Contribution of Calcium Influx on Trichocyst Discharge in Paramecium caudatum

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Contribution of Calcium Influx on Trichocyst Discharge in *Paramecium caudatum*

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ABSTRACT—Trichocyst discharge in *Paramecium* spp. is known to be mediated by rapid exocytosis. Applied stimuli induce fusion of the trichocyst membrane and plasma membrane within 30 ms. Both Ca^{2+} release from intracellular store(s) and Ca^{2+} influx from extracellular region have been suggested to be related to the trichocyst discharge.

We constructed a new system in which to record intracellular levels of Ca^{2+} ($[Ca^{2+}]_i$) and microscopic images simultaneously without changing the optical path. With this system, we recorded $[Ca^{2+}]_i$ at 2 ms intervals and microscopic images of trichocyst discharge at video rate (33 ms intervals) simultaneously in *Paramecium caudatum*.

Simultaneous application of Ca^{2+} chelator at 100 mM with secretagogue onto *Paramecium* cells resulted in only a slight increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$). Furthermore, no extrusion of trichocysts occurred. In contrast, application of secretagogue concomitant with Ca^{2+} chelator at 20 mM induced a $\Delta[Ca^{2+}]_i$ composed of two phases. In this case, extrusion of trichocysts occurred. These observations directly indicated that Ca^{2+} influx from the extracellular medium in addition to Ca^{2+} release from intracellular store(s) contributes to $\Delta[Ca^{2+}]_i$ during trichocyst discharge.

INTRODUCTION

In many organisms, it is well known that $[Ca^{2+}]_i$ has very important roles in regulating various physiological activities. For example, exocytosis is triggered by $\Delta[Ca^{2+}]_i$. To study the regulatory role of $[Ca^{2+}]_i$ in exocytosis, it is important to measure $[Ca^{2+}]_i$ during cell function *in vivo*. For this purpose, it is first necessary to load the cell with some Ca^{2+} indicator. Ciliated protozoans loaded with Calcium Green dextran show quite normal behavior regulated by Ca^{2+}; *Didinium nasutum* discharges toxicysts to attack *Paramecium*, while *P. caudatum* discharges trichocysts as defense against the attack (Iwadate *et al.*, 1997, 1999a, b), and *Vorticella* sp. contracts its cell body in response to mechanical stimulation (Katoh and Kikuyama, 1997). In *Paramecium* spp., it is known that trichocyst discharge includes an exocytotic event. Thus, *Paramecium* spp. is one of the most appropriate systems in which to investigate the relationship between exocytosis and $[Ca^{2+}]_i$.

In the resting state of *Paramecium* spp., trichocysts are enclosed within the trichocyst membrane just beneath the plasma membrane. The process of trichocyst discharge has

been suggested to be composed of three steps; (1) fusion of the trichocyst membrane with the plasma membrane; (2) extrusion of trichocyst; and (3) resealing of the fusion pore (Knoll *et al.*, 1991). Knoll *et al.* (1991) studied the time course of trichocyst discharge in detail by quenched flow analysis and demonstrated that the trichocyst membrane and plasma membrane begin to fuse within 30 ms after stimulation by the chemical secretagogue aminoethyldextran (AED), and that the fusion pore is resealed within 350 ms after AED stimulation. On the other hand, trichocyst extrusion takes less than 1 ms (Plattner *et al.*, 1993).

Many investigators have demonstrated the contribution of Ca^{2+} to trichocyst discharge in *Paramecium* spp. (Plattner 1974, 1976; Bilinski *et al.*, 1981; Gilligan and Satir 1982, 1983; Garafolo *et al.*, 1983; Satir *et al.*, 1988; Kerboeuf and Cohen 1990, 1996; Knoll *et al.*, 1991, 1993; Erxleben and Plattner 1994; Länge *et al.*, 1995; Erxleben *et al.*, 1997; Klauke and Plattner 1997, 1998; Iwadate *et al.*, 1997, 1999a). Fig. 1 shows a brief summary of hypothetical Ca^{2+} behavior during trichocyst discharge. (1) Ca^{2+} release from the cortical alveolar sacs (vast compartment for Ca^{2+} storage (Stelly *et al.*, 1991); ca in Fig. 1) takes place during trichocyst discharge. This was suggested by electron spectroscopic study in which the Ca^{2+} signal was detected within the alveolar sacs only before trichocyst discharge. In contrast, the Ca^{2+} signal became detectable outside the alveolar sacs after trichocyst discharge (Knoll *et al.*,

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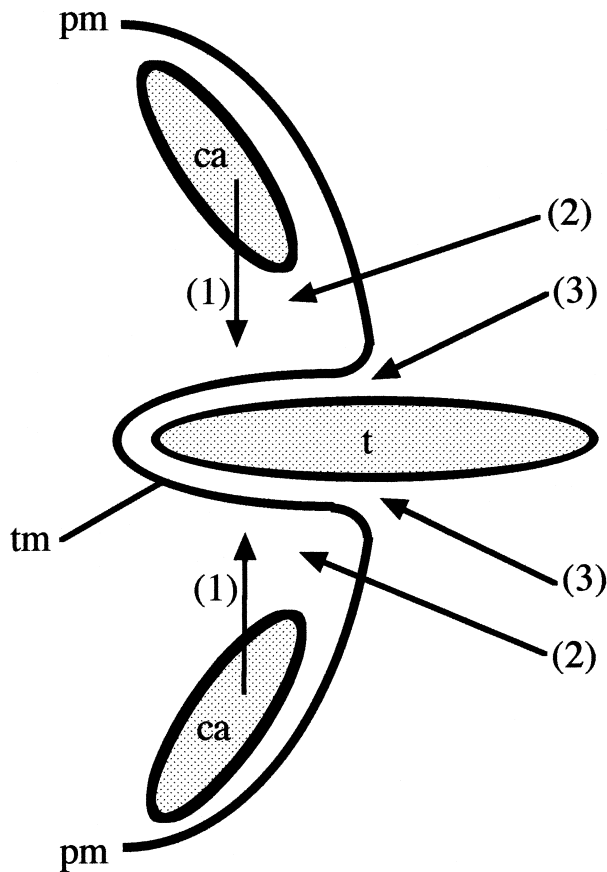
Fig. 1. Three hypothetical categories of Ca^{2+} actions related to trichocyst discharge. (1) Release from cortical alveolar sacs (ca) beneath the plasma membrane (pm). (2) Influx from extracellular medium into the cell. (3) Influx from extracellular medium into the trichocyst (t) vesicle through the fusion pore.

1993). (2) Ca^{2+} influx from the external medium ((2) in Fig.1) takes place during trichocyst discharge (Kerboeuf and Cohen, 1990; Knoll *et al.*,1992). (3) Extracellular Ca^{2+} flows into the trichocyst vesicle through the fusion pore ((3) in Fig. 1) and induces a trichocyst extrusion (Bilinski *et al.*, 1981). This suggested that trichocyst extrusion does not require cytosolic but extracellular Ca^{2+}, because trichocysts should directly face the extracellular medium after membrane fusion. Ca^{2+}-dependent trichocyst extrusion was also demonstrated *in vitro* (Lima *et al.*, 1989).

Although there have been many studies of the role of Ca^{2+} in trichocyst discharge in *Paramecium* spp., direct evidence of Ca^{2+} behavior during the trichocyst discharge in living *Paramecium* has not been reported. Thus, the relationship between two Ca^{2+} transients, release from intracellular store(s) and influx from extracellular region, and the trichocyst discharge has been ambiguous. Recently, Plattner and coworkers (Erxleben *et al.*, 1997; Klauke and Plattner 1997, 1998; Klauke *et al.*, 1998; Klauke *et al.*, 2000) and Iwadate *et al.* (1997) adopted a calcium imaging method with confocal microscopy to detect Ca^{2+} transients during trichocyst discharge. Iwadate *et al.* (1997) recorded $\Delta[Ca^{2+}]_i$ during trichocyst discharge at video rate (33 ms). Plattner and co-workers

(Erxleben *et al.*, 1997; Klauke and Plattner 1997, 1998; Klauke *et al.*, 1998; Klauke *et al.*, 2000) recorded both $\Delta[Ca^{2+}]_i$ and images of trichocyst discharge, by switching the optical path for each video frame (33 ms). It should be stressed in all these reports that the Ca^{2+} transient was composed of only one phase, indicating that Ca^{2+} release from intracellular store(s) and Ca^{2+} influx from the extracellular medium were not distinguished (cf. Fig. 1).

Ca^{2+} imaging of trichocyst discharge provides important information about the spatial distribution of $[Ca^{2+}]_i$, although it gives little information about temporal changes in $[Ca^{2+}]_i$. This is because one frame interval of the video recording is 33 ms, whereas fusion between the trichocyst membrane and plasma membrane begins within 30 ms after stimulation. Thus, it is necessary to use other methods with higher time resolution than Ca^{2+} imaging of video rate that can record both $\Delta[Ca^{2+}]_i$ and image of trichocyst discharge simultaneously.

We constructed a new system in which fluorescent signals reflecting $[Ca^{2+}]_i$ are detected by a photomultiplier tube (PMT), not by video-imaging. This system provides highly improved time resolution of about 0.5 ms (maximum speed) although spatial information about $[Ca^{2+}]_i$ is diminished. Moreover, the system can simultaneously record not only information about $[Ca^{2+}]_i$ but also microscopic images of the specimen without changing the optical path as describes later in detail. Using this system, we revealed in live *Paramecium* cell that Ca^{2+} influx from the extracellular medium in addition to Ca^{2+} release from intracellular store(s) occurs just before trichocyst discharge.

MATERIALS AND METHODS

Culture of *Paramecium*

Wild-type *Paramecium caudatum* cells (kyk402) were cultured in hay infusion at room temperature (20–25°C). Prior to the experiments, *Paramecium* cells were transferred into a standard saline medium containing 1 mM KCl, 1 mM $CaCl_2$ and 20 mM PIPES-Tris (pH 7.0).

Micromanipulation

The method applied for holding the *Paramecium* cell was the same as described previously (Iwadate *et al.*, 1997). Briefly, the standard saline medium containing dispersed *Paramecium* cells was mixed with the same volume of a highly viscous medium (1% methylcellulose, 1 mM KCl, 1 mM $CaCl_2$ and 20 mM PIPES-Tris pH 7.0) about 2 min before microinjection of Ca^{2+} indicator. A *Paramecium* cell swimming slowly in the medium was caught at the tip of a suction pipette (about 50 μm in inner diameter).

Medium containing 1 mM Calcium Green 1 dextran 10000 MW (Molecular Probes, Eugene, OR, USA), 150 mM KCl and 0.5 mM HEPES-KOH (pH 7.0), designated as CaG medium (c in Fig. 2), was injected into the *Paramecium* cell according to the method of "braking micropipette" (Hiramoto, 1974). After injection of CaG medium, the micropipette in which ethanol still remained was pulled out of the cell and ethanol (et in Fig. 2) was applied onto the cell surface (Fig. 2A). In some experiments, calcium chelator was applied onto the cell surface prior to ethanol application. In this case, EGTA medium was used; the composition of the medium was 20 or 100 mM Glycoletherdiaminetetraacetic Acid (EGTA), 1 mM KCl and 20 mM PIPES-Tris (pH 7.0). EGTA medium and ethanol were put into one micropipette (Fig. 2B). Since CaG medium, EGTA medium and ethanol were placed in one pipette in this order (Fig. 2A, B), injection of
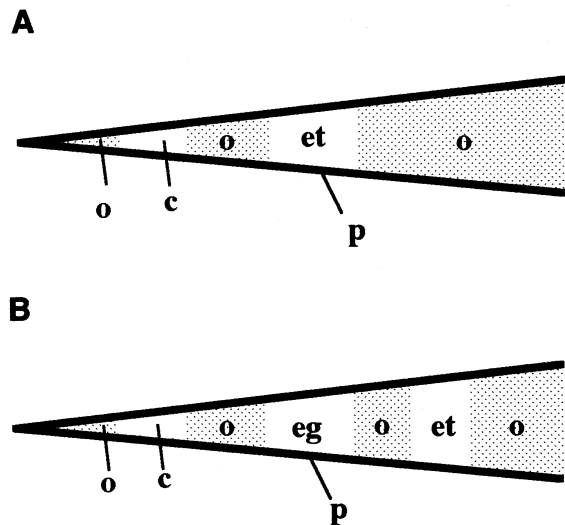
A

B

Fig. 2. Microinjection of CaG medium and application of EGTA medium and ethanol. (A) About 5 pl CaG medium (c) and about 10 pl ethanol (et) were put in one injection pipette (p), sandwiched between small amount of silicone oil (o). CaG medium was injected into a *P. caudatum* cell, and ethanol was then sprayed onto the surface of the cell. (B) In the case of spraying EGTA together with ethanol, about 5 pl CaG medium (c), about 10 pl EGTA medium (eg) and about 10 pl ethanol (et) were put in one injection pipette (p), separated by small amounts of silicone oil (o). CaG medium was injected into a *P. caudatum* cell, then EGTA medium and ethanol were sprayed onto the surface of the cell.
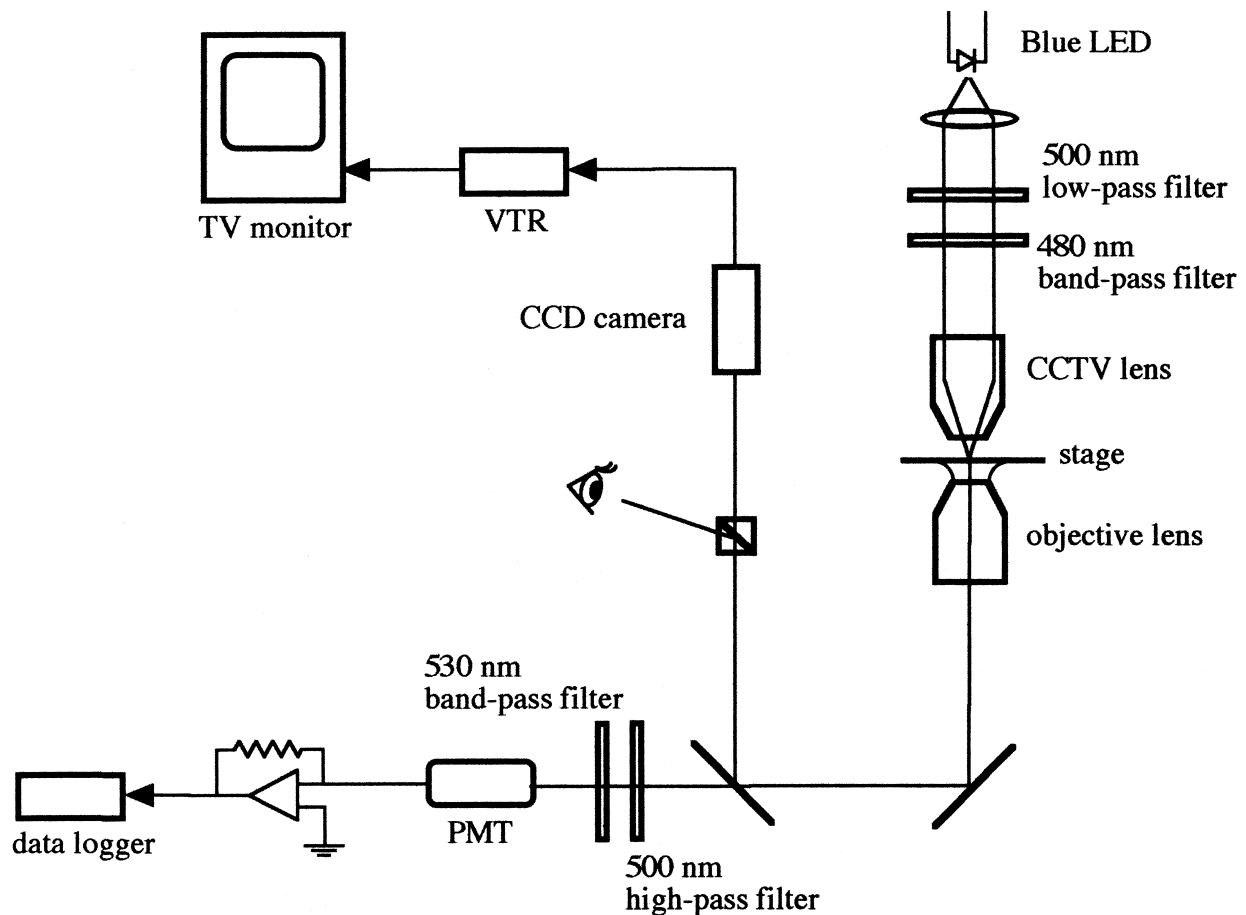

Fig. 3. Schematic representation of the measuring system. A blue LED was attached to an inverted microscope. The light from the LED, which passed through a 500 nm low-pass filter and 480 nm band-pass filter, was condensed at the focal point of an objective lens through a CCTV lens and used as a light source not only for observation of trichocyst discharge but also for excitation of Calcium Green. Fluorescence from Calcium Green, which passed through a 500 nm high-pass filter and 530 nm band-pass filter, was measured with a PMT attached to the side port of the microscope. The output of the PMT was stored with a data logger at 2 ms intervals. Trichocyst discharge was detected with a CCD camera attached to the top port of the microscope and recorded on VHS videotape. Although no filter to cut off the fluorescence of Calcium Green was placed in front of the CCD camera, the camera did not respond to changes in fluorescence intensity of Calcium Green because the intensity was very weak.

CaG medium into the cell body and application of EGTA and/or 99.5% ethanol onto the cell surface were also carried out in this order. The injected volume of CaG medium was approximately 5 pl, corresponding to about 1% of the whole cell volume. The volumes of EGTA medium and ethanol were both 10 pl, respectively.

Micromanipulations of the suction pipette and micropipette were carried out using two micromanipulators (MO-102N and WR-60, Narishige, Tokyo, Japan) and injection through the micropipette was performed using a microinjector (IM-5A, Narishige, Tokyo, Japan).

Simultaneous recording of $[Ca^{2+}]_i$ and trichocyst discharge

An inverted microscope (TE300, Nikon, Tokyo, Japan) was used throughout the experiments. A blue light-emitting diode (blue LED ; LSPB500S, Nichia, Tokushima, Japan), which emits blue light (λ_{max}=473 nm), was placed at the top of the microscope (Fig. 3). The light from the blue LED was filtered through a band-pass filter of 480 nm (full-width at half-maximum (FWHM) 7.4 nm) (35-3441, Coherent, Tokyo, Japan) and a low-pass filter of 500 nm (35-5289, Coherent, Tokyo, Japan) to pass 480 nm light. The LED was focused on the focal point of the objective lens (CFI S Fluor 40xH (N.A. 1.30, Oil), Nikon, Tokyo, Japan) by a closed-circuit television (CCTV) lens (16 mm F1.6, Akizuki Denshi, Tokyo, Japan). In this system, the blue LED was used as an excitation light source for Calcium Green on one hand and as a light source for microscopic observation of the specimen on the other (Fig. 3). Trichocyst discharge in *P. caudatum* was detected with a CCD camera (XC-ST50, Sony, Tokyo, Japan) and recorded on VHS videotape with a videotape recorder (HR-VX200, Victor, Tokyo, Japan). The images were then transferred onto a computer (PC-9821Nr13, NEC, Tokyo, Japan).

Fluorescence intensity of Calcium Green was detected with a PMT (R374, Hamamatsu Photonics, Hamamatsu, Japan), equipped with a high-pass filter of 500 nm (SC-50, Fuji Photo Film, Tokyo) and band-pass filter of 530 nm (FWHM 7.2 nm) (35-3607, Coherent, Tokyo, Japan) and recorded on a data logger (JJ.Joker E-1, Nippon Filcon, Tokyo, Japan) at 2 ms intervals. Recording with the VHS videotape recorder and with the data logger were controlled by a personal computer (PC-9821 Nd/340w, NEC, Tokyo, Japan).

RESULTS

Response of the measurement system to $[Ca^{2+}]_i$

An experiment was carried out to determine whether our measurement system could actually detect $[Ca^{2+}]_i$ as followings. First, the CaG medium was injected into a *Paramecium* cell and fluorescence intensity reflecting $[Ca^{2+}]_i$ at the resting level in the *Paramecium* cell was measured. Klauke and Plattner (1997) demonstrated that the free Ca^{2+} concentration at the resting level in *Paramecium* cells is less than 100 nM. Then, a Ca^{2+} buffer, composed of 70 mM Ca(OH)$_2$, 100 mM EGTA and 100 mM PIPES-KOH (pH 7.0), was injected into the same *Paramecium* cell and the fluorescence was measured in the same manner. The free Ca^{2+} concentration in the Ca^{2+} buffer was estimated as 1 μM (Iwadate *et al.*, 1999b). The injected volume of Ca^{2+} buffer was approximately 50 pl, corresponding to about 10% of the whole cell volume. A typical result of 3 measurements is shown in Fig. 4 in which the PMT current, reflecting $[Ca^{2+}]_i$, rose significantly after injection of Ca^{2+} buffer. This indicated that the measurement system detected the $\Delta[Ca^{2+}]_i$.
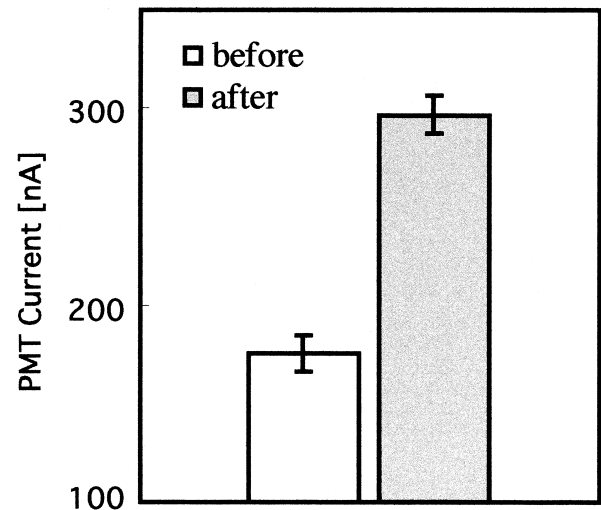

Fig. 4. Fluorescence intensity of Calcium Green before and after the injection of Ca^{2+} buffer into *P. caudatum* cells measured with our system. Before injection of Ca^{2+} buffer containing 10^{-6} M free Ca^{2+}, i.e. at the resting revel (less than 100 nM free Ca^{2+} in the cytoplasm), the PMT current was around 170 nA (left column). After injection, the current rose significantly (right column) indicating that the measurement system detected the $\Delta[Ca^{2+}]_i$. Each vertical bar at the top of each column indicates standard deviation of one measurement for 30 s.

Time course of calcium transient and trichocyst discharge in response to ethanol

Ethanol has been routinely used to deciliate *Paramecium* cells (Ogura, 1981; Nelson, 1995). In the deciliation procedure, it is necessary to incubate the cell in a medium containing 5% ethanol for 2–3 min. It is known that not only deciliation but also trichocyst discharge occur during this incubation process. Therefore, we used ethanol as a secretagogue for trichocyst discharge in the following experiments.

When 5% ethanol is used it takes 2–3 min to induce trichocyst discharge even by the incubation method as mentioned above. Furthermore, in the case of spray application, the sprayed medium diffuses immediately just after application. Therefore, we applied 99.5% ethanol by spraying in the following experiments.

After loading Calcium Green into the *Paramecium* cell, 99.5% ethanol was applied by spraying onto the cell surface. As shown in Fig. 5A, the $[Ca^{2+}]_i$ rose rapidly in response to the application of ethanol. Just after the beginning of $\Delta[Ca^{2+}]_i$, trichocyst discharge occurred (n=7). Fig. 5B, in which a derivative of the PMT current recording is shown, clearly demonstrates the temporal correlation between $\Delta[Ca^{2+}]_i$ and the trichocyst discharge.

To describe the behavior of $\Delta[Ca^{2+}]_i$, we measured the period of $\Delta[Ca^{2+}]_i$ as the time interval between the time when the $[Ca^{2+}]_i$ in the rising phase reaches the half-maximum of $\Delta[Ca^{2+}]_i$ and the time when $[Ca^{2+}]_i$ in the falling phase again reaches the half-maximum of $\Delta[Ca^{2+}]_i$ (full-width at half-maximum time; FWHM time). The FWHM time of $\Delta[Ca^{2+}]_i$ was 3.7 ± 0.8 s (n=4). This time course of $\Delta[Ca^{2+}]_i$ induced by application of ethanol agreed with that induced by application of

AED, which is considered a physiological secretagogue of trichocysts (Erxleben *et al.*, 1997; Klauke *et al.*, 1998, 2000).

Two-phase calcium transient in trichocyst discharge

As described in the Introduction, $\Delta[Ca^{2+}]_i$ may be composed of two different processes; Ca^{2+} release from the intracellular store(s) and Ca^{2+} influx from the extracellular medium (Fig. 1). Despite this, Fig. 5A, in which recording of $[Ca^{2+}]_i$ was carried out with 2 ms time intervals, shows that the Ca^{2+} transient was a simple increase of $[Ca^{2+}]_i$ followed by a gradual decline. This suggested that (1) $\Delta[Ca^{2+}]_i$ is actually composed of only one phase, or (2) measurement of $[Ca^{2+}]_i$ with some
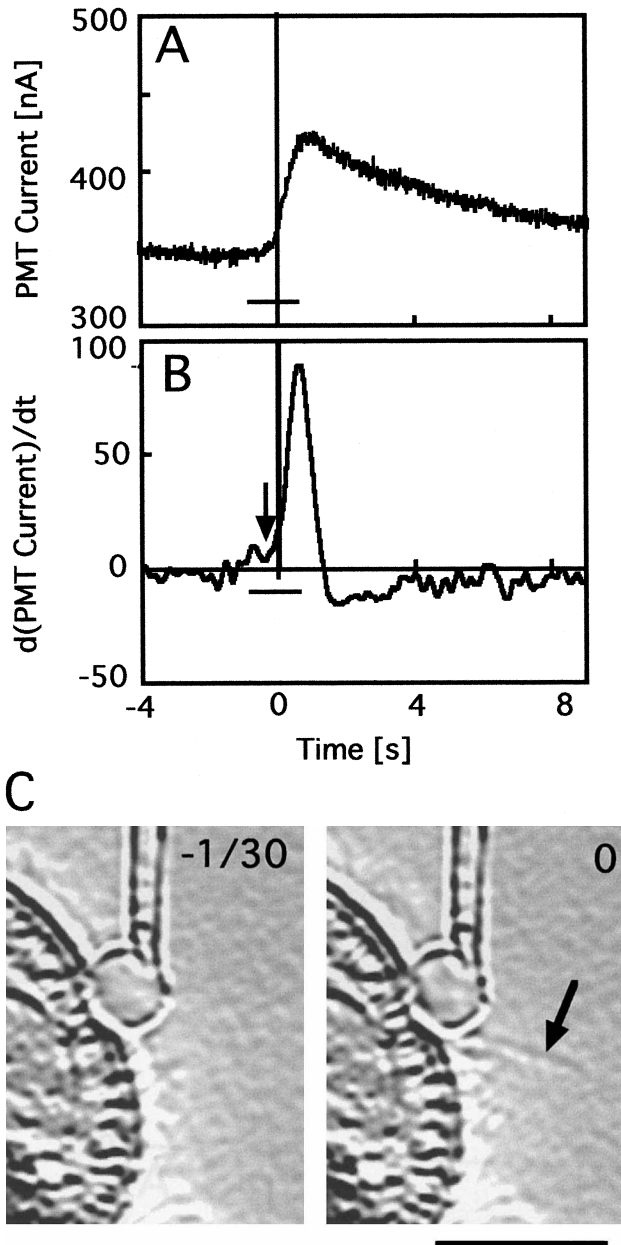

Fig. 5. Simultaneous recording of $[Ca^{2+}]_i$ at 2 ms intervals and images of trichocyst discharge at 33 ms intervals in response to application of ethanol to the anterior part of a *P. caudatum* cell. Application of ethanol onto the cell was continued for the period shown by the horizontal bars both in A and B. Perpendicular lines at time zero in A and B indicate the time when trichocyst discharge occurred. (A) Change of $[Ca^{2+}]_i$ in *P. caudatum* cell. Fluorescence intensity of Calcium Green is expressed as PMT current. (B) First-order time derivative of the PMT current shown in A. Trichocyst discharge took place 26 ms after the beginning of the rise of the derivative (arrow). This indicated that the rise of $[Ca^{2+}]_i$ significantly preceded the trichocyst discharge. (C) Micrographs of trichocyst discharge recorded simultaneously with the record shown in A. Time under the each photograph coincides with that shown in A and B. Discharged trichocysts are indicated with an arrow in each photograph. The sphere near the tip of the injection pipette is silicone oil drop. Scale bar, 20 μm.

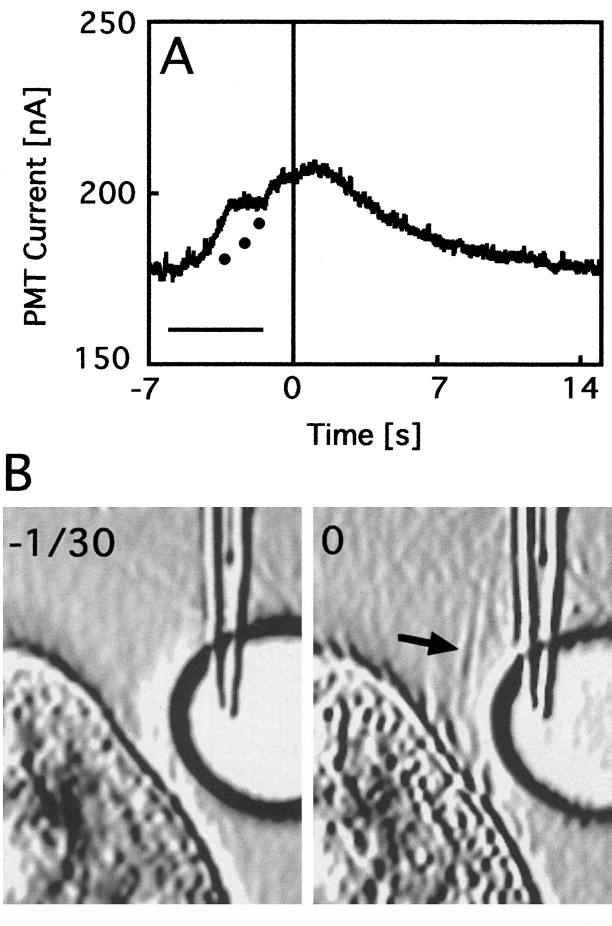**Fig. 6.** Simultaneous recording of $[Ca^{2+}]_i$ at 2 ms intervals and images of trichocyst discharge at 33 ms intervals in response to application of 20 mM EGTA and ethanol to the anterior part of a *P. caudatum* cell. (A) Changes of $[Ca^{2+}]_i$ in *P. caudatum* cell. Fluorescence intensity of Calcium Green is expressed as PMT current. The horizontal bar represents the period of spraying of 20 mM EGTA and ethanol. The perpendicular lines at time 0 s indicates the time at which trichocyst discharge occurred. The $\Delta[Ca^{2+}]_i$ was composed of two phases. Trichocyst discharge did not take place during the first phase but occurred during the second phase. The dotted line represents extension of the second phase of $\Delta[Ca^{2+}]_i$. (B) Micrographs of trichocyst discharge recorded in the same experiment as A. Time under the each image coincides with the time in A. Discharged trichocyst are indicated with an arrow in each photograph. The sphere near the tip of the injection pipette is a silicone oil drop. Scale bar, 20 μm.

sophisticated strategy is indispensable to examine the two-phase $\Delta[Ca^{2+}]_i$.

We highly reduced Ca^{2+} level outside the cell ($[Ca^{2+}]_o$) for only a short period by applying a small amount of EGTA onto the surface of *Paramecium* cells. This procedure should be expected to cause a delay in the start of Ca^{2+} influx from the external medium (Fig. 1). If contact of trichocysts with the external Ca^{2+} triggers the extrusion of trichocysts as suggested by Bilinski *et al.* (1981), trichocysts should not expand for at least the low $[Ca^{2+}]_o$ period even after fusion of the trichocyst membrane and plasma membrane (Erxleben *et al.*, 1997).

We applied 20 mM EGTA medium just before application of ethanol (Fig. 2B). As shown in Fig. 6, the $[Ca^{2+}]_i$ rose in response to ethanol application even in the presence of 20 mM EGTA. Trichocyst extrusion did not take place during this first $\Delta[Ca^{2+}]_i$, indicating that low $[Ca^{2+}]_o$ was generated by EGTA. Thus, the initial $\Delta[Ca^{2+}]_i$ should be of intracellular origin. About 1 s after the end of EGTA- and ethanol-application, the $[Ca^{2+}]_i$ began to rise again and, during this period $\Delta[Ca^{2+}]_i$,

Fig. 7. Simultaneous recording of $[Ca^{2+}]_i$ at 2 ms intervals and images of a *P. caudatum* cell at 33 ms intervals in response to application of 100 mM EGTA and ethanol to the anterior part of the P. caudatum cell. (A) Changes of $[Ca^{2+}]_i$ in a *P. caudatum* cell. Fluorescence intensity of Calcium Green is expressed as PMT current. The horizontal bar represents the period of spraying of 100 mM EGTA and ethanol. Ethanol treatment in the presence of 100 mM EGTA caused a small $\Delta[Ca^{2+}]_i$. (B) Micrographs in the same experiment before and after the application of ethanol concomitant with 100 mM EGTA. Trichocyst extrusion did not occur. Time under each image coincides with the time in A. The sphere near the tip of the injection pipette is a silicone oil drop. Scale bar, 20 μm.

trichocyst extrusion occurred (Fig. 6). In the 4 other cells tested, essentially the same results were obtained; pre-treatment with EGTA significantly delayed the process of trichocyst discharge and revealed that the Ca^{2+} transient was composed of two steps. This indicated that the second $\Delta[Ca^{2+}]_i$ occurred as a result of promoted influx of Ca^{2+} that became evident upon recovery of $[Ca^{2+}]_o$ after disappearance of EGTA by diffusion.

To obtain more complete depletion of $[Ca^{2+}]_o$, we applied 100 mM EGTA medium just before application of ethanol. Even when 100 mM EGTA was used, $\Delta[Ca^{2+}]_i$ occurred although the amplitude was small (Fig. 7). Despite this, trichocyst extrusion was not observed in any of the cells tested (n=4) and the second rise of $[Ca^{2+}]_i$ also did not occur. This result clearly demonstrated that the second $\Delta[Ca^{2+}]_i$ in Fig. 6 was the result of influx of Ca^{2+} from the extracellular medium.

DISCUSSION

The spatial distribution of $\Delta[Ca^{2+}]_i$ during trichocyst discharge has been demonstrated by several investigators (Iwadate *et al.*, 1997; Erxleben *et al.*, 1997; Klauke and Plattner, 1997, 1998; Klauke *et al.*, 1998; Klauke *et al.*, 2000). The $[Ca^{2+}]_i$ rises at the peripheral area in response to the secretagogue stimulus. The area of high $[Ca^{2+}]_i$ spreads along the cell surface and into the deeper cell region within several seconds. However, the time interval of video rate recording (33 ms) is too slow to precisely discuss the time course of the Ca^{2+} transient. Furthermore, we could not ascertain whether trichocyst discharge actually occurred simultaneously with the $\Delta[Ca^{2+}]_i$ (Iwadate *et al.*, 1997). Therefore, the temporal correlation between $[Ca^{2+}]_i$ and trichocyst discharge has remained ambiguous.

To study exocytosis of trichocysts, we constructed a new device with much higher time resolution of $[Ca^{2+}]_i$ measurement. To simultaneously detect both fluorescent signals and microscopic images, we placed a blue LED (luminous intensity; 3.00 cd) as the light source opposite the objective lens, which was used not only as the source of emission for Calcium Green but also as the light source for microscopic observation. To detect the weak fluorescence of Calcium Green excited by the light from the blue LED and to heighten the time resolution of $[Ca^{2+}]_i$ measurement, we used a PMT as a detector, although spatial information concerning $\Delta[Ca^{2+}]_i$ is completely lost in this method. Using the new system (Fig. 3), we simultaneously recorded $[Ca^{2+}]_i$ with PMT and microscopic images of trichocyst discharge with a CCD camera as shown in Figs. 5–7. The time interval of the recording of $[Ca^{2+}]_i$ was 2 ms.

Ethanol has been routinely used to deciliate *Paramecium* cells (Ogura, 1981; Nelson, 1995). For this, the cell is incubated in a medium containing 5% ethanol for 2–3 min. This procedure is known to induce not only deciliation but also trichocyst discharge. After deciliation, the *Paramecium* retains its viability and reproduces new cilia. This suggests that ethanol is not lethal for *Paramecium* and that it may be used as a secretagogue for trichocyst discharge. In the present study,
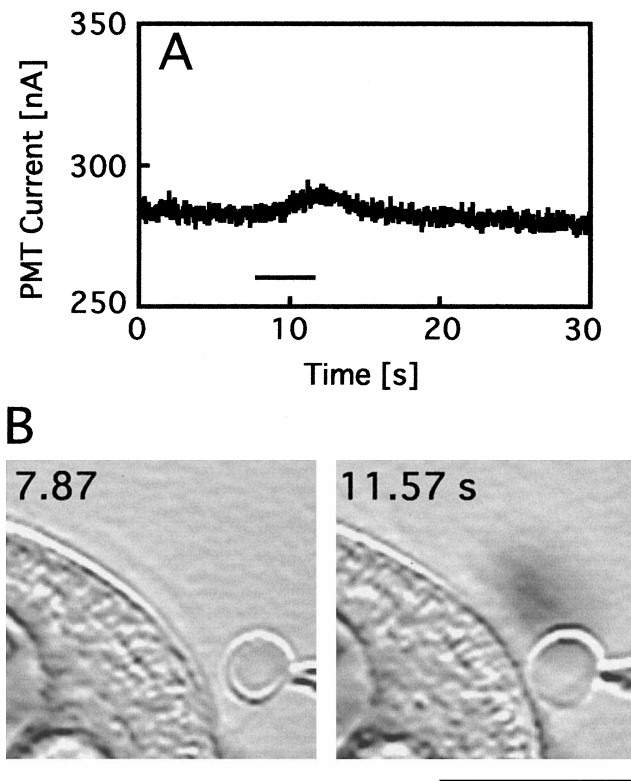
we applied 99.5% ethanol to induce trichocyst discharge by spraying onto the *Paramecium* cell surface because spray application of 5% ethanol did not induce trichocyst discharge probably due to its low concentration. Application of 99.5% ethanol induced trichocyst discharge in *Paramecium* and after the discharge, the *Paramecium* showed normal swimming behavior with normal ciliary beating as is the case of stimulation by a predator (Iwadate *et al.*, 1997) and by AED. The time course of $\Delta[Ca^{2+}]_i$ induced by the application of ethanol was coincident with that induced by application of AED. Thus, trichocyst discharge induced by the ethanol spray should be physiological, at least with regard to intracellular calcium response.

Ethanol is a membrane-permeable compound that affects Ca^{2+} channels. Thus, we cannot exclude the possibility that the trichocyst discharge induced by ethanol spray may not be a result of physiological Ca^{2+} response as follows. (1) Sprayed ethanol injures Ca^{2+} channels at the cell membrane and increases Ca^{2+} influx from extracellular medium via the channels, and finally may induce trichocyst discharge. (2) Sprayed ethanol may permeate into the cell and injure Ca^{2+} channels on the membrane of intracellular Ca^{2+} stores and increase Ca^{2+} release from the stores. The increased release of Ca^{2+} from the stores may induce trichocyst discharge.

In the present study, application of ethanol concomitant with 20 mM EGTA induced $\Delta[Ca^{2+}]_i$ with two phases, and trichocysts were discharged during the second phase of $\Delta[Ca^{2+}]_i$ (Fig. 6). We could not completely exclude the possibility that the first phase of the $\Delta[Ca^{2+}]_i$ may not be a physiological calcium rise because ethanol may damage Ca^{2+} channels as suggested above. Despite this, the second phase definitely reflected influx of Ca^{2+} from the extracellular medium. The first phase of the $\Delta[Ca^{2+}]_i$ probably induces the second phase, i.e. Ca^{2+} influx from the extracellular medium. Ca^{2+} influx should be an indispensable and physiological process to trigger trichocyst discharge.

It is important to investigate the mechanisms of action of ethanol which induces the first phase of $\Delta[Ca^{2+}]_i$. If ethanol attacks Ca^{2+} channels on the intracellular Ca^{2+} store(s) or cell membrane directly, detailed studies of trichocyst discharge induced by ethanol may provide information about signal transduction pathways from extracellular stimulation to Ca^{2+} release from intracellular Ca^{2+} store(s). If ethanol acts as a physiological secretagogue similarly to AED, ethanol may become one of the most useful secretagogues the trichocyst discharge.

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