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Role of Ca^{2+} and cAMP in a cell signaling pathway for resting cyst formation of ciliated protozoan *Colpoda cucullus*

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Summary

Resting cyst formation (encystment) of *Colpoda cucullus* is caused by an increase in an external Ca^{2+} concentration or overpopulation of *Colpoda* vegetative cells. The Ca^{2+}-mediated or overpopulation-mediated encystment was suppressed by Ca^{2+} channel blockers (Cd^{2+}, La^{3+}, Ni^{2+}), Ca^{2+}-chelating reagents (EGTA, BAPTA), calmodulin antagonists (W-7, trifluoperazine), *R*p-cAMPS (an cAMP analog antagonist) and 2'-deoxyadenosine (a P-site inhibitor of adenylate cyclase). On the other hand, by the addition of Ca^{2+} ionophore A23187, IBMX (an inhibitor of phosphodiesterase), cAMP or its membrane-permeable derivative, the encystment was prominently induced even in the Ca^{2+}-free medium. These results suggest that *Colpoda* encystment may be mediated by an increase in cAMP concentration through the activation of adenylate cyclase whose activity is possibly regulated by Ca^{2+}/calmodulin

Key words: cAMP, Ca^{2+}/calmodulin, cyst formation, *Colpoda*

Introduction

As already described by Gutiérrez et al. (2001), Matsusaka (2006) and perhaps other protozoologists, resting cysts of protozoa is one of the cryptobiotic forms, the process of whose formation involves a gene-regulated cyto-differentiation (Matsusaka, 1979; Matsusaka et al., 1989; Martín-González et al., 1991; Gutiérrez et al., 2000; Izquierdo et al., 2000) and cytoplasmic dehydration prior to a stop of metabolic activity. To survive on the soil surface where the puddles temporarily appear and rapidly dry out, the terrestrial colpodid ciliates transform into resting cysts resistant to drying, freezing and higher temperatures (Taylor and Strickland, 1936; Foissner, 1993; Gutiérrez, 2001; Maeda et al., 2005), and they excyst to proliferate when favorable conditions are regained. The resting cyst formation (encystment) is induced by an increase in mainly external Ca^{2+} concentration (a signal for coming desiccation) in *Colpoda cucullus* (Yamaoka et al., 2004) or the overpopulation of vegetative cells in *C. duodenaria* (Strickland, 1940) and *C. cucullus* (Maeda et al., 2005). The Ca^{2+}-induced encystment

of *Colpoda* is, on the other hand, suppressed by components released from bacteria (a signal indicating the existence of food) (Yamasaki et al., 2004) and components contained in plant leaves such as peptides, porphyrins, etc. (Tsutsumi et al., 2004; Yamasaki et al., 2004).

The purpose of the present study is to elucidate the signaling pathway leading to *Colpoda* encystment which is activated by the addition of Ca^{2+} in the surrounding medium or a cell-to-cell stimula-tion with *Colpoda* vegetative cells. We revealed, by means of Ca^{2+} channel blockers (Kasai and Neher, 1992; Mlinar and Enyeart, 1993; Leech et al., 1994; Lee et al., 1999; Triggle 2006), Ca^{2+} chelating reagents and Ca^{2+} ionophore A23187 (Reed and Lardy, 1972), calmodulin antagonists such as W-7 (Hidaka et al., 1981) or trifluoperazine (Vandonselaar et al., 1994), that the signaling pathway leading to encystment might be activated by an Ca^{2+}/calmodulin (Ca^{2+}/CaM). In addition, we obtained results suggesting that an increase in intracellular cAMP concentration was involved in encystment induction. In the present paper, a partial process of an encystment-inducing signaling pathway including Ca^{2+}/CaM and cAMP will be discussed.

Material and methods

Colpoda cucullus was cultured in 0.05% (w/v) cereal infusion inoculated with bacteria (*Enterobactor aerogenes*) as food. The bacteria, which were supplied by the Institute for Fermentation, Osaka, Japan, were cultured on agar plates containing 1.5% agar, 0.5% polypepton, 1% meat extract and 0.5% NaCl. For encystment induction, vegetative cells cultured for 1-2 days were collected by centrifugation (1,500 g, 2 min), rinsed twice in 1 mM Tris-HCl (pH 7.2), and then transferred into 200 µl of test solutions at cell density of 500-1,000 cells/ml or 5,000~10,000 cells/ml (overpopulation).

The number of encysted cells were counted in 80-120 randomly chosen per measurement, and were expressed as a percentage of the total number of cells (80-120 cells). Columns (points) and attached bars correspond to the means of 5-7 identical measurements (the number of measurements shown in the figures) and standard errors. All test solutions contained at least 1 mM Tris-HCl (pH 7.2) (Figs 1, 2, 3B, 4, 5, 6A) or both 1 mM Tris-HCl (pH 7.2) and 0.1% DMSO (Figs 3A, 3C, 6B). Other components contained in the test solutions were labeled in the figures. The rates of encystment (%) were measured at 7-8 hr after the onset of induction. Asterisks (*) and double asterisks (**) in figures show significant differences among columns at $p < 0.05$ and $p < 0.01$, respectively (Mann-Whitney test).

N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W-7 hydrochloride), verapamil hydrochloride, diltiazem hydrochloride, 3-isobutyl-1-methylxanthine (IBMX), 2'-deoxy-adenosine monohydrate (2'-dA), forskolin and adenosine 3', 5'-cyclic monophosphothioate were purchased from Wako Pure Chemical Industries, A23187 (free acid), ethylenebis (oxy-2, 1-phenylenenitrilo) tetraacetic acid tetra (acetoxy-methyl) ester (BAPTA-AM) from Calbiochem, N^6, 2'-O-dibutyryladenosine 3', 5'-cyclic mono-phosphate sodium salt (db-cAMP), N2, 2'-O-dibutyrylguanosine 3', 5'-cyclic monophosphate sodium salt (db-cGMP), adenosine 3', 5'-cyclic monophosphate sodium salt monohydrate (cAMP), guanosine 3', 5'-cyclic monophosphate sodium salt (cGMP) from Sigma-Aldrich, Inc., *R*p-isomer (*R*p-cAMPS) from Biolog Life Sci. Inst. and trifluoperazine dihydrochloride (TFP) from MP Biomedicals, LLC. BAPTA-AM, forskolin and A23187 were dissolved in dimethyl sulfoxide (DMSO) to give 10 mM, 10 mM and 1 mM stock solutions, respectively, and 10 µl of each stock solution was added into 10 ml of 1 mM Tris-HCl (pH 7.2) to produce final concentrations of test solutions at 10 µM, 10 µM and 1 µM, respectively (final concentration of DMSO at 0.1%).

Results and Discussion

Ca^{2+}-mediated encystment induction and effects of Ca^{2+} channel blockers

It has been previously reported that 1 mM Ca^{2+} could induce encystment of *C. cucullus* (Yamaoka et al., 2004), but the minimum Ca^{2+} concentration for inducing encystment is still unknown. In the present study, first, the encystment-inducing effects of several salts ($CaCl_2$, NaCl, KCl, $MgCl_2$) in the surrounding medium were investigated at concentrations raging from 1 µM to 1 mM. At concentrations less than 1 mM, the external NaCl, KCl or $MgCl_2$ hardly showed an encystment-inducing activity, while much lower concentrations of $CaCl_2$ had marked encystment-inducing activity (even 10 µM $CaCl_2$ was effective for encystment induction) (Fig. 1). As previously suggested (Yamaoka et al., 2004), the results also indicate that the effect of $CaCl_2$ may not be due to Cl^-, but to Ca^{2+} at least in these concentrations. In order to examine whether or not the increase in external Ca^{2+} concentration may cause inflow of Ca^{2+} through Ca^{2+} channels,

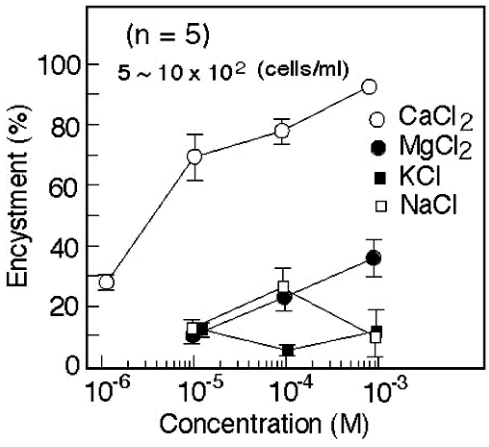

Fig. 1. Effects of various salts (ions) on the induction of *Colpoda* encystment. Every solution contained 1 mM Tris-HCl (pH 7.2) and various concentrations of salts.

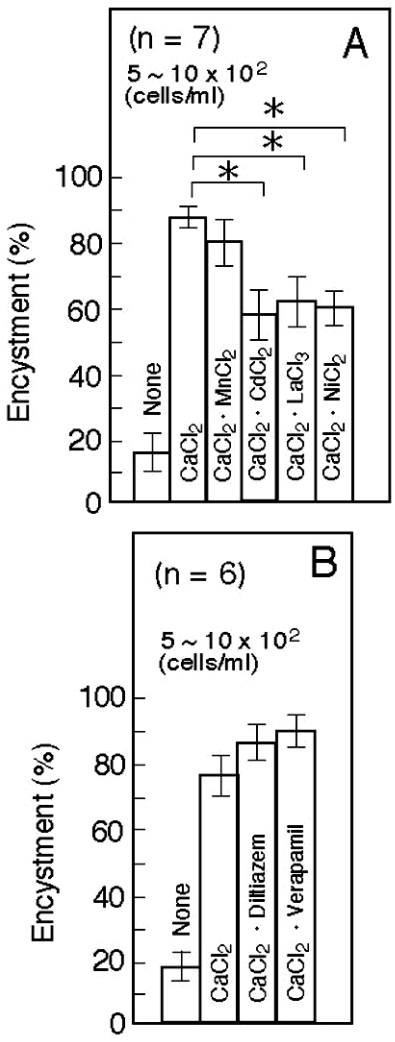

Fig. 2. Effects of Ca^{2+} channel blockers on the induction of *Colpoda* encystment. A - divalent (100 μM Mn^{2+}, 1 μM Cd^{2+}, 1 μM Ni^{2+}) and trivalent cations (1 μM La^{3+}); B - specific blockers (10 μM diltiazem and 10 μM verapamil) for L-type Ca^{2+} channels. The drug concentrations used in this assay are critical for the survival of the cells.

the effects of Ca^{2+} channel blockers such as divalent or trivalent cations (Mn^{2+}, Cd^{2+}, La^{3+}, Ni^{2+}, La^{3+}), verapamil and diltiazem on encystment induction were examined (Fig. 2). As shown in Fig. 2, Ca^{2+}-mediated encystment was significantly suppressed by the addition of Cd^{2+}, La^{3+} or Ni^{2+} into the surrounding medium, while Mn^{2+}, verapamil and diltiazem did not affect encystment. The inhibitory effects of divalent and trivalent metal ions against several types of Ca^{2+} channels are not so specific (Kasai and Neher, 1992; Mlinar and Enyeart, 1993; Leech et al., 1994; Lee et al., 1999), while verapamil and diltiazem are specific blockers against a subclass of voltage-gated Ca^{2+} channels, L-type channels (Triggle, 2006). In consequence, the Ca^{2+} channels involved in the induction of encystment of *Colpoda* may not be L-type Ca^{2+} channels.

Effects of chelating reagents or ionophore for Ca^{2+} on encystment induction

The hydrophobic compound BAPTA-AM permeates into the cell interior, and rapidly splits by means of esterases contained in the cells to form the hydrophilic Ca^{2+} chelator BAPTA which is trapped by the cells. Loading cells with BAPTA-AM, therefore, is expected to suppress a rise in intracellular Ca^{2+} concentration. If the intracellular Ca^{2+} concentration is assumed to be less than 5×10^{-6} M, the presence of 10 μM BAPTA in the cytoplasm will reduce the free Ca^{2+} concentration to less than 2×10^{-7} M. As shown in Fig. 3A, Ca^{2+}-mediated or overpopulation-mediated (Fig. 3A, right two columns) encystments were significantly suppressed by the addition of 10 μM BAPTA-AM in the surrounding medium. If the concentration of contaminating Ca^{2+} in the surrounding medium is assumed to be 10^{-6} M, the addition of 10 μM EGTA (final concentration) reduces the free Ca^{2+} concentration to about 10^{-8} M. The addition of 10 μM (final concentration) EGTA in the external medium also significantly suppressed overpopulation-mediated encystment (Fig. 3B). These results suggest that overpopulation with *Colpoda* vegetative cells may cause encystment through an increase in intracellular Ca^{2+} concentration that is resulted from an inflow of Ca^{2+} across the plasma membrane.

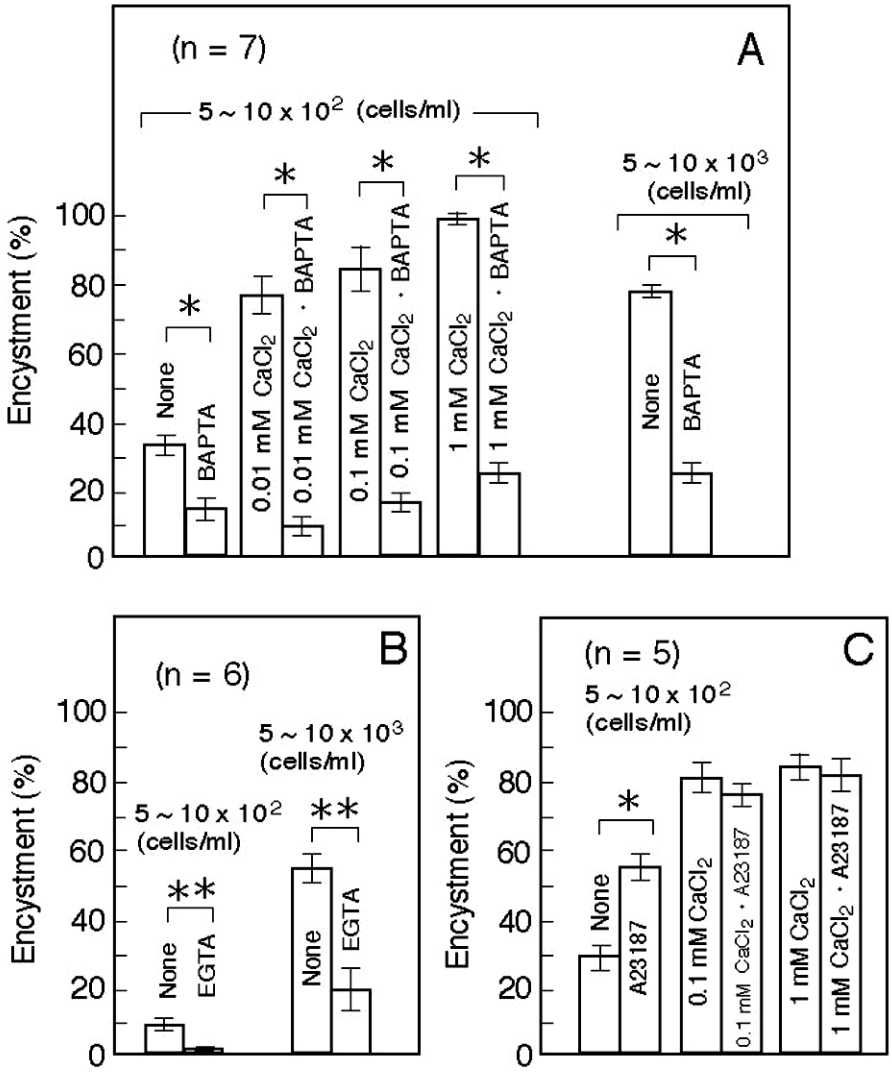

Fig. 3. Effects of intracellular (BAPTA), extracellular Ca^{2+} chelator (EGTA) or Ca^{2+} ionophore A23187 on the induction of *Colpoda* encystment. A - BAPTA-AM (10 μM); B - EGTA (10 μM); C - Ca^{2+} ionophore A23187 (1 μM).

Some of the *Colpoda* encysted even in Ca^{2+}-free Tris-HCl buffer (pH 7.2) (Figs 3A, B; leftmost columns labeled 'None'). Such spontaneous encystment was significantly suppressed by BAPTA or EGTA (Figs 3A, B, leftmost two columns), implying that the spontaneous encystment is possibly mediated by a few Ca^{2+} contaminating the surrounding medium. When *Colpoda* vegetative cells were suspended in a Ca^{2+}-free Tris-HCl buffer (pH 7.2), encystment induction was promoted by the addition of ionophore A23187 (Fig. 3C). On the other hand, encystment induced by the addition of 0.1 mM or 1 mM Ca^{2+} was not further promoted (Fig. 3C). Presumably, in the presence of 0.1 mM or 1 mM Ca^{2+}, enough Ca^{2+} may be supplied into the cytoplasm for the maximum encystment induction.

Effects of calmodulin antagonists on Ca^{2+}-mediated encystment induction

The effects of calmodulin antagonists W-7 and trifluoperazine (TFP) on Ca^{2+}-mediated encystment were examined. As shown in Fig. 4, the encystment-suppression effect of W-7 was rather prominent, although TFP significantly suppressed Ca^{2+}-mediated encystment. These results (Figs 1-4) suggest that a rise in free Ca^{2+} concentration in an external medium elicits an inflow of Ca^{2+} through Ca^{2+} channels which may activate Ca^{2+}-binding

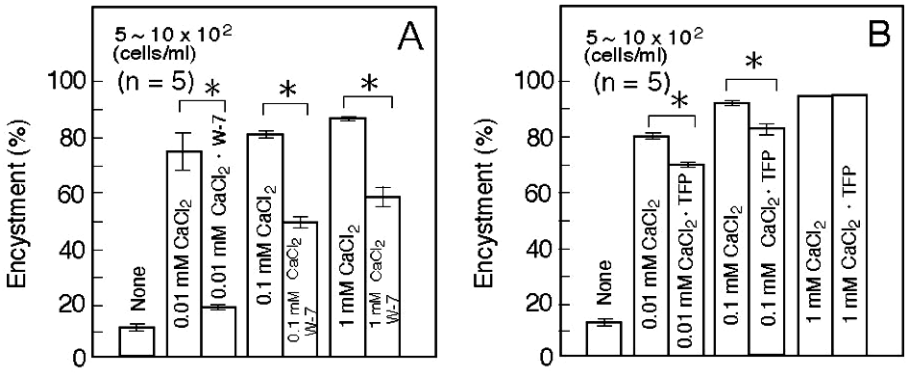

Fig. 4. Effects of 1 μM W-7 (A) and 0.5 μM trifluoperazine (B) on the induction of *Colpoda* encystment. The drug concentrations used in this assay are critical for the survival of the cells.

proteins such as calmodulin. It has been reported that the induction of the encystment of *Entamoeba* is Ca^{2+}/calmodulin (Ca^{2+}/CaM) dependent (Makioka et al., 2001). It is presumed that a signaling pathway leading to the encystment of protozoans may involve a common Ca^{2+}/CaM dependent process.

Role of c AMP in encystment induction

In the presence of 3-isobutyl-1-methylxanthine (IBMX), which is a non-selective inhibitor of phosphodiesterases (Mehats et al., 2002), encystment was markedly promoted even in a Ca^{2+}-free medium

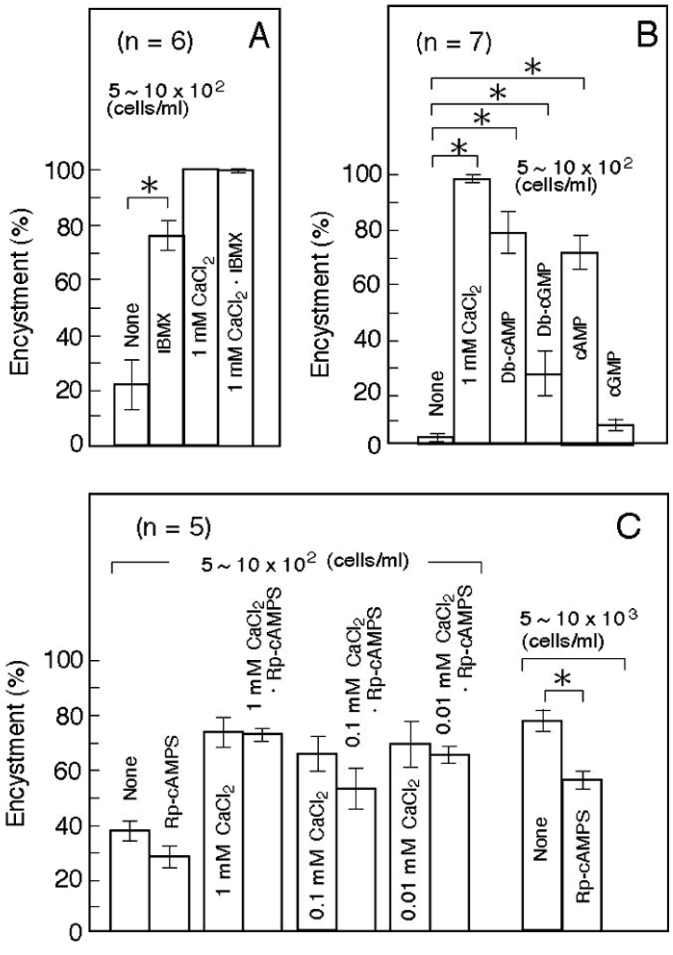

Fig. 5. Role of cAMP in *Colpoda* encystment induction. A - Effects of IBMX (500 μM); B - effects of cyclic nucleotides and their derivatives (500 μM); C - effects of 500 μM *R*p-cAMPS.

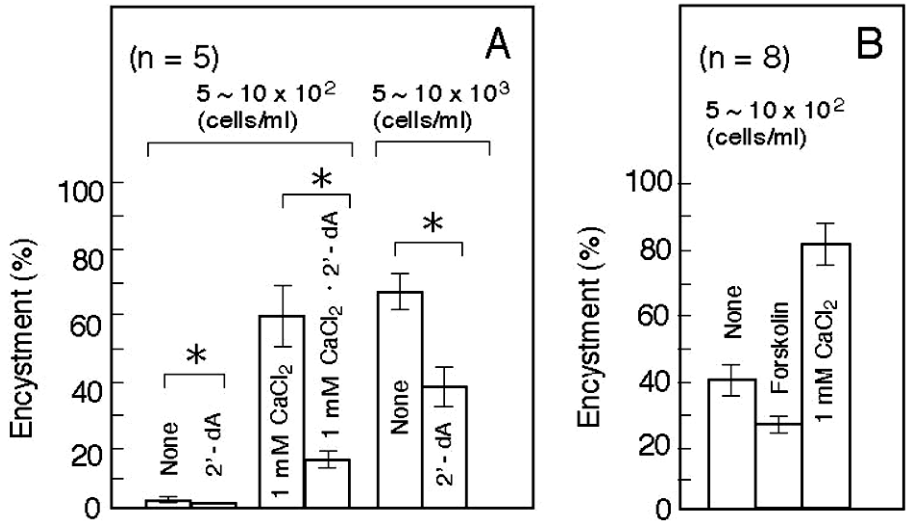

Fig. 6. Effects of a P-site inhibitor or an activator of adenylate cyclase on *Colpoda* encystment induction. A - Effects of 10 μM 2'-deoxyadenosine (2'-dA) which is a P-site inhibitor; B - effects of 10 μM forskolin (an activator of adenylate cyclase).

(Fig. 5A). This result indicates that a certain cyclic nucleotide may be involved in encystment induction. Therefore, the encystment-inducing effects of cAMP, cGMP or their membrane-permeable derivatives (db-cAMP, db-cGMP) were examined. As shown in Fig. 5B, db-cAMP and cAMP remarkably promoted encystment in the Ca^{2+}-free medium. On the other hand, *R*p-cAMPS which is a cAMP antagonist (Rothermel et al., 1983, Botelho et al., 1988) tended to suppress the encystment (Fig. 5C). The results suggest that an increase in the intracellular cAMP concentration may occur in a signaling pathway leading to the encystment. Some mammalian adenylate cyclase isoforms are activated by Ca^{2+}/CaM (Sunahara and Taussig, 2002). In a signaling pathway for the encystment induction of *Colpoda*, Ca^{2+}/CaM may also stimulate adenylate cyclase. Actually, as shown in Fig. 6A, the Ca^{2+}-mediated or overpopulation-mediated encystment was significantly suppressed by the addition of 2'-deoxyadenosine (2'-dA) which is a P-site inhibitor of adenylate cyclase (Fujimori and Pan-Hou, 2005). However, encystment was not promoted by the addition of forskolin which is a stimulator of adenylate cyclase (Seamon et al., 1981). One of the mammalian membrane-bound adenylate cyclase isoforms and a soluble adenylate cyclase are not potently stimulated by forskolin (Sunahara and Taussig, 2002). The putative adenylate cyclase of *Colpoda* may be insensitive to forskolin, although it cannot be concluded whether it is of the membrane-bound or soluble type.

Fig. 7 shows a presumably early process of the signaling pathway leading to the encystment induction of Colpoda, drawn based on the present results. When the external Ca^{2+} concentration is raised, Ca^{2+} probably flows into the cell interior through Ca^{2+} channels. The fact that overpopulation-mediated encystment is suppressed by an intracellular Ca^{2+} chelator BAPTA (Fig. 3A, right two columns) suggests that cell-to-cell mechanical stimulation (Maeda et al., 2005) with *Colpoda* cells may increase the intracellular Ca^{2+} concentration by promoting an inflow of Ca^{2+} or a release of Ca^{2+} from intracellular stored vesicles. Intracellular Ca^{2+} is expected to activate calmodulin through the formation of Ca^{2+}/calmodulin complex (Ca^{2+}/CaM), which may raise the cAMP concentration by the activation of adenylate cyclase (Sunahara and Taussig, 2002), although the possibility that the Ca^{2+}/CaM suppresses phosphodiesterase activity cannot be eliminated. On the other hand, it is possible that cAMP production and following activation of cAMP-dependent proteins may be located upstream of Ca^{2+}/CaM in the signaling pathway for the encystment induction. That is, the cell-to-cell stimulation due to overpopulation with *Colpoda* may evoke an elevation of intracellular Ca^{2+} concentration through proteins regulated by cAMP such as PKA or Epac (Bos, 2003). Therefore, further work will involve determining, by means of in vitro quantitative cAMP assays, changes in the intracellular cAMP concentration caused by the addition of Ca^{2+} to the external medium. In addition, changes in intracellular Ca^{2+} concentration must be visualized when the encystment is induced by the addition of Ca^{2+} to the external medium, by overpopulation with *Colpoda* cells, or

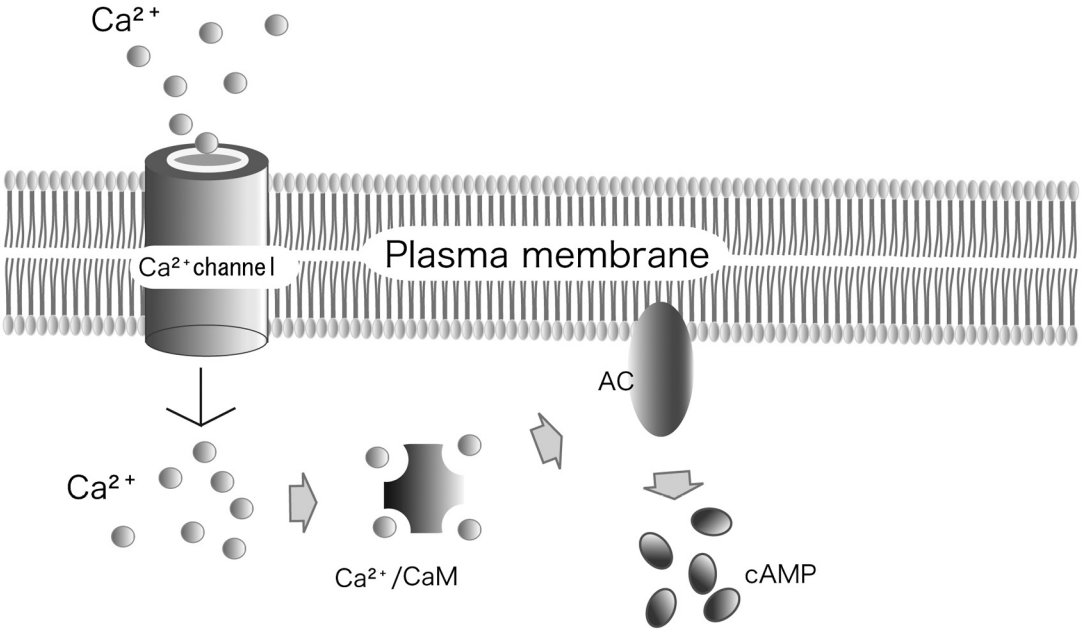

Fig. 7. A schematic diagram showing an early signaling pathway for encystment induction.

by the addition of membrane-permeable cAMP derivatives.

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