. All other reagents were purchased from standard sources and were of the highest quality available commercially.

Cell growth and isolation of microsomes

A thermotolerant strain of *Tetrahymena pyriformis*, NT-1, was grown at 39.5°C in an enriched proteose-peptone medium [14]. Cells were harvested during the early stationary phase. Microsomes were isolated by differential centrifugation essentially as described previously [10]. After the last centrifugation step, isolated microsomes were suspended in 10 mM Mops-Tris (pH 7.2) containing 100 mM KCl and stored at −80°C until used. The microsomal preparation showed 15-fold enrichment of the endoplasmic reticulum marker glucose-6-phosphatase and less than 4% of total adenylate cyclase (plasma membrane marker) as described previous paper [10]. When examined by transmission electron microscopy, the preparatin exhibited vesicular structures and was free of mitochondria.

Protein was determined by the method of Lowry et al. [15], with bovine serum albumin as standard.

Measurement of Ca^{2+} uptake and release

Uptake and release of calcium were measured spectrophotometrically using arsenazo III as an indicator of extravesicular Ca^{2+} concentration in the suspension medium, and its differential absorbance was measured kinetically with a time-sharing dual wavelength spectrophotometer (Hitachi-356 model) at wavelength pair of 685–675 nm [16]. Unless specified otherwise, the standard 3-ml reaction media contained 100 mM KCl, 5 mM $MgCl_2$, 60 μg of creatine phosphokinase, 5 mM creatine phosphate, 0.5 mM ATP, 10 μM $CaCl_2$, 30 μM arsenazo III, 220 μg/ml microsomes and 20 mM Mops-Tris (pH 7.2). Recordings were calibrated by serial additions of $CaCl_2$ as described in Ref. 16 and reactions initiated by addition of ATP at 37°C. The small baseline shift caused by ATP addition was subtracted from all recordings.

Ca^{2+} uptake was also measured by means of an isotope technique as described previously [10]. Briefly, approx. 0.1–1 mg protein/ml was incubated at 37°C in 1 ml medium containing 20 mM Mops-Tris (pH 7.2), 100 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.2 mM EGTA, 5–10 μCi $^{45}Ca^{2+}$ and various amounts of $CaCl_2$ to yield the desired free Ca^{2+} concentration. The free Ca^{2+} concentrations were calculated according to the method of Pershadsingh and McDonald [17], using an apparent association constant of $1.73 \cdot 10^7\ M^{-1}$ for the Ca^{2+}-EGTA complex at pH 7.2. The Ca^{2+} content in the reaction mixture measured by atomic absorption spectroscopy (Hitachi 308) was 3.5 μM and this amount of Ca^{2+} was taken into consideration in the calculation [17]. At the required time-intervals, 0.1-ml aliquots were removed and filtered rapidly through 0.45 μm HAWP Millipore filters. The filters were washed rapidly with 20 mM Mops-Tris (pH 7.2), 5 mM $MgCl_2$, 5 mM EGTA,

100 mM KCl at 4°C, and counted for radioactivity.

Results

ATP-dependent Ca^{2+} uptake in Tetrahymena microsomes

A time-course of ATP-driven Ca^{2+} uptake in *Tetrahymena* microsomal vesicles is shown in Fig. 1a. In the presence of Mg^{2+} and ATP-regenerating system, addition of ATP to the reaction mixture caused a rapid decrease in ΔA (at 675–685 nm), which was due to the net reduction in Ca^{2+} concentration by sequestration into the microsomes. A steady-state level of approx. 27 nmoles Ca^{2+}/mg of microsomal protein was reached within several minutes. No ATP-dependent uptake occurred when magnesium was omitted from the reaction medium. The finding that the accumulated calcium could be rapidly released into the medium upon addition of the Ca^{2+} ionophore A23187 (Fig. 1a), strongly suggested that calcium is actively transported against its concentration gradient [18]. When the experiments were carried in the presence of A23187 (5 μM) or orthovanadate (100 μM) which inhibits microsomal (Ca^{2+} + Mg^{2+})-ATPase [10], the addition of ATP resulted in no stimulation of Ca^{2+} uptake (Fig. 1 b, c). These observations were in good agreement with the previous results obtained using $^{45}Ca^{2+}$ [10].

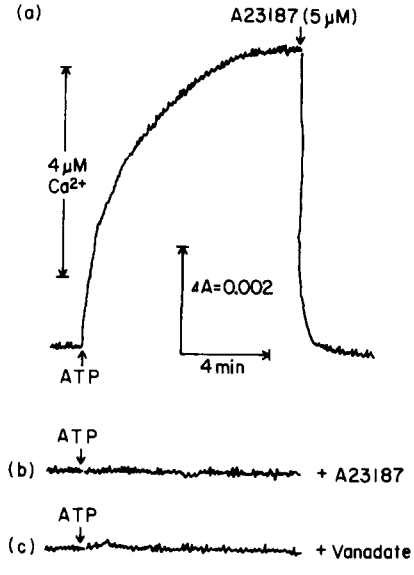

Fig. 1. ATP-dependent uptake of Ca^{2+} by microsomes from *Tetrahymena*. Ca^{2+} uptake was measured spectrophotometrically as described in Materials and Methods using arsenazo III as Ca^{2+} indicator. In figures where Ca^{2+} uptake and release were determined spectrophotometrically, the absorbance is proportional to extravesicular Ca^{2+} concentration. Thus, the net Ca^{2+} uptake by the microsomes is accompanied by a decrease in absorbance (upward deflection in the figure) and the net release is reflected by an increase in absorbance (downward deflection in the figure). 0.5 mM ATP was added (arrow) to initiate Ca^{2+} uptake in the presence of the ATP-regenerating system. The medium was supplemented with 5 μM A23187 (b), 100 μM orthovanadate (c) and without any addition (a).

Kinetic and other characteristics of Ca^{2+} uptake

The rate of ATP-dependent Ca^{2+} uptake by *Tetrahymena* microsomes was measured as a function of ATP concentration. Fig. 2 shows a double-reciprocal plot of initial velocities which were determined from the slopes for ΔA decrease (Fig. 2 inset). From Lineweaver-Burk analysis of the data, the K_m for ATP of the Ca^{2+} uptake was approx. 59 μM and V_{max} was about 40 nmol/mg protein per min. The Ca^{2+} uptake was specific for ATP and other nucleotides could not substitute for ATP. The rates of Ca^{2+} uptake in the presence of GTP, UTP, ITP or CTP were all $\leq$ 10% of the ATP-stimulated rate (data not shown).

The initial rate of Ca^{2+} uptake was also de-

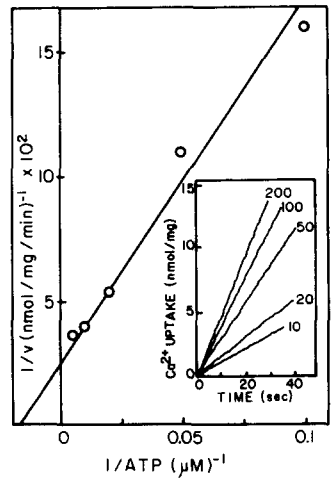

Fig. 2. Dependence on ATP concentration of ATP-stimulated Ca^{2+} uptake in *Tetrahymena* microsomes. ATP-dependent Ca^{2+} uptake was measured spectrophotometrically as described in Materials and Methods, except that ATP concentration was varied as shown with μM in the inset. Initial velocities of Ca^{2+} uptake were determined from the slopes for ΔA decrease (inset) and then plotted in double-reciprocal plot.

termined as a function of the free external Ca^{2+} concentration (Fig. 3). In this case, the Ca^{2+} uptake was measured by isotope technique with $^{45}Ca^{2+}$ and the desired free Ca^{2+} concentration was adjusted using the Ca^{2+}-EGTA buffer system as described in Materials and Methods. The rate of Ca^{2+} uptake increased over the concentration range of 0.1–1 μM free Ca^{2+}, and reached a maximum at approx. 2 μM free Ca^{2+}. A double-reciprocal plot (Fig. 3 inset) yielded an apparent K_m of 0.4 μM free Ca^{2+} and a V_{max} of 7 nmol/mg protein per min. Although the V_{max} value was significantly lower than that derived from the data of ATP kinetics (Fig. 2), this may reflect an underestimation of Ca^{2+} uptake by the isotope technique. For example, $^{45}Ca^{2+}$-$^{40}Ca^{2+}$ exchange, leakage of Ca^{2+} from the vesicles and inefficiency of membrane recovery during filtration could all attribute to the lower rate of Ca^{2+} uptake measured by $^{45}Ca^{2+}$. Ca^{2+} uptake was not inhibited by mitochondrial ATPase inhibitors such as oligomycin (5 μg/ml), dicyclohexylcarbodiimide (100 μM) or NaN_3 (10 mM) and $(Na^+ + K^+)$-ATPase inhibitor ouabain (1 mM).

Ca^{2+} release from Tetrahymena microsomes

In terms of control of cytoplasmic Ca^{2+} concentration, efflux of calcium from microsomal vesiscles is likely to be of equal importance to the uptake process. Therefore, we next examined the Ca^{2+} efflux from *Tetrahymena* microsomes in the ATP-depleted Ca^{2+} uptake medium [19]. As shown in Fig. 4a, in the absence of ATP-regenerating system, addition of limiting concentration of ATP (10 μM) induced a significant Ca^{2+} uptake followed by the release of calcium. However, since subsequent addition of ATP (10 μM) reinitiated Ca^{2+} sequestration with its subsequent release (Fig. 4a), deterioration of microsomal membranes did not appear to occur during Ca^{2+} flux measurement. Furthermore, addition of an ATP-regenerating system after Ca^{2+} release was found to stimulate Ca^{2+} sequestration with no net Ca^{2+} release (Fig. 4b). These observations suggest that Ca^{2+} accumulated during ATP-dependent uptake is released when ATP is exhausted and the steady-state level of accumulated Ca^{2+} concentration in the presence of ATP-regenerating system (Fig. 1) should be determined by the balance of uptake rate against the efflux process.

Effect of trifluoperazine on Ca^{2+} fluxes in Tetrahymena microsomes

It was demonstrated in the previous report [10] that $(Ca^{2+}Mg^{2+})$-ATPase of *Tetrahymena* microsomes was inhibited by the phenothiazine drug,

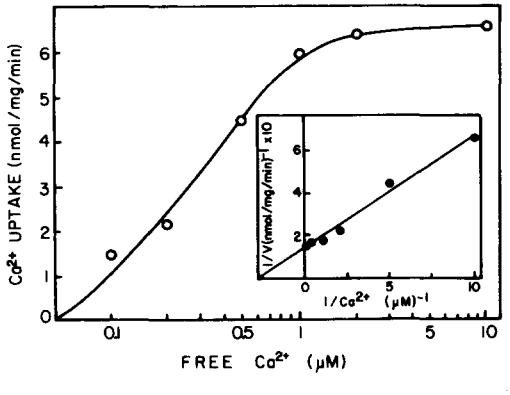

Fig. 3. Effects of Ca^{2+} concentration on ATP-dependent Ca^{2+} uptake of *Tetrahymena* microsomes. ATP-dependent Ca^{2+} uptake was assayed by millipore filtration technique using $^{45}Ca^{2+}$ as described in Materials and Methods. The reaction was started by addition of ATP and the free Ca^{2+} concentration was adjusted by Ca^{2+}-EGTA buffer [17]. The inset represents a double-reciprocal plot of the ATP-dependent Ca^{2+} uptake.

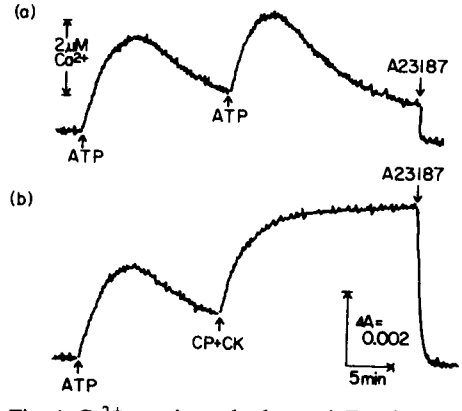

Fig. 4. Ca^{2+} uptake and release of *Tetrahymena* microsomes. Ca^{2+} transport was measured spectrophotometrically as described in Materials and Methods, except that ATP-regenerating system was omitted from the reaction medium. Ca^{2+} uptake was initiated by the addition of 10 μM ATP, and after calcium release 10 μM ATP (a) or 2.5 mM creatine phosphate (CP)+30 μg of creatine phosphokinase (CK) (b) were added (arrows). 5 μM A23187 was added (arrow) to determine the sequestered Ca^{2+}.

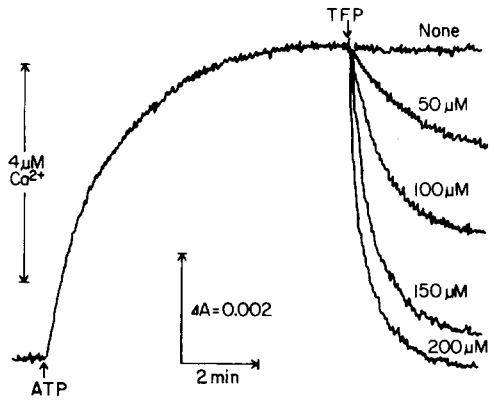

Fig. 5. Effects of various concentrations of trifluoperazine on the Ca^{2+} release of *Tetrahymena* microsomes. Microsomes were incubated as described in the legend to Fig. 1 in the medium containing the ATP-regenerating system. Ca^{2+} uptake was started by addition of 0.5 mM ATP and the concentrations of trifluoperazine (TFP) added (arrow) are reported in the figure.

trifluoperazine. This finding prompted us to investigate whether the agent modifies Ca^{2+} fluxes across the microsomal membranes. Fig. 5 shows that addition of trifluoperazine to microsomal vesicles filled with calcium as a consequence of ATP-dependent uptake, caused an immediate release of the accumulated Ca^{2+} in a dose-dependent fashion. Moreover, if trifluoperazine was added before ATP, the initial rate of Ca^{2+} uptake and the steady-state level of Ca^{2+} accumulation were considerably reduced (Fig. 6). Trifluoperazine is known to act in many cells by interfering with the actions of calmodulin. Addition of calmodulin purified from *Tetrahymena* did not affect Ca^{2+} fluxes in microsomes, even after the microsomal preparation was extensively washed with 5 mM EGTA to remove endogenous calmodulin (data not shown). Therefore, the effects of the drug on Ca^{2+} fluxes demonstrated here may be due to interaction of the drug with component(s) other than calmodulin [20].

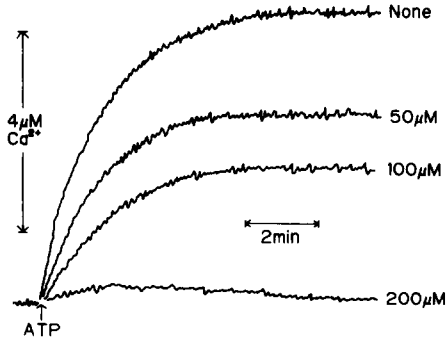

Fig. 6. Effects of various concentrations of trifluoperazine on ATP-dependent Ca^{2+} uptake of *Tetrahymena* microsomes. Ca^{2+} uptake was measured as described in the legend to Fig. 1, except that different concentrations of trifluoperazine were included in the reaction mixture before ATP addition. The reaction was initiated by addition of 0.5 mM ATP in the presence of the ATP-regenerating system.

Discussion

In this study, an ATP-dependent Ca^{2+} transport system has been characterized in microsomal vesicles derived from *Tetrahymena* cells. These vesicles were able to accumulate calcium against an electrochemical gradient, as evidenced by the ability of Ca^{2+} ionophore A23187 to rapidly dissipate the vesicle-associated calcium and by the requirement for MgATP. The Ca^{2+} uptake process was saturable and the steady-state level of approx. 27 nmoles Ca^{2+}/mg of microsomal protein was reached in the presence of ATP-regenerating system. The activity of ATP-dependent Ca^{2+} uptake is considered to be principally localized in the endoplasmic reticulum, since the microsomal preparation used here was not contaminated by other membrane organelles such as plasma membrane and mitochondria as evidenced by marker enzymes and electron microscopy (Materials and Methods). Furthermore, IC_{50} (the concentration required for half-maximal inhibition) of the ATP-dependent Ca^{2+} uptake for vanadate was determined to be 30 μM [10], which was rather similar to that for the Ca^{2+} transport system in the sarcoplasmic reticulum [21]. In addition, insensitivity to calmodulin of the Ca^{2+} uptake also suggests that the endoplasmic reticulum is the origin of the preparation [13].

The ATP-dependent Ca^{2+} uptake was observed to exhibit similar kinetic properties to those of the $(Ca^{2+} + Mg^{2+})$-ATPase [10]. The initial velocities of both Ca^{2+} uptake and $(Ca^{2+} + Mg^{2+})$-ATPase [10] were saturated in the micromolar range of free Ca^{2+} and both activities showed similar affinities for Ca^{2+}. Also, the K_m values for ATP were almost identical; approx. 63 μM [10] and 59 μM

for $(Ca^{2+} + Mg^{2+})$-ATPase and ATP-dependent Ca^{2+} uptake, respectively. In addition, both activities were inhibited by trifluoperazine and vanadate in the similar concentration range [10]. These findings suggest compellingly that the Ca^{2+}-stimulated ATP hydrolysis and the ATP-stimulated Ca^{2+} uptake in *Tetrahymena* microsome are catalyzed by the same enzyme, i.e. the $(Ca^{2+} + Mg^{2+})$-ATPase, as clearly verified in the system of the sarcoplasmic reticulum [22] and erythrocyte plasma membrane [23].

Ca^{2+} sequestered by *Tetrahymena* microsomes during the ATP-dependent uptake process was released when the ATP was exhausted in the absence of the ATP-regenerating system. At present, the molecular mechanism by which ATP depletion induces Ca^{2+} release remains unknown. Many reports have demonstrated that the depletion of ATP induces Ca^{2+} release from sarcoplasmic reticulum [19,24,25] and it was suggested that Ca^{2+} would be released through specific 'channels' in the membrane, the properties of which are altered by a high concentration of accumulated Ca^{2+} [25]. It is thus possible that Ca^{2+} release induced by ATP depletion in *Tetrahymena* microsomes might be mediated through unidentified 'channels' other than the Ca^{2+} translocation sites involved in active transport. In addition, the results obtained in this study showed that addition of trifluoperazine to microsomal vesicles filled with calcium by active transport (in the presence of ATP-regenerating system) induced sudden release of calcium (Fig. 5). Since the drug inhibited both ATP-dependent Ca^{2+} uptake (Fig. 6) and $(Ca^{2+} + Mg^{2+})$-ATPase activity [10] in the similar concentration range, the drug-induced Ca^{2+} release from microsomal vesicles might be due to inhibition of the Ca^{2+} pump in microsomes, resulting in the efflux of calcium along its electrochemical gradient as observed in sarcoplasmic reticulum vesicles [26,27]. Alternatively, the effect of trifluoperazine may be related to its interaction with membrane phospholipids. Previously, we have shown that trifluoperazine affected the physical state of liposomes as estimated by ESR analysis [20]. It is thus possible that trifluoperazine may have some effects on the lipid environment of microsomes and, thereby, alter the permeability for Ca^{2+} and induce Ca^{2+} release. At present time, the precise mechanism of Ca^{2+} release caused by ATP depletion or added trifluoperazine in *Tetrahymena* microsomes remains to be explored. Further characterization of these processes will give more insight into the physiological mechanism of Ca^{2+} release which has been clearly evidenced, such as 'Ca^{2+}-induced Ca^{2+} release' in sarcoplasmic reticulum [28,29].

Several lines of evidence have been provided indicating that Ca^{2+} is critically required for the regulation of ciliary motion in ciliated protozoa [1,30,31]. The increase of intracellular Ca^{2+} has been shown to be responsible for the depolarization-induced reversal of ciliary beat [3,4], and the renormalization of Ca^{2+} concentration is considered to be correlated with the recovery of normal ciliary beat [32]. The microsomal Ca^{2+} transport system described here is thought to be essential for calcium homeostasis in *Tetrahymena*, when intracellular Ca^{2+} concentration is altered during physiological processes such as ciliary reversal. In addition, the present observations also suggest the possibility that *Tetrahymena* microsome may serve as a Ca^{2+}-releasing site which is able to respond to various stimuli, in analogy to sarcoplasmic reticulum [13]. In this context, it is interesting to note that Suzuki et al. [33] reported that the addition of trifluoperazine resulted in ciliary reversal in *Tetrahymena* in vivo. Since the drug could rapidly release Ca^{2+} from microsomes as demonstrated here, the trifluoperazine-induced ciliary reversal might be caused by the increased cytoplasmic Ca^{2+} level as a result of release from the microsomal compartment. On the other hand, there are several reports on the Ca^{2+} influx process [34–36] and Ca^{2+}-stimulated ATPase [37–41] of surface membrane (plasma membrane) in *Paramecium*, and its possible implication in the regulation of the intracellular concentration of Ca^{2+} has been proposed [34,42]. The surface membrane also would be involved in the regulation of the intracellular Ca^{2+} concentration in *Tetrahymena*. At present, although the relative contribution of the microsomal Ca^{2+} transport system in controlling the cytoplasmic Ca^{2+} concentration in *Tetrahymena* remains to be assessed, this system may play a certain role in Ca^{2+} mobilization associated with various cellular actions including ciliary reversal.

Acknowledgment

This work was supported by a Grant-in-Aid for Special Project Research on Molecular Mechanism of Bioelectrical Response (59223007) from the Japanese Ministry of Education, Science and Culture.

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